



Production and Function of a Soluble c-Kit Molecule

By

Stuart Hamilton Read, B.Sc. (Hons)

Enrolled through the Department of Microbiology and Immunology,
University of Adelaide.

Research conducted at the Department of Haematology,
Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science

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STATEMENT

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August, 2001

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To all of you who have asked, finally I can say: Yes, I have finished!!

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APAAP	Alkaline Phosphatase Anti-Alkaline Phosphatase
Asn	asparagine
Asp	aspartate
ATL	acute T cell leukaemia
AU	absorbance units
Az	azide
BFU-E	blast forming unit-erythrocyte
BFU-MK	blast forming unit-megakaryocyte
BM	bone marrow
bp	base pairs
BSA	bovine serum albumin
bSLF _y	biotinylated SLF (recombinant, produced in yeast)
cDNA	complementary deoxyribonucleic acid
Cat. No.	catalogue number
CFU-GEMM	colony forming unit-granulocyte, erythrocyte, monocyte and macrophage
CFU-GM	colony forming unit-granulocyte macrophage
CFU-S	spleen colony forming cells
CHO cells	Chinese Hamster Ovary cells
CIP	calf intestinal phosphatase
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CNTF(R)	ciliary neurotrophic factor (receptor)
COS	African Green Monkey kidney cells
CSF-1(R)	colony stimulating factor-1 (receptor)
dhfr	dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECF	enhanced chemifluorescence
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF(R)	epidermal growth factor (receptor)
EIA	enzyme immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
Epo	erythropoietin
EtBr	ethidium bromide
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
G-CSF(R)	granulocyte-colony stimulating factor (receptor)

G418	geneticin
GIST	gastrointestinal stromal tumour
Gly	glycine
GM-CSF (R)	granulocyte macrophage colony stimulating factor (receptor)
GNNK	glycine asparagine asparagine lysine
gp(130)	glycoprotein(130)
GST	glutathione S-transferase
GVHD	graft versus host disease
HBS	Hepes Buffered Saline
HBSS	Hank's balanced salt solution
HEPES	N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid
HIV	human immunodeficiency virus
HPP-CFU	high proliferative potential-CFU
HPV	human papilloma virus
HRP	horseradish peroxidase
Hu	human
HUVEC	human umbilical vein endothelial cell
HZ4-FeSV	Hardy-Zuckerman 4 feline sarcoma virus
ICC	interstitial cell of Cajal
IFN- γ 1(R)	interferon gamma 1(receptor)
Ig	immunoglobulin
IL-(3)	interleukin-(3)
IPTG	isopropylthio- β -D-galactoside
KIT ^S	soluble c-Kit
LIF	leukaemia inhibitory factor
LTC-IC	long term culture-initiating cells
Lys	lysine
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MDS	myelodysplastic syndrome
<i>me</i>	motheaten locus
MFI	mean fluorescence intensity
MOI	multiplicity of infection
MPS	myeloproliferative syndrome
mRNA	message ribonucleic acid
Msx	L-methionine sulphoximine
MTHC	myb transformed primary foetal liver cells
Mtx	Methotrexate
NGF	nerve growth factor
NHS	normal human serum
NRS	normal rabbit serum
O.D. _{280nm}	optical density at 280 nanometre wavelength
OPD	o-phenylenediamine dihydrochloride
OSM	oncostatin M
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF(R)	platelet derived growth factor (receptor)
PE	R-phycoerythrin
PI3'K	phospho-inositol-3'-kinase

PKC	protein kinase C
PLC- γ 1	phospholipase C-gamma 1
PMA	phorbol 12-myristate 13-acetate
PVDF	polyvinylidene difluoride membrane
Rh123	rhodamine 123
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RTK	receptor tyrosine kinase
SA-HRP	streptavidin coupled to horseradish peroxidase
SA-PE	streptavidin-R-phycoerythrin
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
ser	serine
SH2	src homology domain 2
SH3	src homology domain 3
<i>Sl</i>	steel locus
SLF	Steel factor
SLF _b	Steel Factor produced in bacteria
SLF _y	Steel Factor produced in yeast
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylene diamine
TGF- β	transforming growth factor- β
TNF(R)	tumour necrosis factor (receptor)
Tpo	thrombopoietin
VEGF(R)	Vascular endothelial cell growth factor (receptor)
VLA	very late antigen
<i>W</i>	dominant white locus
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
Y821	tyrosine at position 821 of c-Kit

CONFERENCE PRESENTATIONS

1994
Poster Title: 6th Lorne Cancer Conference, Victoria, AUSTRALIA
Construction and purification of the soluble Kit protein
S. Read and L.K. Ashman

1995
Poster Title: 7th Lorne Cancer Conference, Victoria, AUSTRALIA
Soluble c-Kit as an anti-tumour agent
S. Read and L.K. Ashman

ABSTRACT

The receptor tyrosine kinase c-Kit and its ligand Steel Factor (SLF) are important in normal haemopoiesis. SLF acts as a growth and differentiation factor for haemopoietic stem and progenitor cells, and its membrane-associated form also appears to be important in the adhesion of stem cells to the stroma. In addition, it has been shown that a subgroup of AML patients that did not respond to chemotherapy had AML cells that expressed a high level of c-Kit on the cell surface. Similarly, elevated levels of other receptor tyrosine kinases have been implicated in carcinogenesis, for example, the overexpression of the *HER2/neu* proto-oncogene has been shown to be a prognostic indicator in a subset of breast and ovarian cancers. It is possible that high expression of c-Kit by the leukaemic cells provides them with a growth advantage over their normal counterparts in the bone marrow microenvironment. Thus, a means of inhibiting the interaction of c-Kit on these cells with SLF may remove proliferation and survival signals. Therefore, the main aim of this study was to produce a biological inhibitor of this interaction and evaluate its ability to prevent SLF from binding to c-Kit on live cells.

The extracellular portion of the c-Kit molecule (KIT^S) was chosen as the inhibitor. This soluble receptor was expected to compete with the native membrane bound receptor for the ligand thereby preventing SLF from interacting with the c-Kit expressing target cell. It was envisaged that, if successful, the therapeutic potential of KIT^S may not be limited to AML since KIT^S may play a role in inhibiting the proliferation of certain other tumours which abnormally express c-Kit and proliferate in response to autocrine or paracrine SLF production. For example, it has been shown that SLF producing Small Cell Lung Cancer cells aberrantly express c-Kit providing an uncontrolled autocrine proliferative loop. Mast cells are the only mature haemopoietic cells to express c-Kit on their surface. The interaction of mast cell c-Kit with SLF may have important repercussions in mast cell mediated diseases such as asthma. Another possible application of KIT^S would be to manipulate the interaction of primitive haemopoietic cells with the bone marrow microenvironment, thereby enhancing the mobilisation of stem cells into the peripheral blood.

The cDNA encoding the extracellular domain of c-Kit was generated by polymerase chain reaction using oligonucleotide primers with sequences based on that published by Yarden and

coworkers (Yarden *et al.*, 1987, EMBO J, 3341-3351). After confirmation of the sequence, the KIT^S cDNA was ligated into several different vectors to determine the best method of producing and purifying the recombinant protein. These included eukaryotic, bacterial and insect cell expression vectors. Several KIT^S constructs were generated and their activity studied. A monomeric form of KIT^S containing a 6xhistidine tag at the 3' end of the molecule was expressed in the Baculovirus system. This tag facilitated the initial purification of the recombinant KIT^S molecule using metal chelate chromatography. A dimeric KIT^S molecule was produced in both a eukaryotic cell system using the expression vector pIG and using insect cells infected by a Baculovirus construct encoding the dimeric KIT^S molecule.

Two enzyme linked immunosorbent assays (ELISA) were developed, using anti-c-Kit mAbs generated in this laboratory, to quantitate and measure the production and purification of KIT^S by the various methods described above. The functional activity of purified protein was initially assayed by its ability to block the binding of biotinylated SLF to the c-Kit expressing human erythroleukaemia cell line HEL-DR⁺, measured by immunofluorescence and flow cytometry. KIT^S was shown to inhibit the SLF dependent proliferation, as measured by ³H-thymidine incorporation, of the factor dependent cell lines TF-1 and FDC-P1 (FD^{GNNK⁺#2}) cells expressing low levels of human c-Kit. However, the proliferation of high c-Kit expressing cell lines M07e and FD^{GNNK⁺#1} could not be inhibited by KIT^S at 10 or 80µg/ml respectively. The dimeric form of KIT^S molecule was more effective at blocking the binding of biotinylated SLF to c-Kit expressing cells than the monomeric form, as measured by indirect immunofluorescence assay. It appeared that the dimeric form of KIT^S had a much higher avidity for SLF than the monomeric form. In an assay that measured the adhesion of M07e cells to CHO cells expressing membrane bound SLF, KIT^S was unable to abrogate the c-Kit-SLF interaction.

During the course of this study the existence of a naturally occurring KIT^S molecule was reported by several groups and it has been shown to be present at approximately 300ng/ml in the serum of healthy individuals. The mechanism by which the KIT^S is produced is unknown. It would seem likely that it is generated by proteolytic cleavage of the membrane bound receptor since mRNA species encoding KIT^S have not been demonstrated. There are several naturally occurring isoforms of c-Kit generated by alternate splicing. One pair of isoforms differs by the

deletion or insertion of 12 base pairs encoding the amino acids Gly-Asn-Asn-Lys (GNNK) in the juxtamembrane region of the extracellular domain, which may be near the cleavage region but how the presence or absence of the 4 amino acids affects c-Kit function is unknown. Using an ELISA assay the culture supernatant of several FDC-P1 cell lines expressing various levels of either the GNNK+ or GNNK- isoform of c-Kit were assayed for KIT^S levels. KIT^S was detected in the culture supernatant of both GNNK+ and GNNK- cells suggesting that the four amino acids in question are not essential for the generation of KIT^S.

Treatment of patients with certain cytokines, including G-CSF and SLF, results in the movement of CD34⁺ progenitor cells from the bone marrow and is associated with a down regulation of c-Kit expression. Activation of cells with PMA has been reported to bring about cleavage of membrane bound c-Kit and release of KIT^S. Serum samples from breast cancer patients treated with G-CSF or G-CSF and SLF were analysed for KIT^S levels. While changes in the KIT^S levels were seen in some patients over the time course of treatment, a consistent change was not seen and there was no correlation with the large increase of CD34⁺ cells detected in the peripheral blood of these patients between days 4 and 9 of treatment. This suggests that downregulation of cell surface c-Kit *in vivo* does not result from cleavage of the mature protein and that increases in KIT^S are not responsible for mobilisation.

The function of KIT^S in the serum is also not known but one proposal is that it may act as a chaperone for SLF preventing its degradation prior to reaching its target cells. However, it has not been shown that SLF in serum is complexed with KIT^S. Indeed, if KIT^S in serum is monomeric, it is likely to have a lower affinity for SLF than dimeric KIT^S or membrane bound full length c-Kit. Immunoprecipitations of both SLF and c-Kit from normal human serum were carried out to determine if these molecules were associated. While attempts to immunoprecipitate native serum SLF proved unsuccessful, when biotinylated SLF was added to human serum, it could be immunoprecipitated with endogenous KIT^S using anti-c-Kit monoclonal antibodies. This suggests that the soluble c-Kit detected in serum is capable of complexing with SLF, possibly protecting the SLF from degradation.

Corrections

Abbreviations, to be included, page iii:

FD ^{GNNK+} , FD ^{GNNK-}	cells derived from transfection of murine early myeloid FDC-P1 cells, a factor-dependent cell line, with human c-Kit cDNA
HEL-DR ⁺	a subline of the Human Erythro-Leukaemic cell line, HEL
M07e	factor-dependent human megakaryocytic leukaemia cell line
Sf21	cells derived from the ovary of the worm <i>Spodoptera frugiperda</i> . Used to propagate recombinant baculovirus
TF-1	factor-dependent human erythroleukaemia cell line

Section 1.10.3, Soluble IL-4 Receptor, page 56

The IL-4 receptor is a multimeric complex. The soluble IL-4 receptor referred to in this section is the extracellular IL-4 binding portion of the IL-4 receptor.

Section 1.10.4, Soluble TNF Receptor, page 57

TNF has two distinct but structurally homologous receptors, type I, 55kDa in size, and type II, 75kDa in size. The reference Carter *et al.*, 1994, does not state which receptor they used in their studies. Experiments conducted by Ashkenazi *et al.*, 1991; Van Zee *et al.*, 1992; Howard *et al.*, 1993 and Ammann *et al.*, 1997, all utilised the soluble form of the TNF type I receptor. Higuchi and Aggarwal (1992) studied the effects of both soluble TNF receptor I and TNF receptor II and Trehu *et al.*, 1996 only looked at the effects of soluble TNF receptor II.

Section 1.10.7, Soluble IL-6 Receptor family, page 59

Soluble IL-6 receptor refers to the soluble form of the low affinity, IL-6 binding peptide. The β chain, gp130, also exists as a soluble peptide and this is referred to as soluble gp130 or sgp130.

Table 3.1: Purification of KIT^S batches

	Percent Recovery
KIT ^S -IGc #2	71%
KIT ^S -IGc #3	76%

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1. INTRODUCTION

1.1 Haemopoiesis

The elements of the blood and lymph are derived during foetal and adult life from pluripotent haemopoietic stem cells in a process termed haemopoiesis. Haemopoiesis occurs predominantly in the liver of the foetus and in the bone marrow of adults. The pluripotent stem cells are found in very low numbers in the bone marrow and the majority of these are quiescent or slowly cycling (Bradford *et al.*, 1997). *In vivo* and *in vitro* studies show these stem cells are capable of generating progeny that are able to proliferate and mature into all of the haemopoietic lineages and these cells are capable of reconstituting the bone marrow and blood of lethally irradiated animals. The process of haemopoiesis is a complex one that is tightly regulated by many factors including soluble and membrane bound or extracellular matrix associated growth factors, cytokines, hormones, adhesion molecules and cell-cell contact via these molecules. Each of these factors may have positive or negative influences on haemopoiesis depending on the position of the cells within the bone marrow microenvironment and the combination and extent of synergy of the stimuli the stem and progenitor cells receive (Simmons *et al.*, 1997; Whetton and Spooncer, 1998).

Haemopoietic malignancies occur when the process mentioned above is disrupted in some way. Leukaemias of both the myeloid and lymphoid lineages are defined as the uncontrolled proliferation or expansion of haemopoietic cells that do not retain the capacity to differentiate normally to produce mature blood cells (Sawyers *et al.*, 1991). Some disorders are not strictly leukaemias because they display growth expansion, myeloproliferative syndromes, or a differentiation block, myelodysplasia, but can progress to acute leukaemia, probably after these premalignant cells obtain additional genetic mutations.

An important interaction in the development of haemopoietic cells is that of c-Kit with its ligand Stem Cell Factor. This interaction will be the main topic of the rest of this chapter.

1.2 The discovery of c-Kit

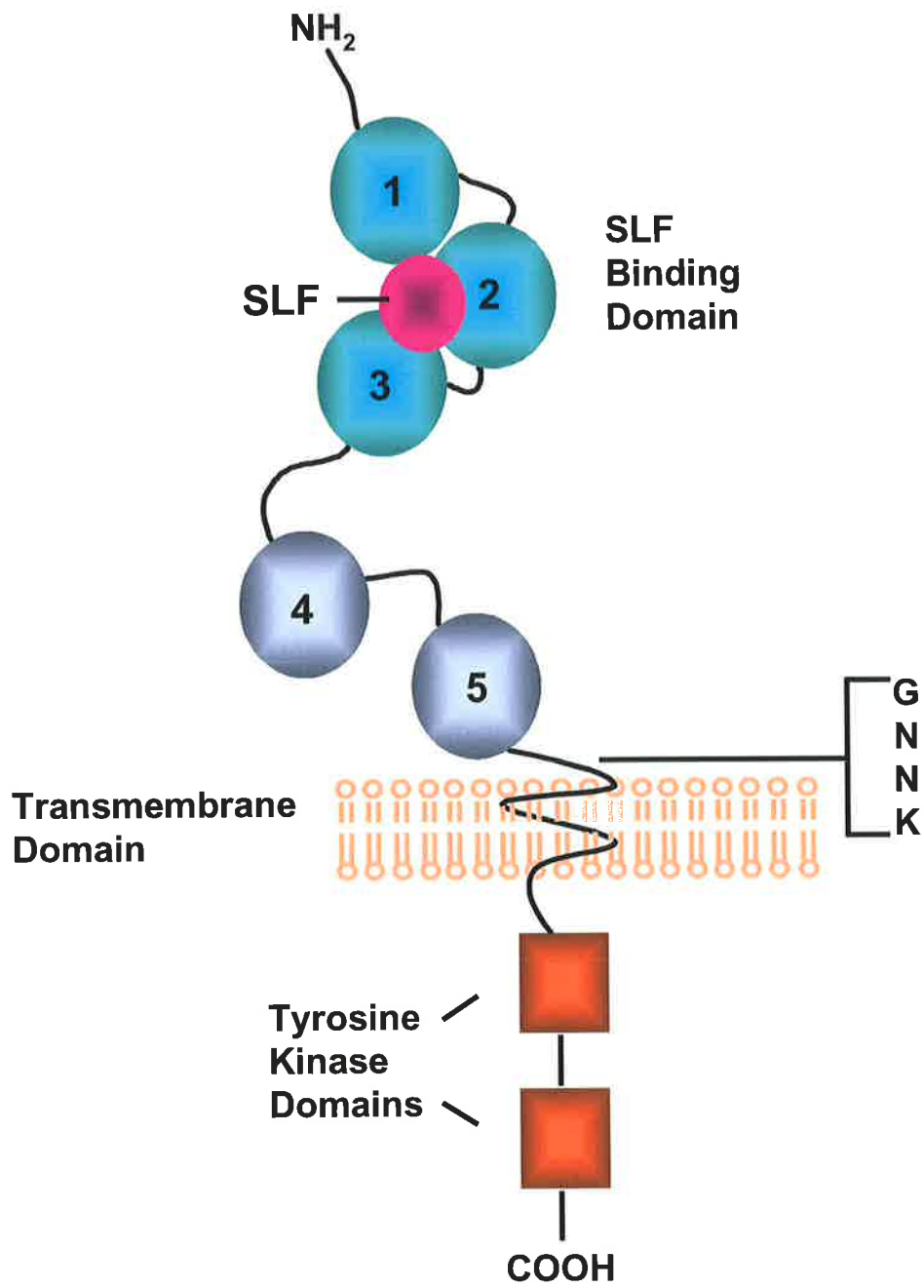
The Hardy-Zuckerman 4 feline sarcoma virus (HZ4-FeSV) was isolated from a male cat with multiple fibrosarcomas. Infectious filtrate isolated from one of the primary tumours was shown to transform feline embryo fibroblasts and mink cells (Besmer *et al.*, 1986). The virus

was replication defective and required a helper virus to generate infectious particles suggesting the retrovirus may be harbouring an oncogene that was disrupting the viral genome. A *Sac/Sal* restriction fragment from HZ4-FeSV contained sequences lacking homology with FeSV sequences. This DNA fragment was presumed to contain the disrupting oncogene that was designated *v-Kit*. Protein analysis using antisera to viral *gag* protein, which has a molecular weight of 27kDa, detected only an 80kDa band by immunoprecipitation and SDS-PAGE, suggesting the *gag* and *v-Kit* products form a fusion protein. The *v-Kit* insert was 1.1 kb in length and encoded a 370 amino acid protein that had 58% homology to *v-fms*, the viral oncogene homologue of *c-fms*, the gene encoding the tyrosine specific protein kinase colony stimulating factor-1 receptor (CSF-1R). Since *v-Kit* did not contain a transmembrane domain it was presumed to associate with the membrane via *gag*-linked myristylation. Human, mouse and cat DNA were probed with *v-Kit* to determine if *v-Kit* was derived from a cellular gene. Weak hybridisation was seen in all cases (Besmer *et al.*, 1986).

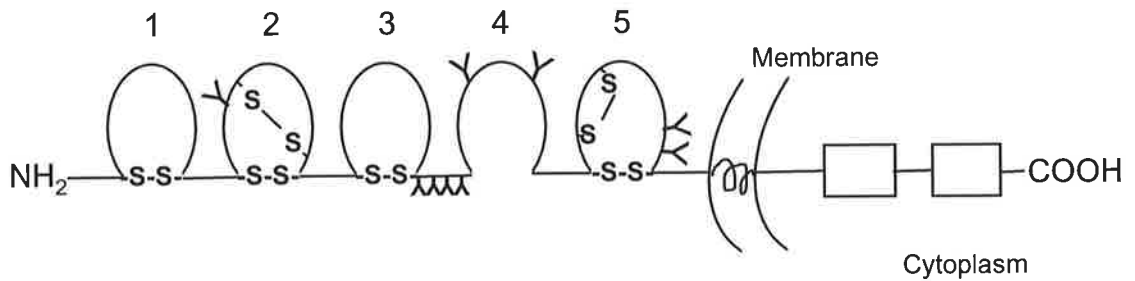
Using oligonucleotides based on the published *v-Kit* sequence, the cDNAs encoding human and mouse *c-Kit* were cloned from placental and foetal brain cDNA libraries respectively (Yarden *et al.*, 1987; Qiu *et al.*, 1988). The cDNA encoded a protein of 976 amino acids. The first 23 amino acids include a stretch of hydrophobic residues that are believed to comprise the signal sequence required for translocation of the protein to the cell surface, and therefore the predicted amino terminus of the mature c-Kit molecule would be the serine at position 24. The mature molecule is 953 amino acids in length, with a calculated molecular weight of 109740 daltons and is encoded by a 5.5kb mRNA transcript. It has a hydrophobic stretch of 23 residues that, by homology, would be predicted to encode a membrane-spanning domain, and the carboxy terminal 439 residues encompass the intracellular domain of the molecule, see Figure 1.1. The extracellular domain shows high homology with CSF-1R, α and β platelet derived growth factor receptor (PDGFR) (Yarden *et al.*, 1987; Qiu *et al.*, 1988) and the more recently described FLK2/FLT3 receptor (Matthews *et al.*, 1991), all having twelve regularly spaced cysteine residues. Comparison of the extracellular domain of c-Kit with other proteins revealed homology with the immunoglobulin superfamily, detecting five repeating units, a common feature of c-Kit, CSF-1R and PDGFR.

Figure 1.1: **A:** Functional model of c-Kit binding to SLF. The Ig-like domains of c-Kit are represented by circles and labelled 1 through 5 starting at the amino terminus (NH₂). The coiled line represents the transmembrane domain. The red boxes represent the split kinase domain. SLF (in bright pink) binds to domains 1 through 3 (in teal). The approximate position of the 4 amino acids that make up the alternate splice variant is depicted with the amino acid abbreviations GNNK. Diagram adapted from Lev *et al.* (1993). **B:** Model for the structure of c-Kit. The five immunoglobulin domains are shown as loops. Proposed intramolecular disulphide bonds are indicated by -S-S-, Y shows proposed sites for N-linked glycosylations, and the split kinase is shown as two open boxes. (Diagram adapted from Majumder *et al.* (1988).

A



B



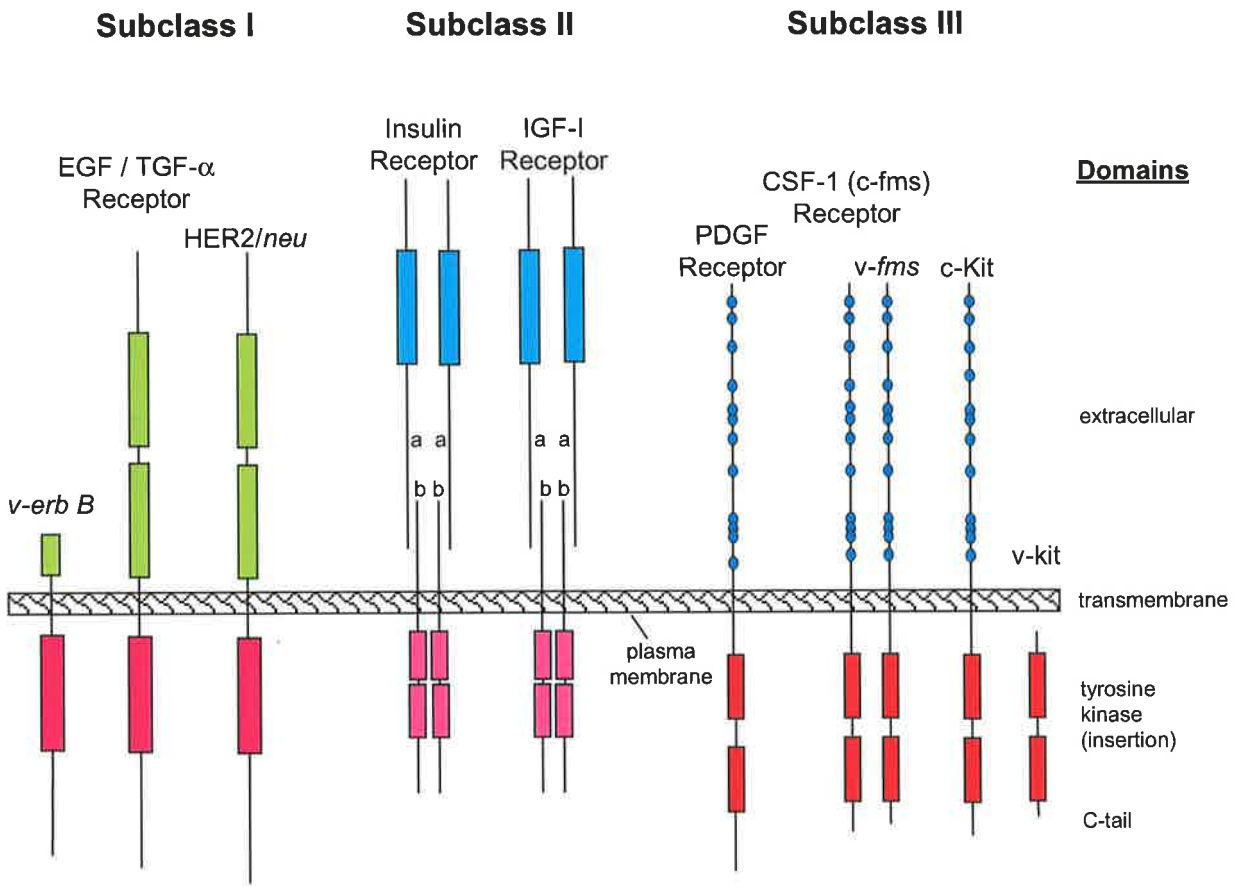
Four of the five domains contain pairs of cysteines that presumably form intramolecular disulphide bonds to secure the immunoglobulin folds. The fourth domain of each of these receptors lacks these cysteines. Four additional cysteines may provide extra disulphide bonds to stabilise domains two and five (Majumder *et al.*, 1988), see Figure 1.1(B). Polyclonal antisera to c-Kit detected a 145000 dalton protein, suggesting post-translational glycosylation of the 9 potential N-linked glycosylation sites (Yarden *et al.*, 1987). The structural similarity of c-Kit, CSF-1, PDGF and FLK2/FLT3 receptors, including a split tyrosine kinase domain (Qiu *et al.*, 1988), groups them into the type three tyrosine kinase receptor family. A schematic representation of the structural domains of the type three tyrosine kinases compared to EGF receptor, a type one tyrosine kinase receptor, and v-Kit is depicted in Figure 1.2.

Comparison of the full-length c-Kit protein to v-Kit revealed that v-Kit had been truncated at both the amino and carboxy termini during transduction. This resulted in the v-Kit product lacking the entire extracellular domain, the transmembrane domain, the first 17 amino acids of the intracellular domain and the last 49-50 amino acids of the intracellular domain which were replaced by five residues as a consequence of fusion with the FeSV polymerase gene (Yarden *et al.*, 1987; Qiu *et al.*, 1988). Other differences also existed and some of these have been shown to contribute to the oncogenicity of v-Kit (discussed in section 1.7).

Using a labelled cDNA probe, human *c-kit* was mapped to human chromosome 4, 4q11-4q21, and mouse chromosome 5, by *in situ* hybridisation (Yarden *et al.*, 1987; Qiu *et al.*, 1988). Some hybridisation to human chromosome 5q23-34 was also seen and this was presumed to be binding to the homologous genes encoding PDGFR and CSF-1R, which reside in this area.

Both the human and mouse *c-kit* genomes span over 80 kilobases and the coding sequence is distributed over 21 exons, ranging in size from >88bp to ~2.3kbp (André *et al.*, 1992; Giebel *et al.*, 1992; Gokkel *et al.*, 1992; Vandenbark *et al.*, 1992). There is a high degree of structural homology between the *c-kit* and *c-fms* genes suggesting that they arose from a common ancestral gene (André *et al.*, 1992).

Figure 1.2: Subclasses and main structural features of the family of receptor tyrosine kinases. Members and their viral counterparts are schematically shown. Light green or light blue regions represent cysteine-rich repeat domains, and red or pink boxes represent the tyrosine kinase domains. Blue circles represent individual cysteine residues in the extracellular portions of subclass III RTKs. (Diagram adapted from Yarden and Ulrich, 1988).



1.2.1 Isoforms

Exon nine has been shown by several groups (Hayashi *et al.*, 1991; Reith *et al.*, 1991; Gokkel *et al.*, 1992; Vandenbark *et al.*, 1992) to be subject to alternative splicing. The use of alternative 5' donor and 3' splice acceptor sites results in two transcripts in both human and mouse tissue and cell lines. These two forms differ by an in-frame insertion/deletion of 12 base pairs that encode the four amino acids Gly-Asn-Asn-Lys (GNNK, codons 510-513) in the extracellular domain of the receptor just prior to the transmembrane domain, see Figure 1.1 and Figure 1.3. The longer form corresponded with that published by Yarden *et al.* (1987), but the mouse sequence published by Qiu *et al.* (1988) represented the shorter form of the two alternate transcripts. Both isoforms are co-expressed in a number of cell types and tissues including mast cells, placenta, brain, lung, foetal liver, ovary, testis, glioblastoma and erythroleukaemic cell lines, normal bone marrow cells, leukaemic cell lines and AML cells (Reith *et al.*, 1991; Giebel *et al.*, 1992; Crosier *et al.*, 1993; Piao *et al.*, 1994, Zhu *et al.*, 1994). The isoform lacking the four amino acids is more abundant in all tissues examined.

The alternate usage of a second splice acceptor site in human exon 15 results in the production of two other isoforms (Crosier *et al.*, 1993). This results in the inclusion or exclusion of 3 base pairs encoding a serine at position 715. This does not occur in the mouse as only one of the 3' acceptor sites is present and all murine c-Kit lack this serine residue. Analysis of several human cell lines and leukaemic samples reveal that both isoforms are co-expressed in normal bone marrow cells, leukaemic cell lines and AML cells, with a predominance of the serine plus isoform (Crosier *et al.*, 1993).

The function of the different isoforms is not understood. To begin to address the function of the isoforms Caruana *et al.* (1999) stably expressed the human GNNK+ and GNNK- isoforms separately in NIH3T3 cells at comparable physiological levels. The GNNK- isoform was more strongly transforming than the GNNK+ isoform. While both induced anchorage independence equally well, the GNNK+ isoform was not able to overcome contact inhibition and did not induce tumour formation in nude mice. To further examine the differences between the isoforms, molecules known to be downstream of the c-Kit signalling cascade were examined. Both exhibited similar affinities for ligand, steel factor (SLF) as had been shown by others (Reith *et al.*, 1991; Williams *et al.*, 1992). A marked difference was seen in

Figure 1.3: Diagrammatic representation of two c-Kit isoforms arising from alternate mRNA splicing, resulting in the addition or deletion of 12 base pairs coding the amino acids glycine, asparagine, asparagine and lysine.



GCA TTT AAA GGT AAC AAC AAA GAG CAA

GNNK+ isoform Ala Phe Lys Gly Asn Asn Lys Glu Gln

G N N K

GNNK- isoform GCA TTT AAA G AG CAA

receptor phosphorylation and internalisation. GNNK- phosphorylation occurred rapidly, peaking at 2-3 mins after SLF addition, followed by down-regulation and internalisation of the receptor whereas the GNNK+ isoform displayed peak phosphorylation at approximately 7.5 mins after SLF addition and was not down regulated or internalised after 20 mins. The amount of phosphorylation was also vastly different with the GNNK- isoform phosphorylated seven fold higher than the GNNK+ isoform (Caruana *et al.*, 1999). Both receptors recruited PI3'K equally well and PI3'K was similarly activated as measured by phosphorylation of its down stream target c-Akt (Reith *et al.*, 1991; Williams *et al.*, 1992; Caruana *et al.*, 1999). The phosphorylation of mitogen-activated protein (MAP) kinase was much stronger in cells expressing the GNNK- isoform (Caruana *et al.*, 1999). The differences in receptor phosphorylation, down regulation and activation of MAP kinase may explain the difference in transforming ability. GNNK- isoform was shown to have low level constitutive receptor autophosphorylation and associate with PI3'K and PLC- γ 1 in the absence of ligand (Reith *et al.*, 1991; Williams *et al.*, 1992) but this was not seen by Caruana *et al.* (1999) and maybe due to a difference in the level of cell surface receptor expression as the earlier workers used transient overexpression systems. The biological roles of the GNNK and Ser isoforms remain unclear. Conservation of the GNNK isoforms between mouse and human and differing signal transduction properties suggest a biological role for the two isoforms. Further analysis of the kinetics of the many proteins known to associate with c-Kit, including phosphatases such as SHP-1 (Yi and Ihle, 1993) and other downstream signalling molecules may provide further insight into their functional relevance.

1.2.2 c-Kit and *W* allelism

Mutations at the dominant white spotting, *W*, locus, located on mouse chromosome 5, result in pleiotropic developmental defects during both embryological development and in the blood forming system of adult life (Russell, 1979; Silvers, 1979). In haemopoiesis, during early development and in adult animals, *W* mutations affect the stem cell compartment, cell populations of the erythroid lineage and mast cells (McCulloch *et al.*, 1964; Gregory and Eaves, 1978; Kitamura and Go, 1978). Most *W* alleles have been identified by their ability to confer a dominant negative phenotype on the melanocyte lineage. The *W* alleles vary in the

overall severity of the phenotype they confer. The most severe mutations produce homozygotes characterised by a partial or complete lack of hair pigmentation, a severe macrocytic anaemia that can lead to death late in gestation or perinatally, and mast cell deficiency. *W* heterozygotes have defects in these areas to varying degrees. The stem cells involved in haemopoiesis, melanogenesis and gametogenesis affected by *W* mutations have an inability to proliferate, migrate and/or survive during embryogenesis.

The molecular basis of the developmental defects associated with *W* mutations became clearer from the work of both Chabot *et al.* (1988) and Geissler *et al.* (1988), when they showed extremely close linkage of both *W* and *c-kit* loci, as the deletion mutant, *W*^{19H}, also showed absence of *c-kit* (Chabot *et al.*, 1988) and rearrangements of the *c-kit* gene in *W*⁴⁴ mutants resulted in decreased *c-kit* mRNA levels (Geissler *et al.*, 1988). In addition, lack of *c-kit* RNA in *W/W* embryo liver compared to that of liver from *W/+* and *+/+* embryos where high expression was detected also indicated a linkage between *W* mutations and expression of *c-kit* (Nocka *et al.*, 1989). The defect exerted by *W* mutations is intrinsic to the haemopoietic stem cells; i.e. the macrocytic anaemia in *W* mice can be cured by syngeneic bone marrow transplantation (Russell, 1970). In agreement with the intrinsic nature of *W* mutations, *c-kit* expression was detected in cellular targets that are affected by those mutations (Nocka *et al.*, 1989). Conversely, *c-kit* expression is not detected in those cell types not affected by *W* mutations (Nocka *et al.*, 1989).

Many *W* mutant mice have been described thus far and these mice display defects in the proliferation and/or migration of primordial germ cells during gametogenesis affecting fertility, and melanoblasts during melanogenesis resulting in lack of hair pigmentation or "white-spotting". Defects in the haemopoietic system of these mice have also been reported manifesting as macrocytic anaemia and mast cell deficiencies (Russell, 1979). The molecular basis for many of the *W* mutations has been elucidated. Mutations at the *c-kit* locus including *W*^Y, *W*³⁷, *W*⁴¹, *W*⁴², *W*⁵⁵ contain missense mutations resulting in single amino acid substitutions in or adjacent to the tyrosine kinase domain of the receptor. These mutations result in reduction or loss of function as detected by kinase activity *in vitro*. The original *W* mutation contains a single base substitution (GT-AT) at the 5' donor site that encodes the transmembrane domain. This results in improper mRNA processing causing a 234 nucleotide

deletion which includes the exon encoding the transmembrane domain and the N-terminal amino acids of the kinase domain, abolishing kinase activity and affecting subcellular localisation (Nocka *et al.*, 1990a; Hayashi *et al.*, 1991). However, in brain cells and mast cells from these animals there are two patterns of splicing, one that results in the deletion of the transmembrane domain and one that results in the deletion of the transmembrane domain as well as the next exon (Hayashi *et al.*, 1991). Thus, there appears to be tissue specificity of splicing.

Blume-Jensen *et al.* (1993) noted that different *W* mutants exert different degrees of penetrance. The severity of the *W* phenotype is dependent upon the nature of the mutation in the *c-kit* gene and whether the animal is homozygous or heterozygous for the mutation. For example, homozygous *W* animals die perinatally from severe macrocytic anaemia however heterozygous animals are viable, have normal blood parameters, are fertile but have pigmentation defects. Mutations that abolish kinase activity due to deletion (*W* and *W^{9H}*) (Chabot *et al.*, 1988; Hayashi *et al.*, 1991) or point mutation (*W³⁷* and *W⁴²*) result in loss of function mutations or "null" mutations (Nocka *et al.*, 1990a; Reith *et al.*, 1990; Tan *et al.*, 1990) and are lethal in the homozygous state. In contrast, mutations that have residual kinase activity are viable as homozygotes (Nocka *et al.*, 1990a; Reith *et al.*, 1990). The *W^V*, *W⁴¹* and *W⁴²* mutations that comprise distinct mutations in the kinase domain of c-Kit cause severe effects in the heterozygous state. This is due to expression of inactive receptor on the cell surface that is still capable of dimerisation (see section 1.5.2) with active receptor, expressed from the normal allele, upon ligand binding. This results in an inactive receptor complex unable to undergo receptor trans-autophosphorylation and initiate the signalling cascade. Mutations that result in no (*W*) or less (*W³⁷*) c-Kit expression on the cell surface are less severe in the heterozygous state (Nocka *et al.*, 1990a). Although there is less receptor on the surface, the receptor present is able to function normally. The severity of *W^V* and *W⁴¹* mutations are less than that of *W* and *W³⁷* due to production of partially active c-Kit in *W^V* and *W⁴¹* animals. *W⁴⁴* and *W⁵⁷* mutations produce c-Kit protein with reduced kinase activity that is associated with reduced levels of mRNA and protein being expressed in the mice. In *W⁴⁴* mice this was due to a rearrangement within the *c-kit* gene (Geissler *et al.*, 1988; Reith *et al.*, 1990). The phenotype displayed by mice carrying the *W^{sh}* mutation is also due to gene

rearrangement. This mutation arises from an inversion of the 5' untranslated region of the *c-Kit* gene (Duttlinger *et al.*, 1995). This inversion affects positive and negative regulatory elements of c-Kit, altering c-Kit expression in a tissue specific manner. c-Kit expression is down-regulated in mast cells, resulting in a lack of tissue mast cells but is up-regulated in somatic dermatomes during melanogenesis. The inappropriate expression of c-Kit during melanogenesis probably accounts for the lack of coat pigmentation (Duttlinger *et al.*, 1995).

A phenotype similar to that caused by *W* mutations in mice is seen in humans. These piebald patients are characterised by autosomal dominant inheritance, white hair forelock and stable areas of hypopigmentation on the chest and extremities. Unlike *W* mutant mice, piebald patients are fertile and do not suffer from defects in the haemopoietic system. Piebaldism appeared linked to mutations on chromosome 4q11-4q12, co-localising with the *c-kit* gene. Confirmation that piebaldism was due to aberrant c-Kit function was shown by detection of a hemizygous deletion that included *c-kit* and *PDGFR- α* genes (Fleischman *et al.*, 1991). Additional proof was provided by other reports that showed different point mutations in different piebald families, causing missense amino acid substitutions in the kinase domain or truncation of c-Kit (Giebel and Spritz, 1991; Fleischman, 1992; Spritz *et al.*, 1992; Spritz *et al.*, 1993; Ward *et al.*, 1995). These mutations mapped to positions similar to those mutated in *W* mice (Fleischman, 1992; Spritz *et al.*, 1992). These studies examined the c-Kit sequence but the activity of the mutant receptors was not investigated. No haematological abnormalities were detected in piebald patients (Spritz, 1992).

Patients with Diamond-Blackfan anaemia have a similar phenotype to *W* mice and piebaldism. Studies evaluating the possibility that these patients also have mutations in the *c-Kit* gene fail to show any abnormalities in the structure or expression of c-Kit (Abkowitz *et al.*, 1992; Drachtman *et al.*, 1992).

1.3 Locating the ligand

Mutations at a separate locus, Steel (*Sl*), on mouse chromosome 10, result in similar phenotypes to the *W* series of mutants, characterised by a reduction of pluripotent haemopoietic stem cells, anaemia, lack of mast cells, defects in gametogenesis and pigmentation. Like *W* mutations there are several *Sl* mutations. Homozygote *Sl* animals

(*Sl/Sl*) die *in utero* while heterozygotes carrying the mutant allele *Sl^d* (*Sl/Sl^d* mice) survive to adulthood, although they are severely anaemic. In contrast to *W* mutations, *Sl* does not affect stem cell function but causes a microenvironmental aberration resulting in an inability of the microenvironment to support the growth of primitive stem cells (Russell, 1979; Silvers, 1979). These conclusions were drawn from transplantation experiments in which normal (+/+) bone marrow, as a source of haemopoietic stem cells, could cure the macrocytic anaemia of unirradiated *W/W^v* mice and also restore the production of spleen colony forming cells (CFU-S) (Russell *et al.*, 1959; Russell and Bernstein, 1968; McCulloch *et al.*, 1964). However, the injection of normal bone marrow cells into *Sl/Sl^d* mice resulted only in partial reversal of the haematologic defects. Injection of *W/W^v* mice with bone marrow from either normal or *Sl/Sl^d* mice resulted in reconstitution of the haemopoietic system (McCulloch *et al.*, 1964, Russell and Bernstein, 1968). Similarly, in long-term bone marrow cultures, *W/W^v* stromal cells were able to support bone marrow cells from *Sl/Sl^d* mice but stroma from *Sl/Sl^d* mice was unable to support the survival and proliferation of *W/W^v* bone marrow cells (Dexter and Moore, 1977). Thus the defect in *W* mice lay within the stem cell compartment while the defect in *Sl* mice appeared to be a function of the cells that constitute the bone marrow microenvironment. Based on these observations it was hypothesised that the ligand for c-Kit was encoded by the *Sl* locus (Russell, 1979; Cabot *et al.*, 1988; Geissler *et al.*, 1988).

Fibroblasts from *Sl/Sl^d* mice were unable to maintain normal mast cells in the absence of IL-3 (Levi-Schaffer *et al.*, 1986; Fujita *et al.*, 1989). Similarly, bone marrow stroma from *Sl/Sl^d* mice cannot maintain survival of IL-3 dependent mast cell lines when co-cultured, whereas stroma derived from normal mice supported the survival of two mast cell lines without addition of growth factors (Boswell *et al.*, 1990). An unknown growth factor was found to be present in the supernatant of normal cells that was not present in the supernatant of *Sl/Sl^d* cells. Flanagan and Leder (1990), hypothesising that the *Sl* locus encoded the ligand to c-Kit, showed that an extracellular domain of c-Kit fused to an alkaline phosphatase tag could bind to fibroblasts derived from normal mice. This binding was diminished on fibroblasts isolated from *Sl/Sl^d* mice suggesting that the genetic defect at the *Sl* locus affects the ligand for c-Kit in some way.

Several groups set out to identify this mystery protein. From partial protein sequences the cDNA encoding the growth factor was cloned by several groups and given many names depending on the activity the factor was presumed to have. These included: Mast Cell Growth Factor, (MGF), purified from a murine stromal cell line, LDA11 (Anderson *et al.*, 1990; Boswell *et al.*, 1990; Williams *et al.*, 1990); Stem Cell Factor, (SCF), purified from Buffalo rat liver-conditioned medium (Zsebo *et al.*, 1990a); Kit Ligand (KL), purified from Balb/c 3T3 fibroblasts (Huang *et al.*, 1990; Nocka *et al.*, 1990b) and Steel factor (SLF) (Williams *et al.*, 1992). Steel Factor or SLF will be used throughout this thesis. Interaction of SLF with c-Kit was shown by crosslinking labelled SLF to c-Kit expressing cells and subsequently immunoprecipitating the complex with antisera that recognise the C-terminus of the *c-kit* gene product (Williams *et al.*, 1990; Huang *et al.*, 1990). The ability of cells to bind and respond to SLF was correlated with *c-kit* mRNA expression (Williams *et al.*, 1990). For example, SLF does not bind to the membranes of cells that do not express c-Kit including the murine factor dependent haemopoietic cell line, 32D, and cells from *W/W* mice. However, SLF does bind to MC-6 cell line, *W/W^f* cells and cells isolated from normal mice, which express c-Kit (Williams *et al.*, 1990; Huang *et al.*, 1990). SLF is capable of binding to COS cells transfected with an expression vector containing *c-kit* but cannot bind to untransfected cells (Zsebo *et al.*, 1990b). The gene encoding for SLF has been mapped to the *Sl* locus of mouse chromosome 10 using somatic cell hybrids, *Sl* deletions and recombination analysis (Zsebo *et al.*, 1990b; Huang *et al.*, 1990) and to human chromosome 12q22-12q24 (Anderson *et al.*, 1991; Geissler *et al.*, 1991; Mathew *et al.*, 1992). DNA probes for *SLF* failed to bind to DNA isolated from *Sl/Sl* cell lines (Zsebo *et al.*, 1990b). The injection of soluble SLF into *Sl/Sl^d* mice resulted in a reduction of the severity of symptoms and an increase circulating numbers of mast cells and erythrocytes (Zsebo *et al.*, 1990b; Flanagan *et al.*, 1991). However, bone marrow from *W/W^f* mice failed to proliferate in response to soluble SLF (Zsebo *et al.*, 1990b). These results support the idea that the *W* and *Sl* mutations are complimentary and that the *Sl* locus codes for a ligand that binds to the *c-kit* gene product.

The cDNA encoding SLF has been sequenced (Anderson *et al.*, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990b) and two mRNA transcripts have been isolated (Flanagan *et al.*, 1991; Huang *et al.*, 1992). The longer of the two encodes

a larger protein of 248 amino acids (SLF²⁴⁸). Although SLF was originally isolated as a soluble molecule, SLF²⁴⁸ is a transmembrane protein comprising a secretion signal peptide of 25 amino acids, a 189 amino acid extracellular domain, a hydrophobic transmembrane domain and a short 36-37 amino acid cytoplasmic tail (Anderson *et al.*, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990b). The second transcript encodes a smaller protein, termed SLF²²⁰. Exon 6 has been spliced out removing 28 amino acids 5' to the transmembrane domain, (Anderson *et al.*, 1990; Flanagan *et al.*, 1991). Within this exon is the recognition site, between amino acids 149 and 177, for a serine protease involved in cleavage, at the cell surface, of SLF²⁴⁸ and releasing a 164-165 amino acid soluble SLF protein (Martin *et al.*, 1990; Nocka *et al.*, 1990b; Williams *et al.*, 1990; Zsebo *et al.*, 1990a; Lu *et al.*, 1991; Huang *et al.*, 1992; Pandiella *et al.*, 1992). This is similar to the production of soluble CSF-1 (Kawasaki *et al.*, 1985; Rettenmeir and Roussel, 1988).

Soluble SLF molecule has the ability to bind to c-Kit expressing cells and induce proliferation. Injection of soluble SLF also reduces the severity of macrocytic anaemia in *Sl/Sl^d* mice (Zsebo *et al.*, 1990b). However, there is evidence that soluble SLF does not fulfil all of the functions of the membrane bound form and that signalling by the two isoforms of SLF through c-Kit appears to be different (see section 1.3.2). In mice, proteolytic cleavage of the SLF²²⁰ form also occurs, although at reduced efficiency (Huang *et al.*, 1992; Pandiella *et al.*, 1992; Majumdar *et al.*, 1994). This is through the usage of a second cleavage site encoded by exon 7, which is present in both SLF²²⁰ and SLF²⁴⁸ isoforms. The human sequence does not contain this second protease site and therefore the SLF²²⁰ form remains associated with the membrane (Majumdar *et al.*, 1994). Proteolytic cleavage of SLF²⁴⁸ is enhanced by phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C (PKC), and this cleavage can be blocked by treating the cells with an inhibitor of PKC activity (Huang *et al.*, 1992). This suggests that protein kinase C may activate the protease.

Soluble SLF has been detected in the serum of normal individuals at an average of 3.3ng/ml (Langley *et al.*, 1993). The partially purified molecule had a deglycosylated molecular weight of 18000 daltons corresponding to the predicted 165 amino acids of the soluble form. Since mRNA encoding the longer form of SLF is found in all tissues expressing

SLF (Huang *et al.*, 1992), all of these sites probably contribute to the high SLF levels found in human serum.

Recombinant *E. coli* and Chinese Hamster Ovary (CHO) cell derived SLF has been found as non-covalently associated dimers at a protein concentration of 400µg/ml (Arakawa *et al.*, 1991; Lu *et al.*, 1991). Similarly, the FLK2 receptor ligand is found in a non-covalently associated form but CSF-1 and PDGF are found in dimers that are disulphide linked (Das and Stanley, 1982; Johnson *et al.*, 1982). Since ligand dimerisation is assumed to mediate the dimerisation and therefore activation of their respective receptors (see section 1.5.2) it was not unlikely that the SLF found in serum would also exist as a dimer. However, it has recently been demonstrated, using sedimentation equilibrium and size exclusion chromatography, that the dimerisation association constant (K_a) for recombinant *E. coli* derived SLF is $2-4 \times 10^{-8} M$. Based on this dimerisation constant, more than 90% of the circulating SLF would be in the monomeric form (Hsu *et al.*, 1997). Unlike serum derived SLF, SLF produced in *E. coli* is not glycosylated. Glycosylation of asparagine residues in SLF has been shown to interfere with SLF dimerisation (Zhang *et al.*, 2000). SLF mutants containing amino acids changes at the presumed dimer interface have substantially reduced mitogenic activity, whereas a mutant resulting in covalent dimerisation of SLF had a 10 fold-increased activity. These data suggests that although SLF is predominantly a monomer in serum, dimerisation of the ligand is required to mediate receptor dimerisation and subsequent signal transduction (Hsu *et al.*, 1997).

1.3.1 Mutations at *Sl* locus

Mutations at the *Sl* locus that result from deletions of the entire SLF gene cause the most severe phenotypes, usually death *in utero* or shortly after birth. Such loss of function alleles include the original *Sl* mutation (Sarvella and Russell, 1956), as well as Sl^j , Sl^{gh} , Sl^{8H} , Sl^{10H} , Sl^{12H} and Sl^{18H} (Russell, 1979; Silvers, 1979; Copeland *et al.*, 1990; Huang *et al.*, 1990; Zsebo *et al.*, 1990b). The degree of SLF alteration matches the severity of the phenotype. Viable alleles of Sl^{17H} and Sl^{pan} show no structural rearrangements of the *slf* gene. Mice homozygous for the Steel-Dickie (Sl^d) allele are viable, with severe macrocytic anaemia, lack of skin pigmentation, mast cell deficiency and sterility (Bernstein, 1960; Kitamura and Go, 1979; Russell, 1979; Silvers, 1979). These mice have been shown to carry unique *slf* DNA

restriction fragment length polymorphisms which result in a shorter transcript (Zsebo *et al.*, 1990b) and this is due to a 4kb genomic deletion (Brannan *et al.*, 1991). The truncated transcript codes for most of the extracellular domain but lacks sequences encoding the transmembrane domain and the cytoplasmic tail (Brannan *et al.*, 1991; Flanagan *et al.*, 1991; Huang *et al.*, 1992). Expression of this transcript in COS cells results in the production of soluble SLF that is capable of inducing mast cell proliferation (Flanagan *et al.*, 1991). The phenotype of the *Sl/Sl^d* allele is almost as severe as that of the mutant *Sl/Sl* allele, which has the entire SLF gene deleted. In contrast to the weak *W/+* phenotype, heterozygous *Sl/+* mice have moderate macrocytic anaemia, pigmentation defects including a ventral spot and their gonads are reduced in size. Thus a 50% reduction in expression of SLF is limiting and the decreased SLF levels are not sufficient for normal c-Kit stimulation. In contrast, 50% gene dosage of *c-kit* is not limiting in most situations (Huang *et al.*, 1990).

Oddly, some of the mutations at the *Sl* locus cause mild phenotypes including mild anaemia, some wild-type coat pigmentation but cause sterility in mice of one sex. *Sl^{pan}*, *Sl^t* and *Sl^{com}* produce female sterility in the homozygous state (Beechey and Searle, 1983, 1985; Kuroda *et al.*, 1988) whereas *Sl^{17H}* produces male sterility in the homozygous state (Peters *et al.*, 1987). SLF is overexpressed in the testis of animals affected by the *Sl^{17H}* mutation and is associated with a reduction in germ cell numbers (Brannan *et al.*, 1992). A point mutation immediately upstream of the 5' boundary of exon 8 in *Sl^{17H}* results in a defect in the splicing mechanism (Brannan *et al.*, 1992). Exon 8 is completely missing resulting in the splicing of exon seven directly onto exon 9. Only the first amino acid of the cytoplasmic tail is present plus 27 unrelated amino acids translated from the resultant frame shift. It has been shown that mice carrying this mutation have greatly reduced melanocyte precursor numbers, explaining the lack of coat pigmentation, and haemopoietic abnormalities including red blood cell deficiency, bone marrow hypoplasia and defective thymopoiesis. *In vitro*, both soluble and membrane associated SLF from *Sl^{17H}* mice exhibit reduced cell surface expression on stromal cells and reduced biological activity. This resulted in decreased erythroid progenitor cell line proliferation and activation of c-Kit (Kapur *et al.*, 1999). Wildtype forms of SLF are localised to and are secreted from the basolateral compartment of epithelial tissues. However, SLF from *Sl^{17H}* mice localises to and is secreted from the apical compartment (Wehrle-Haller and

Weston, 1999). Therefore, loss of melanocytes and germ cells maybe explained by the inability of Sl^{17H} mutant SLF to be targeted to the basolateral compartment of polarised epithelial keratinocytes and sertoli cells (Wehrle-Haller and Weston, 1999). Additionally, the lack of haemopoietic cells may be due to inappropriate localisation of SLF by bone marrow stromal cells. Expression of cDNA encoding Sl^{17H} or several cytoplasmic domain truncated forms of SLF in COS cells demonstrated the importance of the cytoplasmic tail for correct processing and passage of SLF through the endoplasmic reticulum and Golgi complex and expression on the cell surface. Deletion of the cytoplasmic domain did not affect SLF dimerisation on the cell surface but dimerisation of the Sl^{17H} -SLF form was greatly diminished, probably due to an alteration of the tertiary structure of SLF by the unrelated amino acids (Tajima *et al.*, 1998). In addition, cultures from Sl^{17H}/Sl^{17H} bone marrow were not able to support long-term production of haemopoietic cells and transplantation of normal bone marrow into irradiated Sl^{17H}/Sl^{17H} mice resulted in decreased lodgement/homing of CFU-S progenitors to the spleen. The SLF encoded by the Sl^{17H} allele supports mast cell proliferation similar to that of wild type SLF (Brannan *et al.*, 1992) but deletion or rearrangement of the cytoplasmic tail reduced the adhesion of bone marrow mast cells to the SLF expressing COS cells (Tajima *et al.*, 1998). Furthermore, skin and peritoneal mast cells are decreased by 45% and 85% respectively in Sl^{17H} mice (Tajima *et al.*, 1998). These data suggest the cytoplasmic domain is important in SLF biological activity but is not involved in signalling.

1.3.2 Soluble versus membrane bound SLF

Since most cytokines and growth factors are found in a soluble form only, the question is raised as to why SLF is produced in both a membrane bound and soluble form. A membrane bound SLF may prevent diffusion of the growth factor, ensuring proliferation and differentiation of cells in the correct microenvironment only, may control cellular migration during development, act as an adhesion molecule and allow spatial organisation not easily achieved with soluble factors (Flanagan *et al.*, 1991). Since SLF synergises with other haemopoietic growth factors, membrane bound SLF may ensure proliferation and differentiation signals are transmitted to the intended cells via the induction of cell-cell

contact. In the haemopoietic system this may provide another level of control of cell proliferation and differentiation.

W and *Sl* mutations affect cell types that undergo migration during normal development including germ cells migrating from yolk sac to genital ridges, haemopoietic cells from the yolk sac to foetal liver and then the spleen and bone marrow and melanocytes from the neural crest to skin and hair follicles. The defects in *W* and *Sl* mutant animals become apparent at about the time these cells would normally migrate, so it is possible that the membrane bound SLF-c-Kit interaction is important in the guiding process of migration and homing (Russell, 1979; Silvers, 1979; Flanagan *et al.*, 1991). Indeed, mRNA for SLF is expressed in tissues associated with migratory pathways and homing sites of melanoblasts, germ cells and haemopoietic stem cells (Matsui *et al.*, 1990). *Sl/Sl^d* mutant mice have demonstrated that membrane bound SLF fulfils a role in normal development that cannot be compensated for by soluble SLF. Fibroblasts from *Sl/Sl^d* mice were unable to support the proliferation of IL-3 dependent mast cells in the absence of IL-3 but fibroblast from normal cells could (Fujita *et al.*, 1989). In addition, cell lines expressing the membrane bound isoform supported the long-term production of haemopoietic progenitors from CD34⁺ enriched bone marrow *in vitro* however cells expressing only the soluble form of SLF maintained progenitors only transiently (Toksoz *et al.*, 1992). Soluble SLF was not limiting since the addition of SLF increased colony number initially but did not increase the duration of progenitor maintenance (Toksoz *et al.*, 1992). Co-culture of the factor dependent cell line FDC-P1 with fibroblasts from wild type, *Sl/Sl* or *Sl/Sl^d* mice also showed the importance of membrane bound SLF. *Sl/Sl^d* fibroblasts failed to support FDC-P1 proliferation although they produce the same amount of SLF mRNA as wildtype cells (Caruana *et al.*, 1993). In addition, separation of wildtype fibroblasts from FDC-P1 cells by an agar interlayer prevented GM-CSF-independent growth of the FDC-P1 cells (Caruana *et al.*, 1993). Similar results were observed with megakaryocytes. Incubation of c-Kit expressing megakaryocytes with membrane bound SLF expressing fibroblasts resulted in proliferation of the megakaryocytes and their adhesion to the fibroblasts (Avraham *et al.*, 1992). The adhesion could be blocked by the addition of soluble SLF. Neither effect was seen with fibroblasts isolated from *Sl/Sl* mice. Together these different sets of data imply soluble SLF expressed from the *Sl/Sl^d* allele is not adequate for

normal biological function. However, injection of SLF at 30 μ g/kg/day into *Sl/Sl^d* mice resulted in improvement of their macrocytic anaemia and mast cell deficiency (Zsebo *et al.*, 1990b). Thus, large amounts of soluble SLF can at least in part compensate for the lack of endogenously produced membrane bound SLF, implying the amount of soluble SLF produced by *Sl/Sl^d* cells may be limiting in some experimental systems described above. However, expression of either soluble or membrane bound SLF in transgenic *Sl/Sl^d* mutant mice provides further evidence of specific roles for each form of SLF. The membrane bound form but not the soluble form, partially corrected the anaemia, bone marrow hypocellularity and runting. In contrast, only the soluble form restored myeloid progenitor cell numbers (Kapur *et al.*, 1998).

In addition to the differences in spatial presentation discussed above, soluble and membrane bound SLF may differ in the characteristics of c-Kit signalling they induce. If down-regulation of c-Kit requires internalisation of the receptor-ligand complex, turnover of c-Kit and consequently the duration of signalling after stimulation with membrane bound SLF maybe completely different to that induced by soluble SLF. There is evidence that this is so. The binding of soluble SLF to c-Kit expressing cells initiates a signalling cascade that includes receptor phosphorylation and then internalisation, polyubiquitination and degradation, occurring within 15 minutes of ligand binding (Miyazawa *et al.*, 1994). However, the tyrosine kinase activity persists for a longer period of time when c-Kit expressing cells interact with membrane associated SLF as compared to soluble SLF. The activated c-Kit remains on the cell surface for a longer period of time and would be expected to continue to produce efficient signals for cell proliferation and differentiation (Miyazawa *et al.*, 1995). Similar responses were seen with immobilised anti-c-Kit antibody, which prevents internalisation of the receptor whereas soluble antibody induced internalisation (Kurosawa *et al.*, 1996). However, the biological significance is yet to be determined since the authors did not observe a difference in the proliferation rate of c-Kit expressing M07e cells in response to either soluble or membrane bound SLF.

Similarly, membrane bound and soluble SLF have different effects in melanogenesis. Soluble SLF is required to promote the dispersal, on the lateral pathway, of c-Kit positive melanocyte precursors or to attract them to a local source, but the membrane bound form of

SLF is required for their survival upon arrival at the dermis (Wehrle-Haller and Weston, 1995).

From the experimental evidence discussed it appears that soluble and membrane SLF can promote different cellular outcomes. The difference in cellular response may correlate with the length of time the c-Kit is activated. The signal to induce a cell to move toward a chemoattractant may only require short activation of the receptor and signalling cascade consistent with the action of soluble SLF. However, for cellular processes including survival, proliferation and differentiation, prolonged stimulation of the c-Kit and secondary signalling complexes is required. This can be achieved with a vast excess of exogenously added soluble SLF that may not be achievable *in vivo* or by continued stimulation via membrane associated SLF which decreases the down regulation of the c-Kit and therefore lengthens the time of signalling.

The adhesion of c-Kit expressing cells to cells expressing membrane bound SLF may not be only due to the c-Kit-SLF interaction. The stimulation of c-Kit expressing cells with SLF induces transient adhesion of mast cells, normal CD34⁺ bone marrow cells and cell lines (M07e and TF-1) to fibronectin via the integrin VLA-5 and to some extent VLA-4 (Dastyk and Metcalfe, 1994; Kinashi and Springer, 1994; Lévesque *et al.*, 1995). The level of SLF required to induce the adhesion was less than that required to cause proliferation and these levels matched those required to act as a chemoattractant for mast cells (Meininger *et al.*, 1992). This process requires tyrosine kinase activity since mast cells from *W/W^v* mice failed to bind to fibronectin upon stimulation with SLF but could still bind to COS cells transfected with membrane bound SLF (Kinashi and Springer, 1994) and the addition of genistein, a tyrosine kinase inhibitor, inhibits the adhesive response (Lévesque *et al.*, 1995). The adhesion mediated by c-Kit stimulation was transient and dose dependent. Higher concentrations of soluble SLF, greater than approximately 10-30ng/ml, or stimulation with low SLF concentrations for long periods of time, resulted in down regulation of c-Kit expression (both protein and mRNA) and a subsequent loss of adhesion (Adachi *et al.*, 1995; Lévesque *et al.*, 1995).

1.4 Expression of c-Kit and SLF

Initially c-Kit mRNA and protein expression was detected in mast cells (Mayrhofer *et al.*, 1987; Majumder *et al.*, 1988; Nocka *et al.*, 1989), melanocytes (Nocka *et al.*, 1989), testis (Majumder *et al.*, 1988; Nocka *et al.*, 1989), placenta and ovary (Nocka *et al.*, 1989), brain (Qui *et al.*, 1988; Nocka *et al.*, 1989) and the bone marrow (Wang *et al.*, 1989). Since then more sensitive techniques have detected c-Kit expression by endothelial cells (Broudy *et al.*, 1994) and the interstitial cells of Cajal, which control gut motility (Torihashi *et al.*, 1995; Huizinga *et al.*, 1995). Other cell types including astrocytes, renal tubules, breast glandular epithelial cells and sweat glands have all been shown to express c-Kit (Natali *et al.*, 1992; Lammie *et al.*, 1994). In the haemopoietic system expression of c-Kit is mostly confined to a minor subset of cells that contain haemopoietic progenitor cells and co-express the CD34 antigen (Cambareri *et al.*, 1988; Ashman *et al.*, 1991; Papayannopoulou *et al.*, 1991; Simmons *et al.*, 1994). However, c-Kit expression has also been reported on lymphoid progenitors (Simmons *et al.*, 1994), megakaryocytes, natural killer cells and activated platelets (Avraham *et al.*, 1992; Matos *et al.*, 1993; Grabarek *et al.*, 1994). Mast cells express c-Kit at high levels and are the only haemopoietic cells that do not down regulate c-Kit expression upon maturation (Mayrhofer *et al.*, 1997).

SLF is expressed widely throughout the body. Expression has been detected on stromal cells of murine foetal liver and both murine and human bone marrow (Zsebo *et al.*, 1990b; McNiece *et al.*, 1991a; Aye *et al.*, 1992). Many groups have shown the expression of SLF on fibroblasts (Anderson *et al.*, 1990; Flanagan and Leder, 1990; Nocka *et al.*, 1990b; Williams *et al.*, 1990; Zsebo *et al.*, 1990b; Heinrich *et al.*, 1993) and vascular endothelial cells (Aye *et al.*, 1992; Heinrich *et al.*, 1993). The soluble form of SLF is found in normal human serum at an average of 3.3ng/ml (Langley *et al.*, 1993).

Analysis of mRNA and protein levels in some solid tumours has shown the co-expression of c-Kit and SLF. Co-expression was detected in both small cell lung cancer and non-small cell lung cancer tumours and tumour derived cell lines (Hibi *et al.*, 1991; Krystal *et al.*, 1996; Pietsch *et al.*, 1998), in 2 of 16 female genital tract derived tumours (Inoue *et al.*, 1994), a small percentage of benign prostatic hyperplasia cases (Simak *et al.*, 2000) and 9 of 11 and 7 of 13 breast tumours and breast tumour derived cell lines respectively (Hines *et al.*, 1995).

Co-expression of c-Kit and SLF in these tumours may be responsible for their deregulated growth. However, normal duct cells of the breast have been shown to express c-Kit suggesting that expression of c-Kit in breast cancer may be normal despite the fact that analysis of the whole tissue would suggest it is not (Matsuda *et al.*, 1993). See section 1.9 for further discussion.

1.5 The importance of the c-Kit/SLF Interaction in Haemopoiesis

Mature cells of the peripheral blood are derived in a hierarchical fashion from the pluripotent stem cells of the human adult bone marrow. This stem cell has the capacity to either self-renew (i.e. produce an identical daughter cell) or differentiate into multipotential cells, colony forming unit-granulocyte, erythrocyte, monocyte and macrophage (CFU-GEMM), which in turn give rise to more lineage restricted or committed progenitor cells (CFU-GM, blast forming unit-erythrocyte (BFU-E), BFU-megakaryocyte (BFU-MK)) that are the precursors to the mature, functionally distinct cells of the blood. As discussed earlier, mutations in mice at the *W* and *Sl* loci, encoding for c-Kit and SLF respectively, demonstrate that the interaction of c-Kit and SLF is very important for normal haemopoiesis to occur. Despite the fact that *Sl/Sl* mice die *in utero*, the foetal liver of the *Sl/Sl* mouse still contains Sca-1⁺ stem cells, albeit at 30-40% of the level in normal littermates. These data indicate that the generation, survival and expansion of the most primitive stem cells does not require the interaction of SLF and c-Kit (Molineux *et al.*, 1991, Kodama *et al.*, 1992). However, SLF, G-CSF and IL-3 were required for clonogenic cell production from long-term culture-initiating cells (LTC-ICs) (Sutherland *et al.*, 1993). Injection of a functionally inhibitory anti-murine c-Kit antibody, ACK-2, into mice, resulted in the elimination of all day-8 CFU-spleen (CFU-S) but not all day-12 CFU-S (Ogawa *et al.*, 1991; Okada *et al.*, 1991). Inclusion of ACK-2 in murine bone marrow cultures with PA6 stromal cells caused a reduction of CFU-S to only 6% at day 12 but CFU-S levels returned to normal 4 and 14 days after the removal ACK-2 (Kodama *et al.*, 1992). This suggests that the haemopoietic stem cells with the highest proliferative potential are resistant to the inhibitory effects of ACK-2. These results imply that although c-Kit is expressed on very early haemopoietic stem cells, a more primitive cell exists that either does not express c-Kit or the receptor is not functional.

Indeed, LTC-IC could be maintained by stromal feeder layers isolated from *Sl/Sl* mice, in the absence of SLF (Ikuta and Weissman, 1992; Sutherland *et al.*, 1993). In addition, studies by several groups have shown the human primitive haemopoietic cells from both bone marrow and cord blood are enriched in the c-Kit^{low} or c-Kit⁻ fraction of CD34⁺CD38⁻ progenitor cells (Gunji *et al.*, 1993; Sakabe *et al.*, 1997; Sakabe *et al.*, 1998; Ramsfjell *et al.*, 1999; Xiao *et al.*, 1999). It is possible that other factors such as FLK2/FLT3 receptor and its ligand can be utilised for the maintenance of these cells (reviewed in Lyman and Jacobsen, 1998).

Culturing of progenitor cells from human bone marrow or cord blood in stromal cell dependent or stroma independent, cytokine supplemented liquid cultures has demonstrated the phenotype of the progenitor cell to give rise to the majority of mature blood cells to be CD34⁺c-Kit⁺lin⁻ and also be Rh123^{dull} (Okada *et al.*, 1991; Bernstein *et al.*, 1991; Tsujino *et al.*, 1993; Simmons *et al.*, 1994). Previous studies had shown primitive multipotential blast colony-forming cells and cells that initiate long-term haemopoiesis *in vitro* are restricted to low or undetectable expression of HLA-DR⁻ and CD33⁻CD38⁻ and lacked any lineage markers (lin⁻) (Simmons *et al.*, 1990; Udomsakdi *et al.*, 1991). Simmons *et al.* (1994) demonstrated 76% of CD34⁺ cells express c-Kit and that 98% of CFU-GM, 98% CFU-Mix and 85% BFU-E came from the CD34⁺c-Kit⁺ fraction (170 fold increase in CFU-GM) and all pre-CFU came from CD34⁺c-Kit⁺Rh123^{dull} fraction. It is likely that more cells were derived from the stromal free system because the negative regulatory signals from the stroma had been removed. Gunji and co-workers (1993) separated the c-Kit positive cells according to the level of expression and found that most CFU-GM colonies were produced by the c-Kit^{high} fraction. The blast cell like c-Kit^{low} fraction, which was also CD34⁺CD38⁻, was more primitive since it produced more colonies later in culture. They also demonstrated that the c-Kit^{high} cells were derived from c-Kit^{low} cells in the presence of SLF, IL-6 and erythropoietin (Epo).

Alone, soluble SLF has little effect on haemopoietic progenitor cells. Some observations suggest SLF is essential for maintaining stem cell viability, allowing them to proliferate and differentiate in the presence of other haemopoietic factors (Bernstein *et al.*, 1991) but other studies show that SLF alone does little (see below). However, in the presence of other growth factors SLF displays its greatest potency to induce the stem cells to produce more committed progenitor cells. A vast body of work has demonstrated that SLF synergises with IL-3 and/or

GM-CSF to produce Epo dependent CFU-GEMM colony formation, with GM-CSF to enhance CFU-GM production, with Epo to enhance the production of BFU-E and IL-3 and GM-CSF or IL-6 to produce BFU-Mk (Anderson *et al.*, 1990; Martin *et al.*, 1990; Bernstein *et al.*, 1991; Briddell *et al.*, 1991; Broxmeyer *et al.*, 1991a,b; Carow *et al.*, 1991; Dai *et al.*, 1991; McNiece *et al.*, 1991a; Metcalf and Nicola, 1991; Migliaccio *et al.*, 1991; Heyworth *et al.*, 1992; Gunji *et al.*, 1993; Tsujino *et al.*, 1993). The colony size is larger in the presence of SLF than the other factor alone and the concentration of the second factor required to produce the same number of colonies is reduced when SLF is present. However, the morphology of the colony is dictated by the second factor implying that SLF provides survival and proliferative stimuli but commitment factors such as IL-3, GM-CSF, Epo, or IL-7 determine the lineage potential of the precursor cell (McNiece *et al.*, 1991b; Metcalf and Nicola *et al.*, 1991). SLF synergises with IL-1, IL-3 or IL-6 in the generation of secondary high proliferative potential-colony forming unit (HPP-CFU) (Zsebo *et al.*, 1990a) and rat recombinant SLF synergises with 3 and 4 factor combinations of CSF-1, G-CSF, GM-CSF, IL-1 α and IL-3 to increase HPP-CFU and total colony production by Sca-1⁺lin⁻ cells (Lowry *et al.*, 1991; Williams *et al.*, 1992). The largest expansion of CFU-GM from cyclophosphamide mobilised, peripheral blood CD34⁺ cells was achieved with a combination of SLF and IL-1, IL-3, IL-6, G-CSF and GM-CSF (Haylock *et al.*, 1992) although a combination of IL-3, IL-6 and G-CSF with SLF was later shown to be just as potent (Makino *et al.*, 1997). SLF also synergises with IL-11 to maintain the survival and expansion of progenitor cells (Neben *et al.*, 1994). Surprisingly, thrombopoietin (Tpo), a primary regulator of megakaryocyte and platelet production, has been shown to synergise with SLF and FLT3 ligand to recruit 40% of single CD34⁺CD38⁻ to proliferate compared to 3% by SLF and FLT3 ligand alone (Ramsfjell *et al.*, 1997 and D.N. Haylock, personal communication) and Tpo, IL-3, IL-6 or IL-11 with SLF maximally stimulated CD34⁺ cells isolated from cord blood (Ohmizono *et al.*, 1997). Addition of IL-3, IL-6 and Epo resulted in growth of up to 80% of cells, which had multilineage differentiation potential. The optimal growth *in vitro* of mobilised progenitors cells requires at least a combination of SLF with G-CSF, GM-CSF, IL-3 and Epo (Cesana *et al.*, 1997). The authors went on to suggest that the culture of SLF dependent progenitors from

mobilised blood might be clinically relevant to assess the reconstituting potential of the autograft.

There is conflicting evidence as to whether SLF and CSF-1 are able to synergise (Broxmeyer *et al.*, 1991a; Lowry *et al.*, 1991; Metcalf and Nicola, 1991), although the synergy seen may be due to the presence of autocrine factors. Heyworth *et al.* (1992) saw synergy between the two factors forcing progenitor cells to differentiate into macrophages instead of neutrophils, again suggesting that while SLF induced cell survival and proliferation, CSF-1 determined the lineage of progenitor cell commitment.

The influence of SLF on the lymphoid lineage is less well understood. CD7⁺CD3⁻CD4⁻CD8⁻ lymphoid cell lines derived from the thymus show a synergistic proliferative response to SLF, IL-2 and IL-7. These cells can be induced to differentiate down lymphoid or non-lymphoid lineages (deCastro *et al.*, 1994). c-Kit⁺ cells were able to reconstitute the thymus of an irradiated mouse, presumably because thymic stromal cells express both IL-7 and SLF (de Vries *et al.*, 1992). Together with IL-7, SLF stimulates the proliferation pre-B cells (McNiece *et al.*, 1991b; Billips *et al.*, 1992). SLF also synergises with IL-12 to support the development of lymphopoietic progenitors (Hirayama *et al.*, 1994). Paradoxically, day 13 foetal liver cells from *W/W* mice, which do not express c-Kit, transferred to immunodeficient RAG-2(-/-) mice, gave rise to all stages of immature B cells in the bone marrow and mature B cells in the peripheral blood (Takeda *et al.*, 1997). In addition, while myeloid and erythroid progenitors were eliminated in mice treated with a blocking anti-c-Kit monoclonal antibody ACK2, 90% of the bone marrow cells were B220⁺ B-lineage cells (Ogawa *et al.*, 1991). Thus, c-Kit seems to be non-essential for B cell development.

Administration of SLF and G-CSF to mice resulted in synergistic myeloid hyperplasia in the bone marrow and spleen with increased numbers of circulating neutrophils and lymphocytes, which was most apparent 4-6 days after treatment (Ulich *et al.*, 1991). Treatment with SLF alone resulted in mast cell hyperplasia in both the bone marrow and spleen. These observations were better explained by Andrews *et al.* (1992) and Fleming *et al.* (1993) when baboons and mice respectively were treated with SLF. They too observed an increase in multiple cell lineages in both the bone marrow and peripheral blood including CFU-GM, BFU-E, CFU-Mix and HPP-CFC. Interestingly, they also noted an increase in the

number of CD34⁺ and Sca-1⁺ cells in the peripheral blood of baboons and mice respectively, approaching the levels seen in the bone marrow of some animals. In the mice, the total number of Sca-1⁺ cells did not increase, rather SLF induced a redistribution of the Sca-1⁺ cells from the bone marrow to the peripheral blood and spleen (Fleming *et al.*, 1993). The 20 fold increase of Sca-1⁺ cells in the peripheral blood permitted long term, donor derived reconstitution of 30% of lethally irradiated mice with just 10 μ l of donor blood compared to 250 μ l of blood from control mice. The concentration of SLF (200 μ g/kg/day) required to mobilise CD34⁺ cells in baboons was found to be toxic to humans but at lower levels in synergy with G-CSF similar numbers of mobilised CD34⁺ cells were achieved (Andrews *et al.*, 1994). Baboons transplanted with cells collected from the peripheral blood of animals treated with SLF and G-CSF had significantly more rapid recovery of platelet and neutrophil counts compared to animals treated with G-CSF alone (Andrews *et al.*, 1995). These results provided further impetus for reconstituting transplantations using mobilised peripheral blood. The mechanics of the mobilisation of the progenitor cells into the peripheral blood are still being investigated, however, SLF appears to perturb the microenvironment within which the stem cells reside possibly by inhibiting the adhesive interaction of c-Kit expressed by the stem cells and membrane bound SLF of the stroma or possibly by altering the functional status of other adhesive interactions including those involving integrins (Lévesque *et al.*, 1994; Papayannopoulou *et al.*, 1998). However, since *in vivo* mobilisation the progenitor cells occurs several days after cytokine administration, the effects of SLF and G-CSF on cellular adhesion are probably indirect.

Mast cells are the only mature haemopoietic lineage to express appreciable amounts of c-Kit. SLF was initially identified as a mast cell growth factor and has been shown to regulate many mast cell functions. Experiments using purified SLF showed both immature and mature mast cells were capable of proliferating and differentiating in response to SLF and deprivation of these cells from SLF resulted in their apoptosis (Boswell *et al.*, 1990; Williams *et al.*, 1990; Nocka *et al.*, 1990b; Zsebo *et al.*, 1990a; Tsai *et al.*, 1991a,b; Mekori *et al.*, 1993; Baghestanian *et al.*, 1996). Continuous, low level, SLF induced activation of c-Kit is probably necessary for mast cell survival and the lack of mast cells in *Sl* mutant mice demonstrates the importance of the SLF/c-Kit interaction for mast cell generation and/or

survival (Kitamura and Go, 1979). It has been reported that SLF alone is capable of inducing mast cell differentiation from immature haemopoietic cells from peripheral blood, bone marrow, foetal liver or cord blood (Irani *et al.*, 1992; Valent *et al.*, 1992; Mitsui *et al.*, 1993) but other growth factors are considered to be necessary as-limit dilution cultures are unsuccessful, suggesting a role for feeder cells (G.W. Aylett and L.K. Ashman, unpublished data). The additional growth factors required may include Nerve Growth Factor (NGF) and IL-15. Alone and in combination with SLF, NGF has been shown to induce and regulate human mast cell development and differentiation (Welker *et al.*, 1998, 2000). IL-15 induces the proliferation of mast cells in the absence of IL-2 receptor utilizing a novel receptor and distinct signalling pathway (Tagaya *et al.*, 1996; Waldmann *et al.*, 1998). Culturing 1×10^7 human cord-blood mononuclear cells in the presence of SCF, IL-6 and prostaglandin E2 resulted in the production of 1×10^7 mast cells (Saito *et al.*, 1995), and mast cell development could be induced from murine spleen cells cultured in the presence of IL-6 and TNF α (Hu *et al.*, 1997). In addition, IL-6 appears to prevent mast cells from undergoing apoptosis induced by serum or cytokine withdrawal or by the addition of IL-4 (Yanagida *et al.*, 1995; Oskeritzian *et al.*, 1999). Conversely, the addition of IL-6 to CD34⁺ cord blood cell cultures resulted in a reduction of the number of mature mast cells being produced and cells grown in the presence of SLF and IL-6 had decreased c-Kit expression (Kinoshita *et al.*, 1999). This suggests IL-6 can modulate SLF-dependent human mast cell development. SLF has been shown to upregulate mast cell secretory capacity and directed migration (Bishcoff and Dahinden, 1992; Columbo *et al.*, 1992; Valent *et al.*, 1992; Sperr *et al.*, 1993; Nilsson *et al.*, 1994). SLF is a chemoattractant for mast cells and mast cells derived from *W⁴²* mice, which lack functional c-Kit, fail to migrate in response to SLF (Meininger *et al.*, 1992; Nilsson *et al.*, 1994). The motility of mast cells is stimulated by 100 fold less SLF than is required for their survival (Kinashi and Springer, 1994; Dastyk and Metcalfe, 1994). The interaction of SLF with c-Kit is also required for mast cell attachment to fibronectin or stromal cells. Cultured mast cells isolated from *W/W* mice, or cells lacking the extracellular domain of c-Kit, fail to adhere to fibroblasts, and conversely, mast cells with functional c-Kit fail to adhere to fibroblasts isolated from *Sl* mutant mice (Adachi *et al.*, 1992; Mekori *et al.*, 1997). Excessive exposure of mast cells to SLF results in partial down regulation of c-Kit mRNA and protein expression

preventing the cells from proliferating but these cells are still capable of chemotaxis in response to SLF (Baghestanian *et al.*, 1996). The reason for c-Kit downregulation is not known but it may either be part of a negative feedback loop, preventing excess migration and accumulation of mast cells in SLF rich areas or preventing mast cells from migrating elsewhere in an uncontrolled manner (Baghestanian *et al.*, 1996).

1.6 Biochemistry of c-Kit

As the importance of the interaction of SLF with c-Kit became evident groups set about looking for ways to dissect the events following SLF binding to c-Kit. Similar to other type three tyrosine kinase receptors (Ullrich and Schlessinger, 1990) c-Kit was presumed to undergo receptor dimerisation upon ligand binding, resulting in activation of the kinase domain and induction of the signalling complex.

1.6.1 Ligand Binding Domain

The first step in c-Kit signalling is the binding of SLF to the receptor. The ligand binding site of c-Kit was defined using monoclonal antibodies (mAb), fragments of the extracellular domain of c-Kit and mouse/human receptor chimeras. Several mAbs were shown to inhibit SLF binding to c-Kit expressing cells and one of these prevented SLF induced proliferation of these cells. These antibodies bound to the extracellular domain of the receptor as shown by their binding to soluble portions of the receptor (Kit-X, Kit 1-2 or Kit 1-2-3, which contain all of the extracellular domain or the first two or the first three Ig domains respectively) (Blechman *et al.*, 1993). These antibodies were able to recognise both of the shorter forms of c-Kit, indicating they recognise epitopes on domains one or two or the intervening sequences, implying that the ligand binding site lies in this region. In competitive binding experiments recombinant Kit 1-2 was inefficient at preventing ligand binding to full length c-Kit, whereas Kit 1-2-3 was as effective as the entire extracellular domain (Blechman *et al.*, 1993). This suggests that, while domain 3 may not directly bind SLF, it is involved in and required for stabilisation of the interaction.

Since the murine c-Kit molecule does not bind human SLF it was possible to further localise the ligand-binding site by replacement of individual loops of mouse c-Kit with human

c-Kit creating mouse-human chimeras. This approach identified the second Ig loop of human c-Kit as the core of the binding site but indicated that adjacent domains 1 and 3 were required (Blechman *et al.*, 1993; Lev *et al.*, 1993). The presence of human domain 2 in the mouse receptor was sufficient to confer high affinity binding of human SLF to the mouse chimeric receptor (Lev *et al.*, 1993). However, domain 3 was found to be more critical for murine ligand binding to the mouse receptor (Lev *et al.*, 1993) and the mouse receptor inhibitory mAb ACK-2 was found to bind to domain 3 (Lev *et al.*, 1993).

Human domain 3 increased the affinity of the murine receptor for human SLF by decreasing the rate of ligand dissociation. The authors proposed that ligand binding to domain 2 results in a conformational change that allows the third domain to fold over the binding cleft preventing ligand dissociation. These data suggest that the high affinity binding sites of c-Kit for SLF reside in domains 1 and 2 but determinants in domain 3 are required for the formation of the ligand binding cleft (Blechman *et al.*, 1993).

1.6.2 Dimerisation of c-Kit

Ligand induced dimerisation of a receptor tyrosine kinase (RTK) is seen as the first step in ligand induced signal transduction (Ullrich and Schlessinger, 1990). Dimerisation of the receptors and accompanying conformational changes allow basal kinase activity to phosphorylate a critical tyrosine residue in the activation loop, exposing the kinase domain to phosphorylate other receptors and secondary signalling molecules (Hubbard *et al.*, 1998; Plotnikov *et al.*, 1999). Dimerisation of the EGF receptor is non-covalent but it has been shown that both non-covalent and di-sulphide linked, covalent dimerisation is involved with the signalling through both the CSF-1 and PDGF receptors (Li and Stanley, 1991; Li and Schlessinger, 1991). The covalent dimerisation of the receptors was shown to be part of the downregulation of the receptor (Li and Stanley, 1991). It was proposed that other members of the type three tyrosine kinase family may also covalently dimerise but this has not been determined for c-Kit.

Ligand induced receptor dimerisation of c-Kit was first shown by Blume-Jensen and colleagues (1991) and this dimerisation correlated with activation of the kinase domain. Since then many researchers have set out to establish the mechanism of c-Kit dimerisation but it still

remains controversial. SLF induces dimerisation of c-Kit in a dose dependent manner and can be measured by fluorescence resonance energy transfer (Broudy *et al.*, 1998). Dimerisation was detected within 3 minutes of SLF addition to M07e cells and was maximal 30 minutes after the addition of SLF. Several groups have shown that the extracellular domain of c-Kit is sufficient for ligand-induced dimerisation to occur (Lev *et al.*, 1992a; Lemmon *et al.*, 1997; Philo *et al.*, 1996). This is unlike the situation for EGFR (Gunther *et al.*, 1990) but similar to PDGFR (Duan *et al.*, 1991) where overexpression of the extracellular domain of PDGF receptor in insect cells resulted in spontaneous dimerisation (Herren *et al.*, 1993).

Based on the observation that dimerisation was still maintained in presence of an excess of SLF, Lev *et al.* (1992b) suggested that the dimerisation is not due to the bivalency of the ligand but due to an intrinsic property of the receptor. To this end they went on to demonstrate that heterodimers of human and mouse c-Kit could be formed in the presence of human SLF even though human SLF binds the mouse receptor with very poor affinity (Lev *et al.*, 1992b) suggesting dimerisation occurs even when only one receptor molecule has engaged the ligand. Using mAbs, mouse-human chimeras and cut downs of c-Kit, this group identified Ig domain 4 as the domain responsible for dimerisation. Antibodies able to block ligand induced dimerisation recognised recombinant extracellular domain receptors but did not bind to molecules which lacked Ig domain 4 (Blechman *et al.*, 1995), and these antibodies prevented the proliferation of factor dependent TF-1 cells in response to SLF. The antibodies were not able to bind to full length receptor involved in preformed dimers. In addition, a c-Kit molecule containing only the three most N terminal Ig domains could not be crosslinked in the presence of SLF but the addition of Ig domain 4 allowed dimers to be crosslinked and detected. Cells expressing c-Kit with Ig domain 4 deleted failed to proliferate in response to SLF and the lack of response was shown to be due to an increased rate of SLF dissociation (Blechman *et al.*, 1995). Thus, from these data, it was concluded that dimerisation of the c-Kit was due to a dimerisation site in the fourth immunoglobulin domain and that monovalent binding of SLF to c-Kit caused a conformational change in the receptor, exposing the dimerisation site. It is possible that the receptors for PDGF and CSF-1 behave in a similar way. Several oncogenic mutations of the CSF-1 receptor that activate tyrosine

phosphorylation by stabilising the receptor dimers were mapped to the fourth Ig domain of this receptor (Carlberg and Rohrschneider, 1994).

However, data from other groups suggest that these results are inaccurate. In contrast to Blechman *et al.* (1995), Lemmon *et al.* (1997) found the first three Ig domains of c-Kit were able to dimerise in the presence of SLF by size-exclusion chromatography, dynamic light scattering and sedimentation equilibrium although like Blechman *et al.* (1995), they could not show chemical cross-linking. The authors concluded that the stoichiometry and thermodynamics of a full length extracellular domain and the three domain molecule were the same, with a mean K_d for SLF binding of 54.9nM and 49.2nM respectively. This suggests domain 4 makes no significant thermodynamic contribution to SLF binding or dimerisation and showed one SLF dimer bound to two c-Kit molecules, indicating that dimerisation was indeed a function of a dimeric ligand. In addition, the sequential binding model proposed by Blechman *et al.* (1995), opposes the data of Philo *et al.* (1996). Philo and colleagues (1996) demonstrated even a two-fold excess of SLF substantially reduced the proportion of complexes containing two c-Kit and two SLF molecules in favour of a complex consisting of one c-Kit molecule and two SLF molecules. Furthermore, they saw no evidence of the formation of a complex containing 2 c-Kit molecules and 4 SLF molecules, in the presence of an excess of SLF, as suggested to occur by Lev *et al.* (1992) and Blechman *et al.* (1995). Oddly, SLF induced proliferation of fibroblasts expressing wild-type c-Kit was inhibited by high SLF concentrations (Blechman *et al.*, 1995) suggesting that at high SLF concentrations the dimers are not maintained, contradicting earlier experimental data from the same laboratory (Lev *et al.*, 1992b) and their own model (Blechman *et al.*, 1995). In addition, Blume-Jensen *et al.* (1991) showed high concentrations of SLF reduced c-Kit dimerisation.

It is quite possible that the lack of dimerisation seen in the presence of inhibitory mAbs or the absence of domain 4 could be due to either steric influences or conformational changes, preventing ligand binding and receptor dimerisation (Lemmon *et al.*, 1997). In fact, while the extracellular forms of c-Kit mentioned above all bind SLF with similar affinities to full length receptor, the receptor devoid of domain 4 binds SLF approximately 10 fold more weakly than intact c-Kit (Blechman *et al.*, 1995). Thus, the results gathered so far suggest that in reality it is probably a combination of the two models and that receptor dimerisation is mediated by

both bivalent ligand binding and interactions between other parts of the receptor molecules but not necessarily in domains 4 or 5.

1.6.3 Ligand-Independent Receptor Activation

Reith *et al.* (1991) reported that the c-Kit isoform missing the 4 amino acids (GNNK) just exterior to the transmembrane domain was able to undergo ligand independent phosphorylation and association with PI3'K and PLC γ 1. In more recent experiments using NIH3T3 cells expressing equivalent levels of either the GNNK+ or the GNNK- isoform of c-Kit, negligible ligand independent phosphorylation was observed (Caruana *et al.*, 1999). The difference in reported results may be explained by the massive overexpression of c-Kit in transiently transfected COS cells in the experiments conducted by Reith *et al.* (1991). Indeed, high murine c-Kit surface expression on NIH3T3 cells resulted in factor-independent growth (Caruana *et al.*, 1998). One of two naturally occurring mutations in the cytoplasmic domain of human c-Kit in the mast cell line HMC-1, V560G mutation in the juxtamembrane region, results in ligand-independent receptor dimerisation (Kitayama *et al.*, 1995). A similar mutation, G664V, in the transmembrane domain, results in constitutive dimerisation of the HER2/neu receptor and activation of the intrinsic catalytic function (Weiner *et al.*, 1989; Ben-Levey *et al.*, 1992). Transfected 293T cells expressing the equivalent mutation to V560G in murine c-Kit (V559G) produce large tumours in nude mice that then succumb to leukaemia and die (Kitayama *et al.*, 1995). Expression of the second naturally occurring mutation, D816V in human and D814V in mouse c-Kit, also results in factor-independent growth. D814V c-Kit was also found to be constitutively phosphorylated on tyrosine in the absence of ligand (Tsujimura *et al.*, 1994; Kitayama *et al.*, 1995). Expression of both wild-type and D814V c-Kit in the same cell resulted in tyrosine phosphorylation and autokinase activity independent of SLF stimulation. This suggests that the D814V mutation is a dominant positive activating mutation (Tsujimura *et al.*, 1994). It was originally suggested that D814V c-Kit activation was dimerisation independent. However, more recent data shows the factor independent growth of cells transfected with D814V c-Kit can be virtually abrogated by co-expression of W⁴² c-Kit, a dominant negative form of c-Kit, but not by wild-type c-Kit. This suggests that D814V c-Kit may not function as a monomer but require receptor dimerisation

for inducing factor independent growth (Tsujiura *et al.*, 1999). Thus, alteration of the tertiary structure by mutation probably allows the ligand-independent dimerisation of two receptor molecules providing the initiation of the signalling complex via transphosphorylation. This constitutive activity of the receptor results in continual proliferative signals being delivered to the nucleus, resulting in uncontrolled cell growth, which correlates with the induction of transformation.

1.6.4 Signal Transduction

Ligand induced dimerisation of the receptor tyrosine kinases (RTK) initiates a signalling cascade which ultimately results in cell survival, proliferation, differentiation, chemotaxis or migration, depending on the receptor, the cell type and other signals received. The receptor itself responds to dimerisation by transphosphorylation, on tyrosine residues, of each of the receptor molecules within the complex, often referred to as autophosphorylation. The tyrosine autophosphorylation sites in EGFR are confined to the carboxyl terminal tail (Yarden and Ullrich, 1988), whereas they are scattered throughout the juxtamembrane domain, kinase insert, tyrosine kinase domain and the carboxyl tail of the β PDGFR (Claesson-Welsh, 1994). The tyrosine at amino acid position 821 and 823 in murine and human c-Kit respectively is conserved between RTKs and lies at position Y857 of human β PDGFR (Claesson-Welsh, 1994) and Y807 or Y809 respectively of the murine and human CSF-1R (reviewed in van der Geer and Hunter, 1993). Its autophosphorylation in type III RTKs increases kinase activity and precedes phosphorylation of other sites in the receptor or of substrates (Hanks *et al.*, 1991). Introduction of c-Kit expressing the Y821 mutation into mast cells derived from W^{sh}/W^{sh} mice impaired their proliferation and survival but did not affect their adhesion to fibronectin (Serve *et al.*, 1995). The down stream effects of this mutation are at present unclear but activation of PI3'K, p21ras and MAP kinase were not affected, nor was the induction of early response genes *c-fos* and *c-junB*, *c-myc* and *c-myb* (Serve *et al.*, 1995). This suggests Y821 is essential for c-Kit-mediated mitogenesis and survival but not adhesion. Similar mutations of homologous residues in CSF-1R and β PDGFR impair mitogenic signalling (reviewed in van der Geer and Hunter, 1993; Claesson-Welsh, 1994).

Upon autophosphorylation, many proteins bind to the phosphorylated tyrosine residues, usually outside the kinase domain, via their SH2 domains. These SH2 domains are approximately 100 amino acids in length and folded to create a pocket that binds the phosphorylated tyrosine and surrounding amino acids, which confer binding specificity (Pawson and Schlessinger, 1993; Cohen *et al.*, 1995).

The binding of one such SH2 containing protein to c-Kit has been the subject of many studies. Phosphatidylinositol 3'-kinase, PI3'K, has been shown to bind to the interkinase domain of c-Kit, via its SH2 domain containing p85 subunit, in a variety of cell types (Lev *et al.*, 1991; Rottapel *et al.*, 1991; Shearman *et al.*, 1993; Serve *et al.*, 1994; Serve *et al.*, 1995; Vosseller *et al.*, 1997) and phosphorylation-dependent binding of p85 to peptides containing the kinase insert sequence was shown (Lev *et al.*, 1992c; Rottapel *et al.*, 1991). Deletion of the interkinase domain prevented p85 association and thus inhibited PI3'K activation (Lev *et al.*, 1992c). Association was found to be dependent upon the phosphorylation of human tyrosine residue 721 (Y721) or murine Y719. Mutation of this tyrosine to phenylalanine abolished p85 association (Serve *et al.*, 1994; Serve *et al.*, 1995; Vosseller *et al.*, 1997), preventing PI3'K activation and this resulted in diminished *c-fos*, *c-junB*, *c-myc* and *c-myb* mRNA induction. (Serve *et al.*, 1995). The interkinase domain of other type three tyrosine kinase receptors has been shown to be essential for the association of p85 with the activated receptor. Similar to c-Kit, phosphorylation of tyrosine at positions 740 and 751 in the PDGFR and 721 in the CSF-1R is required for p85 binding (Kazlauska and Cooper, 1989; Escobedo *et al.*, 1991; Fantl *et al.*, 1992; Kashishian *et al.*, 1992; Reedijk *et al.*, 1992). Residues Y740 of human PDGFR and Y721 of human CSF-1R correspond to Y721 of human c-Kit. Phosphorylation of this tyrosine residue is essential for p85 binding since kinase defective mutants (W^{37} and W^{42}) that are not phosphorylated, fail to bind p85 (Reith *et al.*, 1991; Rottapel *et al.*, 1991). Additionally, constitutive phosphorylation of either the shorter naturally occurring c-Kit isoform in transiently transfected COS cells or the mutated c-Kit expressed by P815 cells results in constitutive association of c-Kit with PI3'K (Reith *et al.*, 1991; Rottapel *et al.*, 1991).

Bone marrow derived mast cells from W^{sh}/W^{sh} mice reconstituted with mutant Y719F c-Kit had impaired adhesion to fibronectin and membrane ruffling compared with cells

transfected with wild type c-Kit and were unable to potentiate degranulation or filamentous actin assembly (Serve *et al.*, 1995; Vosseller *et al.*, 1997). Inhibition of PI3'K activity with Wortmannin also inhibited secretory enhancement and cytoskeletal rearrangements caused by ligand activated c-Kit. These results implicate receptor proximal PI3'K activation in c-Kit mediated secretory enhancement, adhesion and cytoskeletal reorganisation (Serve *et al.*, 1995; Vosseller *et al.*, 1997). In M07e cells the binding of p85 to c-Kit allows c-Kit to interact with and possibly phosphorylate several other proteins including CRKL, an adaptor protein, and c-Cbl. The association of these proteins with c-Kit and their tyrosine phosphorylation was SLF dependent and occurred within 1 minute of ligand binding (Sattler *et al.*, 1997). CRKL constitutively associates with p85 via its SH3 domain and its SH2 domain binds c-Cbl after SLF stimulation. The tyrosine phosphorylation of c-Cbl is likely to provide binding sites for the SH2 domains of CRKL and p85, further stabilising the complex. The function of the complex is not known but it could be important in propagating signals involving PI3'K such as gene expression and adhesion (Sattler *et al.*, 1997). Others have also detected tyrosine phosphorylation of c-Cbl after SLF stimulation of M07e and TF-1 cells but could not show any direct association of c-Kit and c-Cbl although c-Cbl was associated with Grb2 (Brizzi *et al.*, 1996; Wisniewski *et al.*, 1996). c-Cbl has been shown to associate with the EGF receptor, the CSF-1 receptor and the oncogenic receptor HER2/neu (Lee *et al.*, 1999; Levkowitz *et al.*, 1998, 2000). Association of c-Cbl with the receptors appears to take place at the recycling endosome stage and is dependent upon the kinase activity of the receptor since kinase defective EGF receptor fails to associate with c-Cbl and the receptor is not degraded (Levkowitz *et al.*, 1998). c-Cbl dependent ubiquitination of the receptor targets it to the degradation pathway. c-Cbl induced ubiquitination of CSF-1 receptor increases the endocytic rate of the receptor thereby reducing the signalling time of the receptor (Lee *et al.*, 1999). Interestingly, the introduction of retrovirally encoded c-Cbl into a Neu transformed neuroblastoma resulted in enhanced down-regulation of the receptor and correlated with tumour regression (Levkowitz *et al.*, 2000). c-Cbl may therefore be involved in the ubiquitination and degradation of c-Kit.

The PI3'K target Akt has been shown to be important in regulating SLF induced survival signals. Activation of Akt results in the phosphorylation and inactivation of Bad, a

proapoptotic molecule (Blume-Jensen *et al.*, 1998). While Bad is phosphorylated on two sites, S112 and S136, only S136 phosphorylation was shown to be important for SLF mediated survival. Deregulation of this pathway may result in cellular transformation.

Many other proteins have been shown to associate with c-Kit after SLF binding. Phospholipase C- γ 1 (PLC- γ 1) associates weakly and becomes phosphorylated after ligand binding (Lev *et al.*, 1991; Rottapel *et al.*, 1991; Blume-Jensen *et al.*, 1994) although greater phosphorylation of PLC- γ 1 was seen in these cells when stimulated with PDGF β (Blume-Jensen *et al.*, 1994). PLC- γ 1 similarly binds to PDGFR (Kunijian *et al.*, 1989; Meisenheider *et al.*, 1989) but was shown not to interact with the CSF-1R (Downing *et al.*, 1989). In contrast to the results of Rottapel *et al.* (1991), phospholipase D rather than PLC- γ 1 was shown to associate with c-Kit in rat mast cells (Koike *et al.*, 1993).

Depending on the cell type studied other proteins may be phosphorylated upon c-Kit activation. These include: Raf-1 kinase on serine and MAP kinase on threonine and tyrosine residues (Lev *et al.*, 1991; Funasaka *et al.*, 1992; Hallek *et al.*, 1992; Okuda *et al.*, 1992; Welham and Schrader, 1992; Blume-Jensen *et al.*, 1994), p95^{vav} in M07e and TF-1 cells (Alai *et al.*, 1992; Blume-Jensen *et al.*, 1993), Tec kinase in M07e cells (Tang *et al.*, 1994) and Grb2/Sem-5 (Blume-Jensen *et al.*, 1994). Grb2 has been shown to bind to phosphorylated tyrosines 703, in the kinase insert domain, and 936, in the C terminal tail, via its SH2 domain (Thommes *et al.*, 1999). Binding of Grb2 to Y703 provides a link of SLF activated c-Kit to the Ras/MAPK pathway. Grb7 was also shown to bind Y936 (Thommes *et al.*, 1999). An unknown protein of 200kDa has been shown to rapidly associate with c-Kit and becomes heavily phosphorylated on tyrosine residues when M07e cells were treated with SLF (Linnekin *et al.*, 1995). Others have reported the detection of a similar protein (Kuriu *et al.* 1991, Miyazawa *et al.*, 1991; Funasaka *et al.*, 1992; deVos *et al.*, 1993; Yi and Ihle, 1993). It has been suggested that this protein is a membrane tyrosine phosphatase but this has not been confirmed (Linnekin *et al.*, 1995). Recent unpublished data of Cambareri and Ashman suggest this protein may actually be ubiquitinated c-Kit as it can be immunoblotted with anti-c-Kit, anti-phosphotyrosine and anti-ubiquitin monoclonal antibodies.

Stimulation of the H526 small cell lung cancer cell line with SLF results in binding of Lck, a Src related tyrosine kinase, to c-Kit, resulting in 3-6 fold increase in Lck activity that

persisted for at least 30 minutes (Krystal *et al.*, 1998). Blocking of Lck activity antagonised both SLF mediated proliferation and inhibition of apoptosis, suggesting Src family kinases may be involved in regulating these processes. Signal transduction via Src family kinases is also required for internalisation of SLF ligated c-Kit. When Src family kinases were blocked with PP1, c-Kit was able to associate with clathrin but was not internalised (Broudy *et al.*, 1999). After ligand stimulation, tyrosines 568 and 570 of c-Kit are phosphorylated (Lennartson *et al.*, 1999). Mutation of these tyrosines to phenylalanine prevented the activation Src family kinases resulting in a decrease in Shc phosphorylation, Ras and Erk2 activation and c-fos induction after SLF binding to the receptor (Lennartson *et al.*, 1999). The main Src family kinase to bind to the region containing these tyrosines is Lyn (Linnekin *et al.*, 1997). Treatment of cells with the Src kinase blocker PP1 or antisense oligonucleotides to Lyn resulted in a dramatic inhibition of SLF induced proliferation suggesting Lyn is critical for SLF induced proliferation in haemopoietic cells (Linnekin *et al.*, 1997).

SLF stimulation of c-Kit results in the association of STAT1a, STAT5a and STAT5b and this association is dependent upon active kinase domain of c-Kit (Brizzi *et al.*, 1999).

Two proteins, protein kinase C (PKC) and a haemopoietic phosphotyrosine phosphatase, SHP-1, appear to downregulate activated c-Kit. Upon activation, PKC, a serine threonine kinase, involved in the control of cell proliferation, differentiation and motility, phosphorylates c-Kit on serine residues 741 and 746 located in the kinase insert region (Blume-Jensen *et al.*, 1995). These sites are phosphorylated constitutively in resting porcine aortic endothelial cells and their phosphorylation is increased in response to SLF as well as the phorbol ester, PMA, a known activator of PKC. In addition, serine residues 959 and 821 are also SLF and PMA induced PKC dependent phosphorylation sites. These sites may not be direct targets as PKC does not phosphorylate them *in vitro*, and they are not constitutively phosphorylated (Blume-Jensen *et al.*, 1995). This serine phosphorylation of c-Kit inhibited SLF induced tyrosine phosphorylation of the receptor but did not affect ligand binding affinity (Blume-Jensen *et al.*, 1993). Substitution of serine 741 and 746 with alanine resulted in a two fold increase in SLF stimulated receptor tyrosine phosphorylation and a 2.2 fold increase in PI3K association compared to wild type receptor and increased SLF induced mitogenicity. In contrast, PKC activity was necessary for SLF induced motility responses including actin

reorganisation and chemotaxis (Blume-Jensen *et al.*, 1993). PKC activation appeared to negatively regulate PI3K by direct or indirect serine and threonine phosphorylation of p85 (Blume-Jensen *et al.*, 1994). PKC may also negatively regulate Src, Grb2 and PLC- γ 1 since inhibition of PKC with Calphostin C increased the association of the SH2 domains of these molecules with c-Kit (Blume-Jensen *et al.*, 1994). The mechanism of PKC activation is unclear although it is assumed to require diacylglycerol (DAG) generation. In this respect, several groups have reported PLC- γ 1 association with c-Kit and phosphorylation after SLF binding (Halleck 1992, Herbst 1991, Reith 1991) but Lev *et al.* (1991) and Blume-Jensen *et al.* (1994) found little tyrosine phosphorylation of PLC- γ 1. Other studies show phospholipase D becomes activated in response to SLF and is the main pathway responsible for production of DAG in rat peritoneal mast cells (Koike *et al.*, 1993). Thus PKC appears to be involved in a negative feedback loop that regulates c-Kit and the activity of PKC and determines whether the effect of SLF will be preferentially mitogenic or motogenic (Blume-Jensen *et al.*, 1993). The EGFR is also negatively regulated by PKC induced phosphorylation of serine and threonine residues inhibiting kinase activity and ligand affinity of the receptor (Lin *et al.*, 1986).

Receptor tyrosine kinases may also be negatively regulated by phosphotyrosine phosphatases. The interaction of c-Kit and SHP-1 *in vivo* was demonstrated by crossing W^f/W^f mice with *me/me* mice. The *me* locus contains the gene encoding the phosphatase SHP-1. The presence of the mutant c-Kit encoded by W^f locus resulted in a decrease in macrophage and granulocyte numbers in *me/me* lung, skin, peripheral blood and bone marrow, thereby decreasing the severity of phenotype (Lorenz *et al.*, 1996). SHP-1 had no effect on the proliferative capacity of bone marrow derived mast cells to SLF or IL-3 *ex vivo*. Nevertheless, tyrosine phosphorylation of c-Kit was substantially increased when SHP-1 was deleted in W^f mice. Thus SHP-1 directly dephosphorylates and regulates c-Kit and negatively regulates signalling from c-Kit *in vivo* but in a cell specific manner (Lorenz *et al.*, 1996). Using glutathione S-transferase (GST) fusion proteins containing either the tyrosine phosphorylated segments of the cytoplasmic domain of c-Kit or the SH2 domain of SHP-1 it was shown SHP-1 interacts with and binds to phosphorylated tyrosine residue 569 and residue 567 contributes to this interaction (Kozlowski *et al.*, 1998). Transfection of Ba/F3 cells with

c-Kit containing a phenylalanine substitution at residue 569 resulted in disruption of SHP-1 binding and hyperproliferative responses to SLF. Mutation of the second site, residue 567 did not reduce SHP-1 binding but did prevent SHP-2 binding suggesting c-Kit maybe negatively regulated by many phosphatases (Kozlowski *et al.*, 1998). Additional evidence of SHP-1 regulation of c-Kit comes from experiments conducted using a cell line expressing a mutant form of c-Kit, containing a Asp to Val mutation at amino acid 814 (D814V). This mutant c-Kit induced the phosphorylation of a 130kDa protein and the degradation of normally expressed SHP-1 via an ubiquitin-dependent pathway (Piao *et al.*, 1996). See section 1.7 below for further detail.

Another negative regulator Socs binds to c-Kit via its SH2 domain. While it does not inhibit the catalytic activity of c-Kit or anti-apoptosis signals, it prevents SLF induced proliferative signals possibly by binding and inhibiting signalling protein Grb2 and the guanine nucleotide exchange factor Vav (de Sepulveda *et al.*, 1999).

The Ras GTP-activating protein (RasGAP) has been shown to associate with c-Kit in human embryonic kidney fibroblast cells, 293T cells (Herbst *et al.*, 1991) and haemopoietic cells (Duronio *et al.*, 1992) but not in mast cells (Rottapel *et al.*, 1991). RasGAP is a negative regulator of Ras activity and may be involved in signal termination. RasGAP has also been shown to possibly link PDGFR (Kaplan *et al.*, 1990; Kazlauskas and Cooper, 1990; Molloy *et al.*, 1989) and EGFR to the Ras signalling pathway but appears to be a poor substrate of the CSF-1R (Reedijk *et al.*, 1990).

Different cellular responses including proliferation, survival, differentiation, chemotaxis, migration, adhesion to ECM and secretion of granules can all be elicited by the binding of SLF to c-Kit expressing cells, depending upon the cell type. As discussed above, activation of c-Kit permits its interaction with many molecules involved in signal transduction resulting in activation of several pathways. Importantly, these pathways are not completely linked. For example, treatment of cells with Calphostin C, a PKC inhibitor, blocked c-Kit mediated adhesion to fibronectin, secretory enhancement, membrane ruffling and filamentous actin assembly. In contrast, Cytochalasin D inhibited c-Kit mediated filamentous actin assembly and membrane ruffling but did not affect secretory enhancement or adhesion, providing evidence that the signals mediated by activated c-Kit induced cytoskeletal rearrangements, that are

dependent on actin polymerisation, can be uncoupled from c-Kit mediated secretory and adhesive responses (Vosseller *et al.*, 1997). Additionally, inhibition of PKC did not affect SLF induced phosphorylation of Raf-1 and the activation of MAP kinase (Blume-Jensen *et al.*, 1994) implying the survival, proliferation and differentiation signals can be separated from the other cellular responses c-Kit can mediate. The effects of the different W mutations in various cell types (discussed in section 1.2.2) may be due to the differences in the abundance of the receptor and/or downstream signalling molecules (Shearman *et al.*, 1993). Additionally, different cells may express alternate isoforms of several signalling molecules including PI3'K. As the ability of ligand to elicit a mitogenic response appears to depend upon the magnitude and duration of the signal evoked within the target cell, then receptor number and substrate availability to the activated receptor could be an important factor underlying cell type variability in response to SLF (Shearman *et al.*, 1993). For example, the stimulation of PC12 cells with EGF induces a proliferative response with transient activation of MAP kinase, with no apparent nuclear translocation. In contrast, nerve growth factor (NGF) induces differentiation of PC12 cells, with sustained activation of MAP kinase and associated nuclear translocation (reviewed in Marshall, 1995). The level of receptor expression can also determine the response of the cell. Overexpression of EGF or insulin receptors in PC12 cells results in sustained MAPK activation, resulting in cellular differentiation instead of proliferation (Dikic *et al.*, 1994; Traverse *et al.*, 1994).

The c-Kit isoforms GNNK+ and GNNK- display differences in receptor phosphorylation, down regulation and activation of MAP kinase as well as differences in biological responses to SLF binding (Caruana *et al.*, 1999). The biological activity and consequences of the isoforms are discussed in greater detail section 1.2.1.

Thus the diversity of responses generated by SLF induced activation of c-Kit can be influenced by the set of signalling molecules available to bind, the degree of stimulation by the ligand or the isoform of SLF, the level and isoform of the receptor expressed on the cell surface, the duration of activation of certain signalling molecules or the combination of signals from several receptors.

1.6.5 Downregulation of c-Kit

Following ligand binding it has been shown that the receptor-ligand complexes are endocytosed via clathrin-coated pits. The receptor is then either recycled to the cell surface, or it is polyubiquitinated and targeted to lysosomes where it is degraded. Internalisation of the receptor-ligand complex can be seen as a way of reducing the life span of the receptor and thereby down modulating ligand-induced activation of the receptor. Down modulation of the c-Kit induced by SLF stimulation has been reported by several groups and has been found to be the result of a number of different but not mutually exclusive mechanisms.

The binding of soluble SLF to c-Kit has been shown to induce c-Kit/SLF complex internalisation in mast cells and the megakaryocytic cell line, M07e (Yee *et al.*, 1993, 1994; Miyazawa *et al.*, 1994; Adachi *et al.*, 1995) resulting in reduced levels of c-Kit on the cell surface. Confocal microscopy showed redistribution of c-Kit to caps at one end of the cell within 3 minutes after SLF addition, the same time as dimerisation of the receptor was detected (Broudy *et al.*, 1998). Protein synthesis was required for the reappearance of c-Kit on the cell surface suggesting that the receptor is not recycled to the surface after internalisation (Broudy *et al.*, 1998). Internalisation could also be induced by PMA, a PKC activator, with concomitant decrease in c-Kit RNA expression (Adachi *et al.*, 1995; Ogawa *et al.*, 1995). Asano *et al.* (1993) demonstrated that decreased c-Kit mRNA expression in a human erythroleukaemia cell line, HEL, upon PMA stimulation, was a result of postranscriptional mechanisms involving induction of an RNA destabilising protein. It was shown that membrane-bound SLF was able to prolong the life span of the c-Kit and induce a more persistent tyrosine kinase activation in comparison to soluble SLF by preventing its internalisation (Miyazawa *et al.*, 1995), which may in part explain the difference in biological activity between the two SLF isoforms (see section 1.3.2).

Internalisation and degradation of the c-Kit/SLF complex is reduced in kinase defective c-Kit expressing cells and receptor ubiquitination was shown to be dependent on kinase activity (Miyazawa *et al.*, 1994; Yee *et al.*, 1994). Similar results have been obtained with the CSF-1 and PDGF receptors (Carlberg *et al.*, 1991; Mori *et al.*, 1992, 1993). In primary macrophages isolated from Cbl^{-/-} mice the CSF-1R fails to be multi-ubiquitinated and the internalisation of the receptor is slower (Lee *et al.*, 1999). Prolonged phosphorylation of the

CSF-1R and slower internalisation resulted in increased proliferation of the macrophages in response to limiting concentrations of CSF-1 (Lee *et al.*, 1999). Since Cbl also associates with activated c-Kit it is possible a similar mechanism occurs to invoke the internalisation of c-Kit.

The shedding of the extracellular domain from the cell surface can also down modulate c-Kit. This latter mechanism is induced by PKC dependent and independent pathways (Yee *et al.*, 1993; Brizzi *et al.*, 1994; Adachi *et al.*, 1995) and is independent of kinase activity, which is in agreement with results obtained with the CSF-1 receptor (Downing *et al.*, 1989; Yee *et al.*, 1994). Serine phosphorylation of c-Kit by PKC has also been shown to downregulate the SLF-induced activation of c-Kit by inhibiting tyrosine autophosphorylation and the phosphorylation of the associated PI3'K subunit p85.

Downregulation of c-Kit can also be induced by several cytokines such as IL-3 and GM-CSF in murine bone marrow derived mast cells and FDC-P1 cells (Welham and Schrader, 1991; Caruana *et al.*, 1993), IL-4 in the human mast cell line, HMC-1, and AML cells (Sillaber *et al.*, 1991), transforming growth factor- β (TGF- β) in CD34⁺ cells (Sansilvestri *et al.*, 1995) and erythroid differentiation factor/activin A in murine erythroleukaemia cells (Hines *et al.*, 1995).

1.7 Overexpression of Receptor Tyrosine Kinases

High expression of c-Kit on a subpopulation of AML cells was first described by Ashman *et al.* (1988) and this expression correlated with poor prognosis. It was postulated that abnormally high expression levels of the receptor assisted with the transformed phenotype. Overexpression of other tyrosine kinases has been associated with malignant disease. Amplification and overexpression of PDGFR α is common in glial tumours and glioblastomas (Smith *et al.*, 2000 and references within). The overexpression of HER2/neu and EGF receptors have widely been reported in a number of human malignancies including breast, ovarian and cervical (Slamon *et al.*, 1987, 1989; Paik *et al.*, 1990; Paterson *et al.*, 1991; Liu *et al.*, 1992; Mitra *et al.*, 1994; Tetu *et al.*, 1994), salivary gland (Press *et al.*, 1994), colon (Paik *et al.*, 1990) and non-small cell lung carcinoma (Kern *et al.*, 1990). The overexpression of HER2/neu in breast and/or ovarian cancers correlates with poor prognosis and chemotherapy

resistant disease (Slamon *et al.*, 1987, 1989). This has resulted in several groups examining possibility of new treatments for these diseases, mainly using antibodies that inhibit proliferative signal transduction through the HER2/neu receptor (further discussion in chapter 6) and kinase inhibiting drugs.

While some of these malignancies are reported to involve receptor mutations, importantly the majority of these diseases result from overexpression of an unaltered receptor from either gene amplification or increased transcription. Several groups using transformation assays involving NIH3T3 cells have confirmed this. The overexpression of HER2/neu, EGF or CSF-1 receptors in NIH3T3 cells resulted in cellular transformation as depicted by colony growth in soft agar or methylcellulose and focus formation (Di Fiore *et al.*, 1987a,b; Hudziak *et al.*, 1987; Rohrschneider *et al.*, 1989; Baasner *et al.*, 1996). Cells carrying only one copy of the introduced gene failed to produce colonies. The elevated levels required to induce NIH3T3 transformation correlated with the expression levels seen in cells isolated from human tumours (Di Fiore *et al.*, 1987a,b). HER2/neu induced cellular transformation was independent of ligand and probably due to constitutive receptor dimerisation and phosphorylation, whereas transformation due to EGF receptor overexpression was dependent upon the presence of ligand either exogenously added (Di Fiore *et al.*, 1987b) or by autocrine stimulation with co-expressed TGF- α which acts through the EGFR (Di Marco *et al.*, 1989). Introduction of these cells into nude mice resulted in tumour formation. Brandt-Rauf *et al.* (1990) suggested ligand independent dimerisation could occur at high receptor levels, and high concentrations of purified HER2/neu do indeed self dimerise in the absence of ligand (Samanta *et al.*, 1994). In addition, *in vitro* and *in vivo* experiments using NIH3T3 cells transfected with HER2/neu, under the control of an inducible promoter, clearly showed that the overexpression of HER2/neu was sufficient to cause transformation of these cells and induce tumours in nude mice. Inhibition of HER2/neu expression in these cells resulted in a reversal of the transformed phenotype, preventing colony and foci formation and either preventing tumour formation in mice or causing the regression of already established tumours (Baasner *et al.*, 1996).

Similar to HER2/neu, overexpression of a chimeric EGF/c-Kit and full length murine c-Kit resulted in factor dependent transformation of NIH3T3 cells (Lev *et al.*, 1990;

Alexander *et al.*, 1991) and some colony growth was seen in the absence of added SLF. High or stable expression of c-Kit in a murine haemopoietic cell line (Ba/F3) resulted in low level transformation as detected by the slow growth of these cells as a tumour when injected into nude mice (Kitayama *et al.*, 1995). The authors suggested that this transformation was still dependent upon SLF, possibly produced by stromal cells. 32D cells constitutively expressing wild type c-Kit were able to survive and proliferate when injected into syngeneic mice. The mice died from a leukaemic like disease that was not induced by cells that did not express the receptor (Hu *et al.*, 1995). Several clones, expressing varying levels of c-Kit on their surface were used. A correlation between the level of c-Kit expression and time of development of disease was seen: mice injected with cells expressing high levels of c-Kit developed leukaemia more rapidly. In a detailed study the effect of murine and human c-Kit density on NIH3T3 cell transformation was studied (Caruana *et al.*, 1998, 1999). A series of clones derived from a pool of infectants (displaying full range of transformation characteristics including, focus formation, anchorage independent growth in soft agar and production of tumours in nude mice) showed clones expressing less than 2.5×10^4 receptors/cell failed to produce colonies. Receptor levels above this resulted in colony growth, focus formation and tumourigenicity, which were generally enhanced as the receptor level increased to 8×10^4 copies/cell. Interestingly, compared to CD34⁺ human progenitor cells which express 2×10^4 receptors/cell (Cole *et al.*, 1996), only marginally higher expression levels than physiological were required to generate anchorage independent growth of the murine c-Kit expressing NIH3T3 cells. Oddly, in this system, cells expressing very high levels of c-Kit failed to display a transformed phenotype. As an extension to this observation, experiments were conducted with another vector system allowing the controlled amplification of the human c-Kit gene and associated receptor expression. Again, cells expressing high levels of receptor failed to display anchorage independent growth (Caruana *et al.*, 1998). These results suggest there is an optimal level of c-Kit expression required to generate the production of anchorage independent growth.

The mechanism by which overexpression of receptor tyrosine kinases leads to transformation or why extreme overexpression of these receptors leads to inactivation of the receptor is not known. However, it has been shown that excessive activation of the

Ras/Raf/MAPK pathway in NIH3T3 cells not balanced by the activation of Rho can lead to p21^{Cip1} induction and cell cycle arrest (Sewing *et al.*, 1997; Woods *et al.*, 1997; Olson *et al.*, 1998).

1.8 Activating mutations of c-Kit

Overexpression is not the only way to induce constitutive activation of the c-Kit. Alteration of c-Kit by the HZ4-FeSV to produce v-Kit enabled it to produce tumours in cats and induce a transformed phenotype in NIH3T3 cells (Besmer *et al.*, 1986). The most striking differences were the deletion of the extracellular and transmembrane domains and replacement of the last 50 amino acids with 5 unrelated ones. Similar deletions occurred in the transduction of the *v-erbB* gene by the avian erythroblastosis virus (Downward *et al.*, 1984; Ullrich *et al.*, 1984) and the removal of the cytoplasmic tail was also seen in the *v-fms* gene of the Susan McDonough feline fibrosarcoma and HX5-Feline Sarcoma Virus (McDonough *et al.*, 1971; Besmer *et al.*, 1986; Coussens *et al.*, 1986). Deletion of the extracellular domain results in ligand independent activation of the receptor and removal of the cytoplasmic tail possibly removes negative regulatory domains.

Along with the gross deletions of c-Kit, v-Kit also contains several amino acid substitutions and deletions including the deletion of Y569 and V570 in the juxtamembrane domain, that were present in human, murine and feline sequence, and a G761D substitution in the kinase insert domain (Herbst *et al.*, 1995). All other changes were due to species divergence. These mutations were introduced into a chimeric receptor containing the extracellular domain of the EGF receptor and the cytoplasmic domain of the human c-Kit. Constructs containing these mutations were expressed in a human kidney embryo cell line, 293T, and NIH3T3 cells, either individually or together (Herbst *et al.*, 1991, 1995). In the absence of EGF the individual mutations did not alter receptor phosphorylation or transforming ability, suggesting the constitutive phosphorylation of v-Kit was due to its loss of the extracellular domain. If both deletions and the substitution were expressed together the autophosphorylation of the receptor was impaired and this was more pronounced if the mutant receptor also lacked the cytoplasmic tail. However, this mutant had increased transforming ability and mitogenic activity. The Y568 and V569 deletion alone had a 3 fold higher ability

to induce anchorage independent growth of NIH3T3 cells in the presence of EGF than the control EGF/c-Kit chimeric receptor. In the PDGF and CSF-1 receptors Y579 and Y559 correspond to the Y568 of c-Kit and in PDGF and CSF-1R these sites have been shown to be involved in the binding of tyrosine kinase substrates belonging to the c-Src family (Courtneidge *et al.*, 1993). Therefore, deletion of Y568 may affect the binding of similar c-Src substrates to c-Kit, possibly contributing to its oncogenic activation.

In addition to the mutations described in v-Kit, two mutations, V560G, in the juxtamembrane domain, and D816V, in the phosphotransferase domain, have been detected in the human mast cell leukaemia cell line, HMC-1 (Furitsu *et al.*, 1993). The equivalent of the second mutation (D814V) has also been detected in the murine mastocytoma cell line, P815 (Tsujimura *et al.*, 1994) and rat mast cell line RBL-2H3 (D817Y) (Tsujimura *et al.*, 1995). A third activating mutation was found in the murine FMA3 mastocytoma cell line, containing a deletion of seven amino acids in the juxtamembrane region of the cytoplasmic domain (Tsujimura *et al.*, 1996). Engineered c-Kit molecules carrying these mutations were found to be constitutively phosphorylated in the absence of SLF but only FMA3 and V599G receptors existed as dimers in the absence of SLF (Kitayama *et al.*, 1995; Tsujimura *et al.*, 1996). Murine receptors carrying the equivalent of the human V560G mutation, V559G, expressed in Ba/F3 or FDC-P1 cells, underwent factor independent dimerisation (Kitayama *et al.*, 1995). Cells expressing these mutant c-Kit molecules grew *in vitro* without the addition of exogenous SLF. Subcutaneous injection of the cells into nude mice resulted in large tumours at the site of injection and the mice quickly succumbed to leukaemia and died (Kitayama *et al.*, 1995). Additionally, 4 of 4 human mastocytoma tumours with associated haematological disorder were found to contain D816V mutations in c-Kit (Nagata *et al.*, 1995). These results suggest that the constitutively activating mutation, D816V, of c-Kit may be partially involved in the neoplastic growth of mast cells. In an attempt to understand the role of this mutation researchers have analysed the function of this mutant receptor in cell lines. Tsujimura and colleagues (1999) transfected Ba/F3 cells with a c-Kit construct with the ligand binding and receptor dimerisation domains deleted and the D814 mutated to Y814. These cells grew independent of factor and the receptor was constitutively phosphorylated on tyrosine. The factor independent growth could be inhibited by co-expression of KITW⁴² that acts as a

dominant negative form of c-Kit but not by co-expression with wild type (WT) c-Kit. The mutated c-Kit could also be co-immunoprecipitated with KITW⁴² or WTc-Kit, and also associated with a chimeric extracellular domain of CSF-1R fused to transmembrane and cytoplasmic domains of the mutated c-Kit. These data suggest that the constitutively activating mutations at Asp814 may cause a conformation change resulting in receptor self association, not via the extracellular domain but via the cytoplasmic domain, which may result in activation of downstream targets required for factor independent growth (Tsujimura *et al.*, 1999). However, it cannot be ruled out that deletion of the extracellular domain of c-Kit in these experiments results in the conformation change. Expression of the D814Y mutation in the IL-3-dependent mast cell line IC2 induced ligand independent mast cell growth *in vitro*, tumourigenicity *in vivo* and mast cell differentiation (Piao and Bernstein, 1996).

Ferrao *et al.* (1997) introduced the D816V mutant form of human c-Kit into factor dependent myb transformed primary foetal liver cells (MTHC). These cells grew in the absence of factor, at a rate higher than parental cells in GM-CSF, and displayed increased clonogenicity. While both mutant and WTc-Kit expressing cells proliferated at the same rate, mutant cells displayed increased cell survival, suggesting the signals transduced by these receptors were not identical. Both WTc-Kit, in the presence of SLF, and V816c-Kit cells were able to produce differentiated cells, displaying a large adherent phenotype, expressing both macrophage and neutrophil esterases and were highly phagocytic. In addition, cells expressing V816c-Kit but not WTc-Kit were shown to produce tumours in mice. These tumours were highly mitotic and invasive and displayed myelomonocytic differentiation. Taken with the data from Tsujimura *et al.* (1999), it is possible that V816c-Kit self association results in the activation of an altered signalling pathway compared to SLF ligated WTc-Kit. The mixed populations derived from the V816c-Kit tumours phenotypically resembled leukaemic cells derived from patients with monocytic leukaemia with histocytic differentiation (Ferrao *et al.*, 1997) and it would be of interest to examine these leukaemic samples for such mutations.

Additional evidence that c-Kit and SHP-1 interact was provided by experiments using the P815 cell line. It had been observed that the mutant c-Kit protein in P815 cells was constitutively phosphorylated, on tyrosine residues, in the absence of ligand and was believed

to contribute to the transformed phenotype of these cells (Rottapel *et al.*, 1991; Tsujimura *et al.*, 1994; Kitayama *et al.*, 1995). The amino acid sequence surrounding Asp 814/816 in murine and human c-Kit is widely conserved in many RTKs including PDGFR, CSF-1R, insulinR and hepatic growth factor receptor (from Tsujimura *et al.*, 1994). This mutant c-Kit induced the phosphorylation of a 130kDa protein and the degradation of a normally expressed SHP-1 via an ubiquitin-dependent pathway (Piao *et al.*, 1996). Thus, the oncogenic potential of the mutant c-Kit expressed by this cell line may be enhanced by the removal of the negative regulator, SHP-1. This mutation caused a marked alteration in c-Kit substrate specificity, as determined by autophosphorylation sites, and the pattern of tyrosine phosphorylated proteins detected as compared to wild type c-Kit (Piao *et al.*, 1996). This mutation is similar to one seen in RET RTK, which alters its substrate specificity (Santoro *et al.*, 1995; Songyang *et al.*, 1995) by changing the amino acid sequence recognised from one of a RTK to a nonRTK, thus altering the signalling pattern (Piao *et al.*, 1996).

A novel D816H mutation was detected in a subset of human germ cell tumours. In transfection experiments this mutated c-Kit was also constitutively activated and phosphorylated on tyrosine residues (Tian *et al.*, 1999). In a case of systemic mast cell disease, another mutation, D820G, located very close to the D816V mutation was demonstrated, suggesting a role for this mutation in disease (Pignon *et al.*, 1997). Interestingly, an equivalent mutation in the CSF-1 receptor, D802V, causes constitutive activation of the receptor and transformation of FDC-P1 cells (Glover *et al.*, 1995). Together these data suggest that any amino acid alteration at or near the 816 site results in a conformational change in the receptor, allowing ligand independent phosphorylation and activation of the receptor.

Sequencing of c-Kit cDNA from gastrointestinal stromal tumours (GISTs) revealed mutations in the juxtamembrane region in five separate cases (Hirota *et al.*, 1998). Expression of cDNAs encoding these mutant receptors in the 293T human embryonic kidney cell line resulted in SLF-independent constitutive tyrosine phosphorylation and kinase activation of c-Kit. Introduction of the mutant c-Kit cDNAs into factor dependent Ba/F3 cells resulted in factor-independent proliferation and tumour production in nude mice (Hirota *et al.*, 1998). See section 1.9 for further details.

1.9 c-Kit and Malignant Disorders

The c-Kit protein was first identified as an acute myeloid leukaemia associated antigen (Gadd and Ashman, 1985) and its expression at high level on leukaemic blast cells was associated with poor prognosis in 25% of adult AML patients (Ashman *et al.*, 1988). This correlation was assessed by the binding of an anti-c-Kit mAb, YB5.B8, prior to the demonstration this antibody recognises c-Kit (Lerner *et al.*, 1991). Since then many studies have been conducted examining the role of c-Kit in haematological malignancies as well as certain solid tumours. c-Kit protein and mRNA expression have been detected in many AML cell lines and 60-91% of malignant blast cells from adult and child patients with AML (Wang *et al.*, 1989; Ikeda *et al.*, 1991; Broudy *et al.*, 1992b; Crosier *et al.*, 1993; Reuss-Borst *et al.*, 1994; Smith *et al.*, 1994; Tohda *et al.*, 1993; Sperling *et al.*, 1997; Bene *et al.*, 1998; Cascavilla *et al.*, 1998; Nomdedeu *et al.*, 1999; Schwartz *et al.*, 1999) but there was no correlation with FAB classification although M5 subtypes expressed little or no c-Kit. A correlation between c-Kit expression and poor prognosis has not been seen by other groups (Reuss-Borst *et al.*, 1994; Schwartz *et al.*, 1999). c-Kit expression was shown to correlate with drug resistance in AML and may account for the previously observed association of high c-Kit expression with poor outcome (Sincock and Ashman, 1997). The c-Kit expressed by the blast cells is presumed to be functional since several studies have shown these cells are capable of proliferation, *in vitro*, in response to SLF and that this proliferation could be increased in the presence of other growth factors including IL-3, GM-CSF, G-CSF and Epo (Carlesso *et al.*, 1992; Goselink *et al.*, 1992; Pietsch *et al.*, 1992; Siitonen *et al.*, 1996). SLF alone stimulated the proliferation of cells from 14 of 20 c-Kit⁺ AML cases and in synergy with G-CSF, GM-CSF or IL-3 could stimulate 18, 17, or 16 of the c-Kit⁺ cases respectively (Hirakawa *et al.*, 1996). c-Kit⁻ AML cells did not respond to SLF. c-Kit expression was detected on high numbers of blast cells from 93% of refractory anaemia patients, 63% of AMLs, majority presenting in AML-M1 and AML-M2 subclasses, and 50% of cases with acute promyelocytic leukaemia (Di Noto *et al.*, 1996). Higher expression levels of c-Kit and increased c-Kit positive cells have been detected in myelodysplastic syndrome (MDS) and myeloproliferative syndrome (MPS) patients as compared to healthy volunteers (Siitonen *et al.*, 1994). There was significant correlation of c-Kit expression and CD34 antigen expression

and with the phase of the disease (Siitonen *et al.*, 1994). However, SLF has been shown to enhance progenitor growth, CFU-GM, in MDS patients and treatment of these patients with SLF may improve their outcome (Glinsmann-Gibson *et al.*, 1994; Piacibello *et al.*, 1994) although SLF has also been shown to induce the proliferation of CD34⁺ MDS cells (Sawada *et al.*, 1999). Recently studies using more sensitive techniques show that very few AML samples express modest overexpression of c-Kit and the majority have lower c-Kit expression levels than normal CD34⁺ bone marrow cells (Cole *et al.*, 1996).

Limited examples exist to suggest mutations in c-Kit or autocrine cycles, utilising co-expression of c-Kit and SLF, are involved in the establishment or progression of AML. The c-Kit mutation D816V, shown to be associated with mastocytosis, see below, was detected in 1 of 30 AML patients studied by PCR and restriction enzyme digestion (Ashman *et al.*, 2000). This patient had been classified as M2 AML and did not display mastocytosis in either the bone marrow or the peripheral blood. A similar mutation, D816Y, was detected in AML cells isolated from a patient with M2 AML and a massive presence of mast cells (Beghini *et al.*, 1998). Co-expression of c-Kit and SLF mRNA was reported in 17 of 30 AML cell lines tested by RT-PCR (Caceres-Cortes *et al.*, 1992; Pietsch, 1993) but was not detected using a quantitative RNase protection assay in 69 AML patient samples (Cole *et al.*, 1996). While a lot of investigation has been conducted, at this point in time, the role of c-Kit in AML remains unclear.

The role of SLF and c-Kit in lymphoid malignancies is less clear. c-Kit expression was detected by RT-PCR in T and B lymphoblastic cells from children with non Hodgkin's lymphoma or ALL. Surface expression of c-Kit was detected, by flow cytometry, on about 40% of fresh T-lymphoblastic cells or cell lines but not on fresh B-lymphoblastic cells or cell lines, or Burkitt's lymphoma (Sykora *et al.*, 1997; Tomeczkowski *et al.*, 1998). However, 10% of T-ALL and 11.5% of B-ALL were found to express c-Kit in newly diagnosed cases of childhood ALL (Legitimo *et al.*, 1999) and functional c-Kit has also been detected on a T-ALL cell line established from a paediatric patient (Kees and Ashman, 1995). All c-Kit positive cells downregulated c-Kit in response to SLF and only a few T-lymphoblastic cells proliferated in response to SLF (Kees and Ashman, 1995; Sykora *et al.*, 1997; Tomeczkowski

et al., 1998). This implies the c-Kit-SLF interaction may play a part in some acute lymphocytic malignancies.

Since activating mutations were found in the juxtamembrane domain of c-Kit in mastocytoma cell lines several groups have examined cells from mastocytoma patients for these same mutations. Elevated levels of mutant c-Kit mRNA expression have been detected in 25%-100% of human and canine mastocytomas (Buttner *et al.*, 1998; Kubo *et al.*, 1998; Nagata *et al.*, 1998; Sperr *et al.*, 1998; Worobec *et al.*, 1998a,b; London *et al.*, 1999; Longley *et al.*, 1999; Ma *et al.*, 1999; Nagata *et al.*, 1998). The majority of these mutations were D816V although 5 of 11 canine mastocytomas had duplications of exons 11 and 12, which are associated with constitutive phosphorylation of the receptor in *in vitro* transfection experiments (London *et al.*, 1999). These exons encode intracellular and juxtamembrane sequences, including part of the first kinase domain. Analysis of two patients with mastocytomas and AML found that the AML cells did not carry the D816V mutation and the authors concluded that the AML did not originate from the mutant mast cells.

In melanoma and cell lines derived from melanomas downregulation rather than overexpression of c-Kit has been reported (Lassam and Bickford, 1992; Natali *et al.*, 1992). In fact, enforced c-Kit expression by highly metastatic c-Kit negative melanomas, inhibited tumour growth and metastasis in nude mice. Exposure to SLF resulted in apoptosis of these cells but not of c-Kit negative cells (Zakut *et al.*, 1993; Huang *et al.*, 1996). These data suggest melanoma cells downregulate c-Kit expression to escape apoptosis induced by the SLF produced by keratinocytes and dermal fibroblasts. Loss of expression of c-Kit by metastatic melanoma cells correlates with downregulation of the transcription factor AP-2 (Huang *et al.*, 1998). The c-Kit promoter was shown to contain three functional binding sites for AP-2 and c-Kit mRNA and protein levels were upregulated in AP-2 transfected cells. Therefore the loss of AP-2 expression in these cells may be the crucial step in the development of malignant melanoma (Huang *et al.*, 1998).

Loss of c-Kit expression has been reported in 80-90% of breast cancers. Introduction of c-Kit gene into breast cancer cell line, MCF-7, inhibited anchorage dependent and independent growth, although not of the entire population (Nishida *et al.*, 1996). Additionally, loss of c-Kit expression by ovarian cancers is associated with poor prognosis.

Patients whose tumours did not express c-Kit had shorter disease free survival than patients who had c-Kit expressing tumours (Tonary *et al.*, 2000).

Several studies have examined c-Kit expression in gastrointestinal stromal tumours (GIST). The majority of GISTs were found to express c-Kit protein. Of the c-Kit positive tumours 20-60% of them contained deletions or point mutations in the cytoplasmic juxtamembrane domain (Ernst *et al.*, 1998; Hirota *et al.*, 1998; Sarlomo-Rikala *et al.*, 1998; Lasota *et al.*, 1999; Moskaluk *et al.*, 1999 and Taniguchi *et al.*, 1999). Expression of these mutant forms in Ba/F3 cells resulted in stable malignant transformation (Hirota *et al.*, 1998). While Ernst *et al.* (1998) found no correlation with tumour size, Taniguchi *et al.* (1999) found GISTs expressing mutant c-Kit were larger and more invasive. All studies found a correlation between mutant c-Kit expression and poor prognosis with fewer than 30% of patients surviving past 3 years. True leiomyomas and gastric schwannomas of the gastrointestinal and periphery did not express c-Kit. Therefore c-Kit expression was suggested to be an important marker of GIST and mutations in c-Kit might be used as a marker for recurrence or minimal residual disease (Sarlomo-Rikala *et al.*, 1998; Lasota *et al.*, 1999). Since GIST express both c-Kit and CD34 they may originate from the interstitial cell of Cajal (ICC) because the development of ICC is dependent on the interaction of SLF and c-Kit (Hirota *et al.*, 1998).

In certain solid tumours, apparently abnormal expression of c-Kit, together with appropriate expression of SLF completes an autocrine cycle that is likely to be involved in the oncogenic process. Co-expression of c-Kit and SLF mRNA and protein has been detected in tumour specimens and cell lines from breast cancer (Hines *et al.*, 1995; Turner *et al.*, 1992), cervical and ovarian tumours (Inoue *et al.*, 1994), small cell lung cancer (SCLC) (Hibi *et al.*, 1991; Krystal *et al.*, 1996; Turner *et al.*, 1992), 15% of primary and 18% of metastatic non-small-cell lung carcinomas (Pietsch *et al.*, 1998), a Schwann cell carcinoma (Ryan *et al.*, 1994), malignant testicular tissue (Strohmeyer *et al.*, 1995), human malignant glioma cell lines (Stanulla *et al.*, 1995), one of thirteen gastric carcinoma cell lines (Hassan *et al.*, 1998) and neuroblastoma tumours where abnormal expression of SLF may provide the autocrine loop (Cohen *et al.*, 1994). Despite the detection of mRNA and protein of both c-Kit and SLF in these cells, attempts to block proliferation using anti-c-Kit or anti-SLF antibodies in assays of soft agar colony formation or tritiated thymidine incorporation have been unsuccessful

except in neuroblastomas (Cohen *et al.*, 1994). Similarly, while five of five rhabdomyosarcoma cell lines express SLF and four of five express c-Kit mRNA, c-Kit protein could not be detected and the addition of anti-c-Kit or anti-SLF antibodies did not prevent *in vitro* cell growth (Landuzzi *et al.*, 1998). Most of the above studies utilised mRNA and protein detection to determine co-expression of c-Kit and SLF. However, since the reporting of c-Kit and SLF co-expression was rare (Ryan *et al.*, 1994; Strohmeyer *et al.*, 1995; Hassan *et al.*, 1998) and in some cases the proteins appeared to be non-functional (Stanulla *et al.*, 1995) the relevance of these findings is uncertain.

Whether or not the expression of c-Kit or SLF can be considered abnormal depends on the accurate knowledge of the cell type from which the tumour originated. In SCLC an autocrine cycle of c-Kit and SLF has been implicated in the disease, however the cell of origin is not known but the c-Kit promoter appears normal (G. Krystal, personal communication). Normal duct cells of the breast express c-Kit (Matsuda *et al.*, 1993), so that the expression of the c-Kit in breast cancer may be normal despite the fact that analysis of the tissue as a whole would suggest it is not. Normal human endothelial cells co-express c-Kit and SLF and the addition of exogenous SLF did not increase the proliferative response of these cells nor did the addition of an anti-c-Kit mAb inhibit cellular proliferation (Broudy *et al.*, 1994), leading the authors to suggest co-expression of c-Kit and SLF is not necessarily a transforming event.

In summary, c-Kit mutations detected in some cancers (e.g. some leukaemias, mastocytomas and GISTs) result in constitutive activation of the receptor and factor independent proliferation and appear to be involved in the pathogenesis of the disease. While autocrine cycles involving c-Kit and SLF have been demonstrated in some solid tumours, notably SCLC and breast cancer, their relationship to tumourigenesis is unclear. In melanomas c-Kit has been reported to be down-regulated on progression of the disease and enforced expression inhibits tumourigenicity. Thus the role of c-Kit in cellular transformation may depend on the expressing cell type.

1.10 Soluble Cytokine Receptors

Growth factors, cytokines and hormones exert potent biological effects on target cells. It is therefore not surprising that the activity of these molecules is highly regulated. A major

component of this regulation is the molecules' own production and secretion. However, other very important regulatory methods of growth factor activity exist. These include the cytokine's receptor expression and whether the cytokine is able to bind to the receptor. One method of preventing the growth factor from binding to the receptor is by inhibitors. These inhibitors can be receptor-binding antagonists that inhibit the binding of a cytokine to its receptor by competing for the same binding site (Eisenberg 1990, Hannum 1990), or cytokine binding proteins that inhibit the binding of a cytokine to its receptor by binding to free cytokine. For example, a family of six insulin-like growth factor (IGF)-binding proteins (IGFBPs) bind with high affinity for IGF-I and IGF-II. All six have been shown to inhibit IGF action by preventing the cytokine binding to the corresponding receptor (reviewed in Baxter, 2000).

In some cases, though not IGFBPs, the cytokine binding proteins are often soluble forms of the receptor that the cytokine would normally bind. These molecules contain the extracellular domain of the receptor but lack the transmembrane and cytoplasmic domains. The soluble molecules have the same tertiary structure as the full-length molecule and therefore bind to the cytokine with similar affinity. Thus, if the soluble receptor is present in sufficient local concentrations, by binding the cytokine, it prevents the cytokine from interacting with the native, cell bound molecule, thus preventing signal transduction. Refer to Table 1.1 for a list of some of the receptors that have been found in a soluble form.

Several methods of soluble receptor production exist. One method is shedding of the extracellular domain of the receptor from the cell surface via proteolytic cleavage. An exception is the ciliary neurotrophic factor receptor, CNTFR, which is derived from cleavage of a glycosyl phosphatidyl inositol linked membrane bound form by phospholipase C (Davis *et al.*, 1991; Davis *et al.*, 1993). The other is by generation of an alternatively spliced mRNA transcript that usually skips the exon encoding the transmembrane domain resulting in an incomplete mRNA that encodes a stop codon shortly after the sequence encoding the extracellular domain. Soluble receptors generated from alternate mRNA species include the GM-CSF receptor, G-CSF receptor, EGF receptor, Epo receptor, and the IL-7 receptor (Ullrich *et al.*, 1984; Fukunaga *et al.*, 1990; Goodwin *et al.*, 1990; Raines *et al.*, 1991; Barron *et al.*, 1994). However, the soluble receptors for IL-4, IL-5 and LIF are generated from

Table 1.1: Partial list of some of the receptors found naturally in a soluble form

Receptors	Generation	Association with disease	Therapeutic uses	References
C-KIT	unknown, some would argue both proteolytic cleavage and alternative splicing		Possible treatment of leukaemia Aid mobilisation of stem cells	
CNTF	cleavage of a glycosyl phosphatidyl inositol linkage by phospholipase C			Davis <i>et al.</i> , 1991
EGF	mRNA splicing	secreted by tumour cell line A431		Gunther <i>et al.</i> , 1990
EPO	mRNA splicing			
p185^{HER2}/neu	proteolytic cleavage	produced by HER2 overexpressing tumour cells, possible indicator of tumour aggressiveness but not a diagnostic marker of breast cancer		Pupa <i>et al.</i> , 1993
Fas	mRNA splicing	systemic lupus, angioimmunoblastic lymphadenopathy; altered lymphocyte development and increased proliferation in response to self antigens	HIV: to prevent HIV-Fas induced apoptosis of lymphocytes	Cheng <i>et al.</i> , 1994
FGF	unknown, many variant transcripts of FGFR detected	Not determined, possibly in pituitary tumours, bladder cancer and others		Hanneken <i>et al.</i> , 1994
G-CSF	mRNA splicing			Fukunaga <i>et al.</i> , 1990
GM-CSF	mRNA splicing			Raines <i>et al.</i> , 1991
IFN-α	mRNA splicing			Abramovich <i>et al.</i> , 1994
IFN-γ	proteolytic cleavage		Treatment of immunological and inflammatory diseases	Fountoulakis <i>et al.</i> , 1995
IL-1 and IL-1RA	proteolytic cleavage		reduce autoimmune disease, immune mediated neurological disorders e.g. rejection of heart	Estrov <i>et al.</i> , 1992; Fanslow <i>et al.</i> , 1990 Symons <i>et al.</i> , 1991

			<p>allografts and IL-1 induced B cell function, IL-1 induced B cell proliferation</p> <p>Inhibit the proliferation of AML and CML blasts in the presence or absence of growth factors, no effect on normal progenitors; but IL-1RA blocks proliferation of CFU-S day 12 in regenerating bone marrow, and reduced the number of CFU-GM and BFU-E and increased the number of CFU-E</p> <p>At conc. of 0.1-100ng/ml enhanced AML cell growth at higher conc. it was inhibitory to effects of IL-1α and IL-1β, no effect in absence of IL-1 β (100-200 fold excess required to negate effects of IL-1</p>	<p>Jovcic <i>et al.</i>, 1996</p> <p>Maliszewski <i>et al.</i>, 1990</p> <p>Symons <i>et al.</i>, 1995</p> <p>Carter <i>et al.</i>, 1994</p>
IL-2	proteolytic cleavage	<p>Inflammatory and neoplastic conditions; myocardial infarction, asthma, myasthenia gravis, acute T-cell leukaemia, acute lymphocytic leukaemia, hairy cell leukaemia, Hodgkin's disease, chronic lymphocytic leukaemia, colon cancer, breast cancer, ovarian cancer, suicide autoimmune disease, rheumatoid arthritis.</p>		<p>Pui <i>et al.</i>, 1988</p> <p>Stasi <i>et al.</i>, 1995</p>
IL-4	mRNA splicing		<p>prevent allograft rejection</p> <p>Block cytokine induced B cell functions: blocked proliferation,</p>	<p>Mosley <i>et al.</i>, 1989</p>

			CD23 and Ia cell surface Ag expression and IgG ₁ and IgE secretion	
IL-5	mRNA splicing			Tavernier <i>et al.</i> , 1989
IL-6	proteolytic cleavage	plasma cell dyscrasias, monoclonal gammopathy, multiple myeloma, AIDS, rheumatoid arthritis		Mullberg <i>et al.</i> , 1993
gp130				Narazaki <i>et al.</i> , 1993
IL-7	mRNA splicing			Goodwin <i>et al.</i> , 1990
IL-9	mRNA splicing			Renauld <i>et al.</i> , 1992
LIF	mRNA splicing			Layton <i>et al.</i> , 1992
M-CSF	proteolytic cleavage			Downing <i>et al.</i> , 1989
NGF	proteolytic cleavage			Zupan and Johnson, 1991
PDGF	proteolytic cleavage			Duan <i>et al.</i> , 1991; Tiesman and Hart, 1993
TNF	proteolytic cleavage	Malignancies: cancer of GI tract, breast, lung, hairy cell leukaemia, chronic lymphatic leukaemia, autoimmune diseases; HIV, gram-negative bacterial sepsis.	Abrogate TNF mediated injury At low concentrations (200-400ng/ml) causes stabilisation of TNF and enhances its effect on AML cells. At high (1-2ug/ml) concentrations inhibits TNF action on these blast cells 200 fold excess does not completely abrogate the TNF effects. Inhibit LPS induced local acute inflammatory reaction, although injected at same time as LPS. As good as IL-1ra. Authors suggest role in treating autoimmune diseases, transplant rejection, GVHD.	Carter <i>et al.</i> , 1994 Ulich <i>et al.</i> , 1993

alternatively spliced mRNA transcripts that include an exon that directs protein chain termination prior to the transmembrane domains (Mosley and Beckmann, 1989; Tavernier *et al.*, 1992; Tomida *et al.*, 1994). It is possible that some soluble receptors may be generated by both alternative mRNA transcripts and proteolytic cleavage where the alternate transcript encodes the proteolytic cleavage site, similar to the generation of soluble SLF (Flanagan *et al.*, 1991).

Despite considerable research into soluble receptors their function remains unclear. The matter is clouded further by the observation that some soluble receptors may have more than one role. Four different models of soluble receptor function have been proposed (reviewed in Heaney and Golde, 1996) and these are depicted in Figure 1.4.

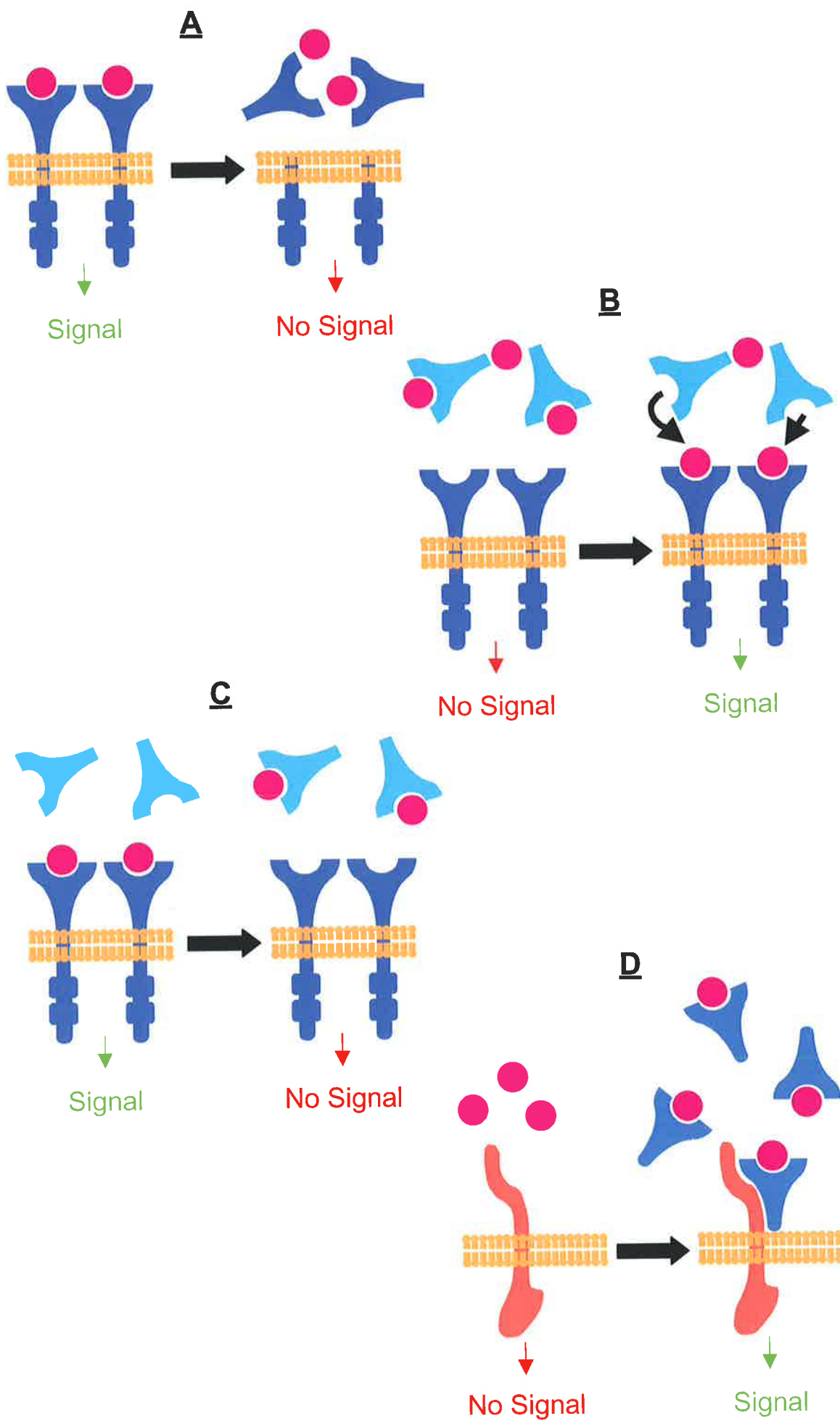
The first model, Figure 1.4 (A), proposes soluble receptors are generated as a by-product of receptor activation and downregulation. Upon binding of the cytokine, aspects of the signal cascade induced by this initial interaction, feedback to the receptor. Proteolytic cleavage of the receptor results, releasing the ligand-receptor complex from the cell surface. This prevents further signalling via the receptor/ligand complex. This method of downregulation of the signalling cascade maybe in addition to internalisation and degradation of the receptor/ligand complex. An example of this is the CSF-1R that is cleaved by a protease induced by PKC activation (Downing *et al.*, 1989).

In another model the role of the soluble receptor is to prevent the degradation of the cytokine in the extracellular environment, including serum (Figure 1.4 (B)). The soluble receptors may act as a chaperone protein that binds the ligand in solution and carries it through the circulation, possibly protecting it from proteolytic degradation thereby prolonging its half-life or ensuring its delivery to the membrane associated receptor expressed by target cells (DiStefano and Johnson, 1988).

The third model hypothesises that the soluble receptor is an antagonist of the receptor ligand interaction (Figure 1.4 (C)). In this model, soluble receptors appear to represent a biological mechanism for selectively and efficiently inhibiting cellular responsiveness to specific mitogenic and differentiation factors. The soluble receptor directly competes with the membrane-associated receptor for ligand binding thus functioning as a specific high-affinity antagonist. If the affinities of the membrane associated and soluble receptors are similar, the

Figure 1.4: Mechanisms of soluble receptor action. **A:** Generation of soluble receptors by proteolytic cleavage to down-modulate membrane-bound receptor numbers and prevent signal generation by ligand-receptor interaction. **B:** Soluble receptors serving as binding proteins to stabilise ligand in the extracellular space. **C:** Soluble receptors compete with membrane-bound receptors for ligand to decrease receptor-mediated signal generation. **D:** Soluble receptors associating with non-ligand-binding receptor units and ligand to confer ligand sensitivity to cells that do not express membrane bound receptor.

Adapted from Heaney and Golde (1993).



ability of the soluble receptor to inhibit signalling depends on the relative concentrations of the two receptors. Many soluble receptors have been shown to be able to prevent the cytokine from interacting with the membrane associated receptor and thereby prevent membrane associated receptor activity. While such inhibition may be a function of soluble receptors of single subunit receptor systems it is uncertain whether this is the case in multiple subunit receptor systems e.g. the IL-3, IL-5 and GM-CSF family. In these cases the soluble receptor generally has a low affinity for ligand and therefore is unlikely to effectively compete for ligand against the multiple subunit high affinity membrane bound receptor.

Not all soluble receptors can be considered inhibitors since some soluble receptors can trigger a biological response. In the fourth model, the soluble receptor is able to bind to the ligand and the signalling chain of the receptor complex (Figure 1.4 (D)). This activity is seen in the IL-6 receptor family. This family comprises a multi-subunit receptor, centred on a common subunit, gp130. Soluble IL-6 receptor has been shown to be able to bind to IL-6 and induce signalling in cells only expressing gp130 (Taga *et al.*, 1989). Soluble CNTF receptor may have similar activity. Hence these factors can stimulate responses in cells that do not express all the components of the signalling receptor, expanding the range of cells that are targets for these factors. The control of activity of IL-6 family members is further complicated by the discovery of a soluble gp130 (sgp130) molecule in serum. The native sgp130 was capable of binding to sIL-6R when IL-6 was added. sgp130 was able to inhibit IL-6 action mediated by sIL-6R and this action could be extended to oncostatin M (OSM), LIF and CNTF (Narazaki *et al.*, 1993).

1.10.1 Inhibitory effects of soluble receptors

The discovery of naturally occurring soluble receptors lead Fernandez-Botran, (1991), to suggest that soluble receptors could be used as specific and potent immunosuppressors without the non-specific affects of drugs currently in use. Fernandez-Botran suggested that these molecules would be superior to anti-cytokine antibodies in many ways including having 100-1000 fold higher affinities and, by being smaller, allowing better distribution. Furthermore, soluble receptors do not have structures that would interact with other cells, e.g. the Fc domain of immunoglobulins, and would not be recognised as foreign by the immune

system as would an antibody generated in another species, although humanisation and genetic manipulation of antibodies has overcome some of these problems. The usefulness of soluble receptors *in vivo* is further illustrated by the discovery that soluble homologues of IL-1R, IFN- γ R and TNFR are expressed by viruses. These molecules increase virulence apparently by depressing the host cytokine response to the invading pathogens (Smith *et al.*, 1991; Spriggs *et al.*, 1992; Upton *et al.*, 1992).

Fernandez-Botran provided examples where soluble receptors could be used as therapeutic tools, including the treatment of some malignancies where the tumour cells use cytokines as autocrine growth factors, e.g. lymphoid malignancies (IL-2) and multiple myelomas, (IL-6); treating hypersensitivity with sIL-4R and inflammation in disease such as rheumatoid arthritis with sIL-1R.

1.10.2 Soluble IL-1 Receptor

It has been shown that autocrine production of IL-1 by AML blasts supports neoplastic growth (Sakai *et al.*, 1987, Cozzolino *et al.*, 1989) and maintains autocrine synthesis of G-CSF and GM-CSF (Cozzolino *et al.*, 1990) by these cells. Researchers have examined the potential clinical benefits of IL-1 inhibition in AML using a soluble IL-1 receptor (sIL-1R). Soluble IL-1 receptor, sIL-1R, is a truncated IL-1 receptor that exists in solution as a monomer and binds 1 mol of IL-1 per mol of soluble receptor. It has been shown that sIL-1R as well as IL-1 receptor antagonist, IL-1Ra, a naturally occurring analogue of IL-1, are capable of inhibiting AML blast colony proliferation in a dose dependent manner ranging from 10 to 500 ng/ml for sIL-1R. When added at 100ng/ml sIL-1R inhibited acute myeloid leukaemia-colony forming unit (AML-CFU) growth 3% to 96% in the absence of growth factors and in the presence of factors such as phytohemagglutinin leukocyte conditioned medium (PHA-LCM), G-CSF, GM-CSF, IL-3, and SLF (Estrov *et al.*, 1992). However, sIL-1R was more effective in the absence of the growth factors suggesting that IL-1 is not essential for cell growth. The inhibitory effect was partially reversed by the addition of IL-1 β , thus proving sIL-1R specificity (Estrov *et al.*, 1992). Another group achieved similar results although the inhibitory effects of sIL-1RI were only seen at concentrations greater than 100ng/ml. At lower concentrations stimulatory effects were seen (Carter *et al.*, 1994). A phase I study of

the effects of sIL-1R in 11 patients with relapsed and refractory AML showed sIL-1R was well tolerated at concentrations up to 1000 $\mu\text{g}/\text{m}^2/\text{day}$ for 13 days and a maximum tolerated dose was not achieved (Bernstein *et al.*, 1999). The half-life of sIL-1R after intravenous dosing was 7-12h and 2-4 days after subcutaneous dosing. However, at the doses administered no apparent anti-leukaemic effect was seen.

In addition to its effects on AML cells, sIL-1R has been used to treat immunologically linked diseases. Soluble IL-1 receptor has been shown to inhibit IL-1 induced B cell proliferation (Maliszewski *et al.*, 1990) and by blocking the inflammatory response invoked by IL-1, sIL-1R was able to prolong the survival of heterotopic heart allografts in mice from 12 days to 17 days, although the authors suggest that co-treatment with antagonists of the IL-2 receptor may improve graft survival time (Fanslow *et al.*, 1990). Experimental autoimmune encephalomyelitis, exacerbated by IL-1 α , could be suppressed by daily administration of sIL-1R but best results were observed when the treatment was initiated at the same time as the encephalomyelitis (Jacobs *et al.*, 1991). IL-1 stimulated glycosaminoglycan accumulation is a characteristic of orbital connective tissues from patients with Grave's ophthalmopathy. Treatment of orbital fibroblasts cultures with either IL-1Ra or sIL-1R inhibited IL-1 (10U/ml) induced glycosaminoglycan synthesis, with complete inhibition observed using sIL-1R at 1 $\mu\text{g}/\text{ml}$, (a 1440 fold molar excess) (Tan *et al.*, 1996). This suggests IL-1Ra and/or sIL-1R may be useful in the prevention or treatment of Grave's ophthalmopathy. Soluble IL-1RI decreased the severity of chills induced by an inflammatory response to endotoxin in humans but did not alter other symptoms associated with this disease. The lack of effect was proposed to be due to interaction with IL-1Ra, a point examined by Burger *et al.* (1995). IL-1Ra described by Hannum (1990) and cloned by Eisenberg (1990) is the only receptor antagonist described so far. IL-1Ra binds specifically to IL-1 receptors preventing either isoform of IL-1 (α or β) from binding to the receptor and subsequently transducing a signal. It has been shown that IL-1Ra and sIL-1RI are able to interact with high affinity and neutralise each other's inhibitory action (Svenson *et al.*, 1993; Burger *et al.*, 1995, Symons *et al.*, 1995). Since IL-1Ra and sIL-1RII do not interact (Symons *et al.*, 1995), Burger *et al.* (1995) suggested that this combination would be the preferred treatment regime.

IL-1 production by peripheral blood mononuclear cells has been shown to up regulate HIV expression *in vitro*, presumably by activation of the HIV reverse transcriptase (Poli *et al.*, 1994). sIL-1R has been shown to inhibit HIV expression in acutely infected primary PBMC and the cell line U1. A phase I/II trial of sIL-1R was conducted in 12 HIV infected individuals with CD4⁺ cell counts lower than 300/ μ l (Takebe *et al.*, 1998). Patients were injected subcutaneously with up to 1250 μ g/m² 3 times a week for 8 weeks. While seven patients reported symptomatic relief and the sIL-1R was safe and well tolerated at the doses tested, no consistent changes in objective markers of HIV disease were observed.

1.10.3 Soluble IL-4 Receptor

Treatment of B cells *in vitro* with soluble IL-4 receptor (sIL-4R), cloned by Mosley *et al.* (1989), resulted in decreased B cell proliferation and IL-4 dependent B cell differentiation as measured by induction of IgG₁ and IgE secretion by LPS blasts, downregulation of IgG₃ secretion and increased Ia and Fc ϵ R expression (Maliszewski *et al.*, 1990). Administration of a sIL-4R prolonged the survival of heterotopic cardiac allografts from 11 days in controls to 15 days in treated mice and inhibited, in a dose dependent manner, popliteal lymph node hyperplasia that occurred as a local response to injected allogeneic cells. This inhibition could be reversed by simultaneous administration of IL-4, demonstrating the effect of sIL-4R was specific (Fanslow *et al.*, 1991). Despite these encouraging results one cannot ignore the failure of shIL-4R transgenic mice to inhibit an IgE response (Devos *et al.*, 1993). This may be due to insufficient concentrations of soluble receptor to block the locally produced high levels of IL-4 (Devos *et al.*, 1993).

Endogenous serum levels of sIL-4R increase during immune responses characterised by T cell activation and increased IL-4 production, consistent with the idea that endogenous sIL-4R is involved in the regulation of IL-4 activity during immune responses (Fernandez-Botran *et al.*, 1996a). This same group demonstrated sIL-4R prolonged the half-life of IL-4 injected into mice, maintained IL-4 activity and reduced its clearance through renal excretion (Ma *et al.*, 1996). These effects are consistent with the ability of sIL-4R to potentiate IL-4 activity *in vivo* (Ma *et al.*, 1996). Thus, sIL-4R has been shown to have both agonistic and antagonistic activities, depending upon the relative concentration of sIL-4R and IL-4.

1.10.4 Soluble TNF Receptor

The activity of soluble TNF receptors (sTNFR) has been studied in a number of systems. Increased circulatory levels of sTNFR have been detected in patients with gastrointestinal tract, breast and lung cancers as well as hairy cell leukaemia and chronic lymphocytic leukaemia (Aderka *et al.*, 1991; Digel *et al.*, 1992). It would appear that the soluble receptor has a contradictory role. At low concentrations recombinant sTNFR binds to the factor and prolongs its half-life. However, at high concentrations the sTNFR inhibited AML cell proliferation *in vitro* (Carter *et al.*, 1994) and TNF induced proliferation of U-937 cells (Higuchi and Aggarwal, 1992). A dimeric sTNFR, was capable of inhibiting TNF α induced cytotoxicity of murine cells and protected mice from induced endotoxic shock depending on the time and dose of injection (Ashkenazi *et al.*, 1991). In humans, increased levels of sTNFR were detected in patients critically ill with endotoxemia and these levels were capable of blocking the *in vitro* cytotoxicity of TNF α (Van Zee *et al.*, 1992). In other experiments, dimeric sTNFR was able to block TNF α induced activation of HIV reverse transcriptase in both monocytes and lymphocytes (Howard *et al.*, 1993). Transgenic mice expressing sTNFR were protected against TNF induced bone loss caused by oestrogen deficiency (Ammann *et al.*, 1997). More recently efforts have concentrated on inhibiting the TNF induced effects of rheumatoid arthritis and the elevated TNF toxicity in patients treated with IL-2. Patients with metastatic cancer treated with high dose IL-2 were subjected to a phase I trial using a sTNFR:human IgG chimera (sTNFR:HuIgG). 21% of the patients showed some form of response, including inhibition of TNF bioactivity and reduction in a polymorphonuclear leukocyte chemotactic defect, suggesting that sTNFR:HuIgG may modulate some of the toxic effects of IL-2 while preserving its antitumour action (Trehu *et al.*, 1996). However, a follow up randomised, placebo controlled trial of this same molecule in patients with advanced melanoma and renal carcinoma failed to show a reduction in the clinical toxicity associated with high dose IL-2 therapy (du Bois *et al.*, 1997).

1.10.5 Soluble PDGF Receptor

In diseases such as atherosclerosis, osteosarcoma and glioblastoma where overproduction of PDGF may be involved in the pathogenesis of the disease, it has been suggested that the

development of PDGF antagonists, for example, soluble PDGF receptor, sPDGFR, may lead to new therapeutic approaches (Duan *et al.*, 1991). The authors demonstrated sPDGFR β was capable of inhibiting the actions of BB-PDGF in fibroblast tissue cultures. The soluble form of the receptor was able to dimerize in a ligand dependent manner and block the binding of ^{125}I -BB-PDGF to membrane bound receptor expressed on CHO cells. sPDGFR β specifically inhibited BB-PDGF induced ^3H -thymidine incorporation into 3T3 fibroblast cells despite the fact that the soluble molecule had a lower affinity for the ligand than native membrane bound form requiring an excess of 10 fold to give half maximal inhibition (Duan *et al.*, 1991). A naturally occurring soluble PDGFR α has been detected in human serum and the conditioned medium of smooth muscle cells although an inhibitory action was not demonstrated (Tiesman and Hart, 1993). At this time, reports of sPDGFR being tested *in vivo* have not been published.

1.10.6 Soluble VEGF Receptor (FLT-1)

Vascular endothelial cell growth factor, VEGF, is a selective mitogen for vascular endothelial cells, promoting angiogenesis and vascular permeability. VEGF expression is elevated in a wide variety of solid tumours and is thought to support their growth by enhancing tumour neovascularisation. Its receptor on these cells is the CSF-1R-like tyrosine kinase, FLT-1. A naturally occurring soluble version of this receptor, sFLT-1, has been detected in the supernatants of cultured human umbilical vein endothelial cells (HUVEC) (Kendall and Thomas, 1993) and is generated by alternate splicing of the mRNA. The endogenous sFLT-1 is chromatographically and immunologically similar to recombinant sFLT-1 and binds ^{125}I -VEGF with a comparable affinity (Kendall *et al.*, 1996). The authors also showed human sFLT-1 is capable of forming a VEGF stabilised complex with KDR, the VEGF receptor 2, *in vitro*, suggesting sFLT-1 is capable of forming ligand induced heterodimeric complexes with mitogenically competent full length KDR receptor and creating a dominant negative complex (Kendall *et al.*, 1996). Initially, cloned recombinant sFLT was shown to inhibit the proliferation of HUVECs *in vitro*, (Kendall and Thomas, 1993) and the authors suggested that sFLT could be used therapeutically to inhibit the vascularization of tumours and perhaps blood-borne tumour metastasis. Since then recombinant murine sFLT-1

has been used to inhibit the growth of transplanted R3230AC cells by 75% and vascular density by 50% when administered concomitantly (Lin *et al.*, 1998). HT-1080 human fibrosarcoma cells transfected with a cDNA construct encoding sFLT-1 were inhibited from implanting and growing in the lungs of nude mice (Goldman *et al.*, 1998). In addition, survival was significantly prolonged in mice injected intracranially with human glioblastoma cells stably transfected with the gene encoding sFLT-1. It was assumed that sFLT-1 was able to form dominant negative heterodimers with membrane bound VEGF receptors. More recently, recombinant adenovirus expressing the entire extracellular domain of human FLT-1 fused to the Fc portion of human IgG was shown to inhibit VEGF induced DNA synthesis in endothelial cells (Takayama *et al.*, 2000). Injection of this construct into skeletal muscle of nude mice prevented the growth, after 10 days, of 5 of 6 lung cancer cell lines. Tumour size decreased, angiogenesis was suppressed and increased apoptosis was evident (Takayama *et al.*, 2000). These results suggest sFLT-1 may play an effective role in anti-angiogenic therapy for cancers and other disease states dependent upon angiogenesis.

1.10.7 Soluble IL-6 Receptor family

In contrast to the predominantly inhibitory effects of sIL-1R, sIL-4R, sTNFR, sPDGFR and sFLT-1R the soluble IL-6 receptor, sIL-6R, has been shown to have both inhibitory and stimulatory effects (Iglesias, 1995). The IL-6 receptor complex consists of a low affinity, ligand binding peptide and a signal-transducing peptide, gp130, which does not bind IL-6 but together these molecules form the high affinity receptor complex. Soluble versions of both the ligand binding peptide and gp130 have been detected in the urine of normal individuals (Novick *et al.*, 1989; Honda *et al.*, 1992; Narazaki *et al.*, 1993; De Benedetti *et al.*, 1994; Iglesias *et al.*, 1995) and have also been found in the conditioned medium of human myeloma cell lines, activated peripheral blood mononuclear cells and HIV sero-positive blood donors (Iglesias *et al.*, 1995), suggesting a role for this molecule in the pathogenesis of diseases. Elevated levels of IL-6 and sIL-6R detected in HIV patients may be involved in the development of AIDS associated Kaposi's sarcoma. The Kaposi's sarcoma cells express gp130 and proliferated *in vitro* when sIL-6R was added to the culture (Murakami-Mori *et al.*, 1996). Addition of IL-6 to the culture further augmented cell growth. sIL-6R significantly

stimulated the proliferation of three human papilloma virus (HPV) immortalized cell lines and four carcinoma cell lines in the presence of IL-6 but inhibited the growth of epithelial cells derived from normal ectocervix. In fact, the cells that respond to sIL-6R also produce it. This suggests that sIL-6R provides a selective advantage for premalignant HPV-immortalised cells and carcinoma cell lines (Iglesias *et al.*, 1995). The mode of action of the sIL-6R may be deduced from the effect of human IL-6 in human soluble IL-6 receptor (hsIL-6R) transgenic mice (Peters *et al.*, 1996). Compared to control animals, much lower levels of injected hIL-6 resulted in an acute phase protein gene induction in transgenic mice. This action was due to the prolonged half-life of the IL-6 molecule suggesting that the sIL-6 receptor exerts its agonistic activity in part by preventing IL-6 degradation. It has also been shown that the IL-6/sIL-6R complex is capable of binding to the gp130 protein and inducing signalling via the gp130 protein (Mackiewicz *et al.*, 1992; Yasukawa *et al.*, 1992). In fact, it has been reported that heart muscle cells only respond to IL-6 in the presence of sIL-6R (Hirota *et al.*, 1995). In contrast to its potential agonistic activity, a therapeutic role for sIL-6R was shown with the administration of IL-6 and sIL-6R having a neuroprotective activity in delaying progression of wobbler mouse motor neuron disease (Ikeda *et al.*, 1996).

1.10.8 Soluble Cytokine Receptors in Disease

The therapeutic potential of soluble receptors may not be as great as first thought. Many studies are reporting the detection of elevated levels of soluble receptors in human disease states. There is increasing evidence that soluble receptors such as sIL-2R, sIL-6R and sTNFR may contribute to disease (reviewed by Fernandez-Botran *et al.*, 1996b; Heaney and Golde, 1996) (see also Table 1.1). Elevated levels of sIL-2R have been detected in a wide variety of conditions including myocardial infarction, asthma, rheumatoid arthritis (Symons *et al.*, 1988), systemic sclerosis (Steen *et al.*, 1996), childhood primary malignant neoplasms (Bodey *et al.*, 1996), parasite infections, acute T-cell leukaemia, hairy cell leukaemia, Hodgkin's disease, chronic lymphocytic leukaemia, colon cancer, breast cancer and ovarian cancer (Heaney and Golde, 1996 and references therein). In CLL, hairy cell leukaemia and Hodgkin's disease increased levels of sIL-2R correlated with disease stage, tumour burden (Ambrosetti *et al.*, 1989; Gause *et al.*, 1991; Musolino *et al.*, 1991) and survival in ATL (Kamihara *et al.*,

1994) but it was not clear whether the soluble receptor was produced by the tumour cells or from associated inflammatory responses. It is possible that the soluble receptor may depress antitumour responses (Pui *et al.*, 1988) and suppress immune responses to infectious diseases and autoimmune disease.

Soluble TNF receptor levels are elevated in the serum of patients suffering several diseases including HIV infection (Kalinkovich *et al.*, 1992) and gram-negative bacterial sepsis (Froon *et al.*, 1994). Initially the sTNFRs are able to neutralise the effects of TNF but this effect is limited and once saturated, sTNFR is unable to oppose TNF activity (Giardin *et al.*, 1992).

Increased levels of soluble IL-6 receptor have been detected in patients with monoclonal gammopathy and early multiple myeloma. In patients with advanced multiple myeloma levels are almost twice that of normal and correlate with poor survival (Gaillard *et al.*, 1993; Kyrtsolis *et al.*, 1996; Pulkki *et al.*, 1996; Papadaki *et al.*, 1997). Treatment of 12 patients with a neutralizing anti-IL-6 monoclonal antibody failed to alter the serum level of either sIL-6R or sgp130 suggesting that there is no correlation between IL-6 activity and the soluble receptor levels (van Zaanen *et al.*, 1998). sIL-6R was shown to induce proliferation of 6 of 16 AML samples and all but one of the 16 also produced sIL-6R (Saily *et al.*, 1999). Since AML cells are provided with exogenous IL-6 and sIL-6R in the bone marrow microenvironment and since many also express both themselves, it is possible that signalling through the IL-6/sIL-6R complex plays a role in maintaining AML growth *in vivo* (Saily *et al.*, 1999). It is also possible that sIL-6R is associated with hypercalcemia and bone lesions of multiple myeloma since murine sIL-6R enhanced IL-6 mediated osteoclast formation in murine bone marrow cultures (Tamura *et al.*, 1993).

1.11 Naturally occurring soluble c-Kit (KIT^S)

Naturally occurring soluble c-Kit was first detected by ELISA in the culture supernatant of HUVEC grown for 2 and 4 days at high density (Broudy *et al.*, 1994). The amount of soluble receptor increased from 40pg/ml (1.4×10^6 cells) to 460pg/ml (1.8×10^6 cells), over this time and exceeded the amount of soluble SLF produced by the cells over the same time period, 78pg/ml and 107 pg/ml respectively (Broudy *et al.*, 1994). Vascular endothelial cells

are not the only cell type to be shown to produce KIT^S. KIT^S was detected in the culture supernatant of several human leukaemia cell lines, OCIM1, M07e, HEL and CMK (Turner *et al.*, 1995; Kawakita *et al.*, 1995). Levels detected, by enzyme immunoassay (EIA), after 96 hours from OCIM1, M07e and HEL cell lines were 12.7 ng/ml, 34.1 ng/ml and 12.3 ng/ml respectively. Production of soluble c-Kit by normal human endothelial cells may serve to regulate the bioactivity of both the soluble and transmembrane forms of SLF within the bone marrow microenvironment (Broudy *et al.*, 1994).

Like other soluble receptors KIT^S can be detected by ELISA in the serum of normal individuals (mean value of 324ng/ml \pm 105ng/ml, range of 163-788 ng/ml, n=112, Wypich *et al.*, 1995). There was no significant difference between males and females but KIT^S levels were higher in those donors less than 30 and lower in those over 50 years (Wypich *et al.*, 1995). KIT^S was also be detected in follicular fluid from adult ovaries (Tanikawa *et al.*, 1998).

The amount of KIT^S in serum of patients with a variety of haematological disorders varied greatly depending on the disease. Patients with lymphoid malignancies showed a decrease in serum KIT^S levels compared to normal individuals. There was also a decrease in KIT^S levels in patients with aplastic anaemia but this was not statistically significant. Patients with acute phase CML had greatly elevated levels. In AML the level of KIT^S in the serum from patients with immature phenotypes of leukaemic blasts was higher than that of controls or patients with more mature phenotypes. It appears that patients with MDS have low levels of KIT^S but as the disease progresses to leukaemia the levels rise (Kawakita *et al.*, 1995). The results are summarised in the Table 1.2 below. In graft versus host disease (GVHD) KIT^S levels in serum were significantly lower in patients with moderate to severe acute GVHD (grade II-IV) compared to those with no or mild acute GVHD (grade 0-I) (Hashino *et al.*, 1995).

Table 1.2: Serum KIT^S levels in patients with haemopoietic disorders (from Kawakita *et al.*, 1995).

Disease	Phenotype	Median (AU/ml) ^{a,b}	Range	Number of patients (n=)
Healthy		199	127-313	51
Lymphoid	ALL, CLL, ATL	106	79-171	14
Aplastic Anaemia		133		15
AML	M0 or M1	361	141-1376	12
	M2	196.5	112-535	14
	M3	233.5	148-315	10
	M4	210	75-332	10
	M6	255	113-258	3
	M7	222	183-533	3
CML	chronic	189.5	52-409	14
	blast crisis	725	221-1470	4
MDS	refractory anaemia	129.5		8
	refractory anaemia with excess blasts	186		5
	refractory anaemia with excess blasts in transformation	273		5
	overt leukaemia	300		5

^aarbitrary units per ml

^bmeasured by ELISA

The wide range of values and small number of patients within each group make these data difficult to interpret.

1.11.1 Origin of serum KIT^S

The KIT^S detected in serum has a calculated molecular weight of 95-98kDa and, when deglycosylated, has an apparent molecular weight of 56kDa (Wypich *et al.*, 1995). The weight lost due to deglycosylation matches that lost by the full length receptor when deglycosylated (Reith *et al.*, 1991). An immunoreactive 80kDa species was also seen and this may be derived from the incompletely glycosylated c-Kit precursor which has a molecular weight of about 125kDa (Blume-Jensen *et al.*, 1991; Funusaka *et al.*, 1992). The molecular weight of 98kDa suggests that KIT^S consists of the entire extracellular domain of the c-Kit. Since no alternate mRNA transcripts that encode only the extracellular domain of c-Kit have been detected, it is presumed that KIT^S is generated by proteolytic cleavage of the full length, membrane bound receptor. It has been suggested that the GNNK sequence that is present in one isoform c-Kit (see section 1.2.1) may form part of the proteolytic cleavage site but at present there is no published evidence for this (Wypich *et al.*, 1995) despite the statements of Heaney and Golde (1996). This hypothesis appears based on the observation that PKC has been shown to activate a specific cleavage of the CSF-1 receptor, a related molecule to c-Kit,

at a site just outside the transmembrane domain (Downing *et al.*, 1989). The idea that KIT^S is formed by post-translational processing is supported by pulse chase experiments which showed an immunoreactive 120kDa protein initially being converted to a 130 kDa protein two hours after biosynthetic labelling, with no 98kDa protein detectable in the supernatant. The 98kDa protein was not detected in the cell lysate by immunoprecipitation with c-Kit specific antibodies but did appear in the culture supernatant 5 hours after pulse chase, and continued to increase over 30 hours (Turner *et al.*, 1995). Their data suggest 98kDa protein is generated from the 130kDa protein.

Several groups have looked at the method of KIT^S production. Experiments were based on the hypothesis that KIT^S is generated as a means of down regulating the receptor. KIT^S was detected in the culture supernatants of murine bone marrow derived mast cells (Yee *et al.*, 1993), and factor dependent cell lines M07e and TF-1 (Brizzi *et al.*, 1994). Although KIT^S could be detected in the culture supernatant of unstimulated mast cells, the amount of KIT^S detected in the supernatant was increased by treatment of the cells with PMA or calcium ionophore (Yee *et al.*, 1993). In contrast, KIT^S was only detected in the culture supernatant of M07e, TF-1 and c-Kit transfected CHO cells, after the cells had been stimulated with PMA (Brizzi *et al.*, 1994). However, the latter observation does not concur with data from another group (Turner *et al.*, 1995). The release of KIT^S from the cell surface therefore appears to be mediated by PKC, since PMA and calcium ionophore are both activators of PKC. PKC activation has previously been shown to induce proteolytic cleavage of CSF-1 receptor (Downing *et al.*, 1989) and SLF (refer to section 1.3).

A 100kDa anti-c-Kit immunoreactive protein, lacking an intracellular kinase domain, was detected in association with M07e cells (Brizzi *et al.*, 1994). This protein had the ability to bind ligand as demonstrated by covalent crosslinking experiments performed on intact cells. The authors suggest there are two forms of truncated extracellular domain of c-Kit, both called p100^{c-kit}. One is the soluble form the other is membrane anchored but does not have an intracellular domain. They also suggest that the soluble form may stimulate SLF expressing cells and the truncated membrane associated form may be involved in adhesion (Brizzi *et al.*, 1994). A 50kDa protein corresponding to the intracellular domain of the cleaved molecule was not detected and they therefore suggest that the soluble c-Kit is not generated by

proteolytic cleavage. However, there are no alternate mRNA transcripts detectable and this is in disagreement with the data of Turner *et al.* (1995). The observation of a membrane associated truncated c-Kit molecule should be treated with caution since a similar molecule has not been reported by other researchers.

Experiments with murine bone marrow derived mast cells carrying mutations in the intracellular domain investigated the requirement for kinase activity for KIT^S release. *W⁴²*, which lacks kinase activity, and two mutants with tyrosine residues replaced with phenylalanine at positions 719 or 821 showed while that kinase activity of c-Kit was required for SLF induced receptor internalisation, it was not required for PMA induced, PKC dependent, proteolytic cleavage of c-Kit and the release of KIT^S into the supernatant (Yee *et al.*, 1994).

1.11.2 Activity of soluble c-Kit

Several groups using different sources of KIT^S have studied the activity of KIT^S. The data are summarised in Table 1.3 below.

Table 1.3: Source and activity of KIT^S used *in vitro*

Source of KIT ^S	Purity	Affinity for SLF	Assay	Author
Full length c-Kit	N.D. ^a	1nM	Scatchard	Huang <i>et al.</i> , 1990
Serum	20%	6nM	Scatchard	Wypich <i>et al.</i> , 1995
Serum	N.D.	300pM	Scatchard	Turner <i>et al.</i> , 1995
Recombinant	80-90%	0.7nM	Scatchard	Lev <i>et al.</i> , 1992
Recombinant	98%	1-2nM	Scatchard	Turner <i>et al.</i> , 1995
Culture supernatant of OCIM1 cells	N.D.	200pM	Scatchard	Turner <i>et al.</i> , 1995

^a N.D. = not determined

The data suggest that at least some of the KIT^S detected in serum is functional. The levels of KIT^S in serum are quite high compared to the levels of soluble SLF (33 fold excess). Wypich *et al.* (1995) propose 85% of soluble SLF will be bound to KIT^S but only 2.5% of total KIT^S will be bound to SLF. The proliferative capacity of M07e cells to SLF was diminished in the presence of culture supernatant of PMA treated M07e cells but not of untreated cells suggesting that the KIT^S released from M07e cells after PMA treatment is capable of inhibiting the c-Kit/SLF interaction. This affect could be abrogated by depleting the supernatant of soluble c-Kit by immunoprecipitation with an anti-c-Kit antibody that

recognises an epitope in the extracellular domain of c-Kit (Brizzi *et al.*, 1994). The results from this experiment indirectly provide evidence that KIT^S detected in culture supernatant of c-Kit expressing cells is functional.

The function of KIT^S in these experiments is made clearer by other researchers. KIT^S inhibited SLF binding to c-Kit expressing CHO cells in a concentration dependent manner. Associated with the inhibition of SLF binding to these cells there was a lack of c-Kit autophosphorylation or the activation of PI3'K and Raf 1, known downstream c-Kit signalling molecules (Lev *et al.*, 1992a). It was suggested that the inhibition of c-Kit signal transduction was due to the prevention of SLF binding to c-Kit. However, it could not be ruled out that the inhibition was due to the formation of non-functional heterodimers between membrane bound and soluble c-Kit as seen in the experiments conducted by Brizzi *et al.* (1994). In agreement with the first hypothesis the formation of KIT^S-membrane bound c-Kit heterodimers have not been detected (Turner *et al.*, 1995). In addition, when a crosslinking agent was added, a band detected on SDS-PAGE of 220kDa was detected as well as one of 110-120kDa. This suggested that KIT^S was capable of dimerisation in the presence of ligand (Lev *et al.*, 1992a). Thus, the activity of KIT^S appears to be due to the formation of heterocomplexes of KIT^S and soluble SLF, preventing SLF from interacting with membrane bound c-Kit and thereby preventing SLF induced signalling cascades in c-Kit expressing cells.

However, the role of KIT^S in serum is still unknown. It may act as a chaperone, binding SLF in serum and preventing its degradation thereby extending the half-life of soluble SLF and, possibly, transporting SLF to its site of action. Alternatively, KIT^S may act as a native antagonist preventing SLF from stimulating c-Kit expressing cells at inappropriate locations or times. Since SLF also has a membrane bound isoform it is possible that KIT^S induces some form of signalling in SLF expressing cells via the membrane bound SLF molecule (Wypich *et al.*, 1995). However, as discussed in more detail in section 1.3.1, mice expressing SLF with the cytoplasmic domain deleted showed a minor haemopoietic disorder, reduced mast cells in periphery, and no stromal disorders (Tajima *et al.*, 1998) suggesting the cytoplasmic domain of SLF does not have a significant role in signalling. The c-Kit/SLF interaction appears to play a role in the adhesion of progenitor cells to the stroma of the bone marrow. Shedding of the extracellular domain of c-Kit may provide a mechanism for the progenitor cells to down

regulate c-Kit, along with other molecules involved in adhesion, allowing their movement from the microenvironment.

1.12 AIM

It had been shown that a subgroup of AML patients that do not respond to chemotherapy had AML cells that expressed a high level of c-Kit on the cell surface (Ashman *et al.*, 1988), possibly providing them with a growth advantage over their normal counterparts in the bone marrow microenvironment. Therefore, the major aim of this study was to produce a soluble form of the c-Kit molecule and assess its biological activity and ability to block SLF induced proliferation of c-Kit expressing cells.

During the course of this work the presence of KIT^S in the serum of normal individuals was reported. Since the long-term objective was to treat patients with KIT^S, an understanding of the functional activity of KIT^S in serum was required. Thus the second part of this study attempted to characterise the binding characteristics of serum KIT^S. Thirdly, serum samples from patients undergoing cytokine induced stem cell mobilisation had been stored at the Hanson Centre for Cancer Research. These serum samples were used to evaluate the KIT^S concentration over the course of cytokine treatment.

2. MATERIALS AND METHODS

All reagents used were of analytical grade unless otherwise specified. All solutions made in the laboratory were made using Milli-Q purified water. This water was generated by de-ionising distilled water using a Milli-Q RO60 system (Millipore Corp., USA) and then further purified by passing through two beds of ion exchange resins, a carbon filter and an organic filter using a Milli-Q system (Millipore, USA).

A list of suppliers of the reagents and materials used appears in appendix 1 (section 8.1).

The composition of commonly used solutions can be found in appendix 2, (section 8.2).

Many of the molecular techniques described here are expertly detailed in Sambrook and Russell (2001).

2.1 Tissue Culture

The components and production of the media used for cell culture work are listed in appendix 2 (section 8.2.2).

2.1.1 Cells

COS African Green Monkey kidney cells (Gluzman *et al.*, 1981) were maintained as adherent cultures in RPMI 1640 supplemented with 5% FBS.

CHO, CHO (*dhfr*-): The Chinese hamster ovary cell line was obtained from American Type Culture Collection (CCL-61) (Puck *et al.*, 1958). Cells were cultured in Ham's F12 supplemented with 5% FBS. CHO (*dhfr*-) (CRL-9096) cells lack the dihydrofolate reductase gene.

FD^{GNNK+}, FD^{GNNK-} cells were derived from transfection of murine early myeloid FDC-P1 cells, a factor-dependent cell line, (Dexter *et al.*, 1980), with human c-Kit cDNA. Transfection of cells with c-Kit cDNA was carried out by Dr G. Caruana (Department of Haematology, Hanson Centre for Cancer Research). Original FDC-P1 cells were obtained from Dr. T. Gonda (Department of Human Immunology, Hanson Centre for Cancer Research, Adelaide). Cultured cells were maintained in DMEM containing 10% FBS supplemented with murine (m) GM-CSF (80 U/ml) or mIL-3 (300 U/ml).

HEL-DR⁺, a subline of the Human Erythro-Leukaemic cell line, HEL, was a gift from Dr Beverly Torok-Storb (Fred Hutchinson Cancer Institute, Seattle, USA). Cells were maintained in RPMI-1640 supplemented with 5% FBS.

M07e, a factor-dependent human megakaryocytic leukaemia cell line (Avanzi *et al.*, 1988), was obtained from Dr. P. Crozier (Department of Molecular Medicine, School of Medicine, University of Auckland, New Zealand). It was maintained at log phase in DMEM supplemented with 5% FBS and conditioned medium produced from CHO cells transfected with either human (h) GM-CSF or hIL-3 cDNA (provided by Dr A. Lopez, Department of Human Immunology, Hanson Centre for Cancer Research, Adelaide, Australia).

Sf21: Recombinant Baculovirus was propagated using the Sf21 cell line. This cell line had been generated from ovary cells from the worm larvae *Spodoptera frugiperda* or Fall Army worm. It was provided in the Clontech Baculovirus kit. Cells were cultured in either Grace's insect medium supplemented with 10% FBS and 2mM glutamine or Sf-900 II SFM (GibcoBRL Cat. No. 10902-088) a complete, serum free medium.

TF-1 is a factor-dependent human erythroleukaemia cell line (Kitamura *et al.*, 1989). It was maintained in RPMI 1640 supplemented with 5% FBS and conditioned medium produced from CHO cells transfected with either hGM-CSF or hIL-3 cDNA, see above.

WCB6F₁ SI/SI 3T3 (VSOP), a fibroblast cell line was obtained from Dr. J. Fujita (Kyoto University, Japan) (Fujita *et al.* 1989) and were maintained in DMEM supplemented with 10% FBS.

2.1.2 Cell Line Maintenance

All tissue culture work was carried out in Class 2 'biohazard' laminar flow hoods (Gelman Sciences). All tissue culture medium was prewarmed to 37°C before use except the insect cell media which was prewarmed to room temperature.

Cell cultures in culture flasks (25cm², 75cm² and 150cm²) (Corning, Falcon, Greiner, Nalge Nunc) were incubated at 37°C in a 5% CO₂ in air atmosphere to maintain the pH, with 97% relative humidity, maintained using incubators purchased from Forma Scientific. After 3 months of culture the cells were discarded and fresh cultures were established from frozen stocks.

Non-adherent cell lines were maintained at a cell density between 1×10^5 and 1×10^6 /ml. Cell densities and viabilities were calculated from haemocytometer counts using trypan blue (ICN Biomedicals, Cat #195532, 0.8% w/v in PBS) exclusion to indicate viability.

Adherent cell lines were maintained under sub-confluent conditions. Cells were harvested from near confluent cultures by aspirating the culture supernatant, washing the cells with HBBS, then incubating the cells with trypsinisation solution in HBBS for 2-5 min. The sides of the culture flask were firmly hit, several times, to dislodge the cells and medium supplemented with 5-10% FBS was added to inhibit the trypsinisation solution. Cell number and viability was determined as outlined above. Sub-cultures were established using cells diluted between 1/10 and 1/30 in fresh culture flasks.

Sf21 cells were maintained in static or suspension cultures. To sub-culture Sf21 cells, the medium from near confluent flasks was removed and replaced with 5mls of medium at room temperature. The sides of the flask were then gently slapped several times to dislodge the cells. Cell number was determined by using a haemocytometer and viability determined by trypan blue exclusion, as above. Sf21 cells were maintained at a temperature of 27-30°C in a dry air incubator without CO₂.

Suspension cultures of Sf21 cells were in sterile 500ml glass Schott bottles containing a magnetic flea, in 100-200mls of medium, supplemented with 2mM glutamine and 1/100 Pluronic F-68 (GibcoBRL, Cat No 24040-032) which helped prevent destruction of the cells by shear forces. Cultures were maintained at a density of 5×10^5 - 2×10^6 /ml. Cells were incubated at 27-30°C away from light and kept in suspension with gentle rotation of the magnetic flea.

2.1.3 Cryopreservation of Cells

Cells were frozen in the presence of 10% dimethyl sulphoxide, DMSO, (MERCK, Cat. No. 10323) to prevent the crystallisation and fracturing of the cell membrane. Cells were harvested at log phase and suspended at 1×10^7 cells per ml. Immediately prior to freezing, an equal volume of freezing mix (20% DMSO, 30% heat-inactivated FBS, 50% RPMI-1640, filter sterilised through a 0.22µm nitrocellulose filter) was added slowly while mixing. The mixture was aliquoted into cryopreservation ampoules (Nalge Nunc, Cat No. 3-66656), and

control rate frozen (Kryo 10, Planer) to -80°C. The cryotubes were then stored in liquid nitrogen filled canisters.

2.1.4 Thawing Cryopreserved Samples

The appropriate medium was prewarmed to 37°C. The sample, after rapid thawing in a 37°C water bath, was transferred to a 10ml tube and an equal volume of medium was added dropwise over 5 min. After a further 10 min, an equal volume of medium was added. The sample was left for another 10 min, and then centrifuged at 200g for 5 min. The sample was washed twice in medium to remove all the DMSO and resuspended at the appropriate density.

Sf21 cells were thawed by a different method. Ampoules of cells were warmed quickly to room temperature in a water bath and the contents transferred to a 25cm² flask, containing 5mls of medium. After 5 hours the medium was replaced by fresh medium and the cells cultured as detailed above.

2.1.5 Cytokines

Purified recombinant human (rhu) SLF produced in *E. coli*. (SLF_b) was supplied by Amgen Corporation, (Thousands Oaks, CA, USA) and rhuSLF produced in yeast (SLF_y) was provided by Immunex Corporation, (Seattle, WA, USA). Biotinylated SLF_y (bSLF_y) was kindly provided by Mr Steve Cole, (Department of Haematology, Hanson Centre for Cancer Research). Conditioned medium containing either recombinant human GM-CSF or IL-3 was obtained from cultured CHO cells transfected with the respective expression plasmids, kindly provided by Dr Angel Lopez, (Department of Human Immunology, Hanson Centre for Cancer Research).

2.2 Immunoassays

2.2.1 Antibody Details

Mouse mAbs against human c-Kit included: YB5.B8 (IgG₁) (Gadd & Ashman, 1985), 1DC3 (IgG₁) (Aylett *et al.*, 1995), Kit4 (IgG_{2a}) (A.C. Cambareri, L. Ngyuen, L.K. Ashman, unpublished), produced in our laboratory; 1C1.HF (IgG₁) (Bühring *et al.*, 1993) and A3C6E2 (IgG₁), both gifts from Dr. H-J. Bühring (Transplantation Immunology and

Immunohematology, University of Tübingen, Germany) and SR-1 (IgG_{2a}) (Broudy *et al.*, 1992a) a gift from Dr V. Broudy (Division of Hematology, Department of Medicine, University of Washington, Seattle).

Mouse mAbs against phosphotyrosine: PY20 (Transduction Laboratories, U.S.A) and 4G10 (Upstate Biotechnologies, USA).

Isotype-matched negative control mAbs included: 3D3.3 (IgG₁), 1D4.5 (IgG_{2a}) and 1A6.12 (IgM) (anti-*Salmonella*; O'Connor & Ashman, 1982) and 1B5 (IgG₁) an anti-*Giardia* mAb provided by Prof. G. Mayrhofer (Dept of Microbiology and Immunology, University of Adelaide).

2.2.2 Purification of Mouse Monoclonal IgG Antibodies

Antibodies were purified as described by Ey *et al.* (1978). A 10ml plastic column was packed with 5ml of Protein A-Sepharose (Pharmacia, Cat. No. 17-0780-01) and was equilibrated with 0.1M phosphate buffer (Na⁺, pH 8.2) supplemented with 0.1% w/v NaN₃ (loading buffer). The column was stored at 4°C in this form until required. If it had been previously used, the column was washed with 20ml 0.5M propionic acid to strip any bound protein, and then equilibrated with 10 column volumes of loading buffer. Prior to loading onto the column hybridoma culture supernatants were adjusted to pH 8.2 and filtered through a 0.45µm filter. Ascites fluid was centrifuged at 100000g for 30 minutes, diluted 1:1 with 0.1M Tris-HCl, pH 8.6 and filtered. The flow rate of the column was approximately 1ml/min. If the volume of the antibody source was less than the void volume of the column then the flow was halted for 30 min to allow antibody binding. The column was washed with loading buffer to remove unbound material until the O.D._{280nm} (1cm light path) of the effluent was less than 0.05 as measured by a spectrophotometer. The antibody was then eluted from the column with 0.1M citrate buffer (Na⁺). The pH of the citrate buffer differed depending upon the isotype of the antibody to be eluted: IgG₁, pH 5.5; IgG_{2a}, pH 4.5; IgG_{2b}, pH 3.5. Initially the elution buffer was added in small volumes, approximately 200µl, to create a sharp interface with wash buffer, and then with a large volume. Fractions of 1-2ml were collected until the O.D._{280nm} was less than 0.05. The eluate was immediately neutralized in an equal volume of 1M Tris-HCl, pH 8.6, + 0.1% w/v azide and gently mixed. Fractions with

O.D._{280nm} greater than 0.1 were pooled. The column was then washed with loading buffer until it was of neutral pH. The purified antibody was dialysed 3 times against PBS/Az. The antibody concentration (mg/ml) was determined using the spectrophotometer, absorbance at 280nm of 1 is equivalent to 1.35mg/ml for IgG (Harlow and Lane, 1988). The antibody could be concentrated to 2-10mg/ml using an Amicon concentrator (Millipore, Cat. No. 4304) and stored at -20°C in the presence of 50% glycerol. The antibody activity was checked by indirect immunofluorescence (see section 2.2.5).

2.2.3 Biotinylation of Monoclonal Antibodies

Monoclonal antibodies were biotinylated as per the protocols in Kendall *et al.* (1983), Updyke and Nicholson (1984) and Gretch *et al.* (1987). Purified monoclonal antibodies stored in 50% glycerol were dialysed against 0.1M carbonate buffer (Na⁺, pH 9.0) and then adjusted to concentrations of 100µg/ml and 1000µg/ml. Fresh stocks of biotin (NHSS-spacer biotin, Pierce, Cat. No. 21217) were made at concentrations 0.185mg/ml, 1.85mg/ml and 18.5mg/ml in H₂O. To determine the maximal level of biotinylation with minimal inactivation of the antibody, biotin:antibody ratios were set up as follows: 0:1, 100:1, 500:1, 1000:1, 2000:1, 5000:1 and 10000:1.

Biotin was added to the antibody, at the molar ratios indicated above, in a total volume of 33µl, mixed and incubated for 1 hour at room temperature. To stop the reaction 27µl of 1M glycine, pH 7.5, was added and this was incubated for a further 1 hour at room temperature. A 5µl aliquot was removed for testing in the spot blot assay (see below) to check biotinylation and the rest was diluted to 5µg/ml in PBS/BSA/Az and titrated in an indirect immunofluorescence assay (see section 2.2.5).

Spot Blot Assay: Aliquots of the biotinylated antibody at the various ratios were serially diluted 1/2 in PBS/BSA/Az and 2µl aliquots of each dilution were spotted onto nitrocellulose and allowed to air dry. The procedure for detecting the biotinylated protein was the same as that outlined in section 2.7.4.

Immunofluorescence: The activity of the antibody was determined by indirect immunofluorescence assay (see section 2.2.5) on cells expressing the corresponding antigen. The level of biotinylation was determined using streptavidin conjugated to Fluorescein or

R-phycoerythrin. Retention of antibody activity was determined by comparing the signals obtained with labelled and unlabelled antibody and anti-mouse immunoglobulin (Ig) conjugated to fluorescein or R-phycoerythrin.

Once the best compromise between the level of biotinylation and activity of the antibody was determined a large batch of labelled antibody was made at this optimal ratio of biotin to antibody.

For the anti-c-Kit antibody 1DC3 the optimal ratio was found to be 1000:1. The biotinylated antibody, b1DC3, was dialysed against PBS/Azide, 3-4 changes, diluted 50% v/v with glycerol and stored at -20°C.

2.2.4 Coupling of monoclonal antibodies to Cyanogen Bromide Sepharose-4B.

Dried cyanogen bromide activated Sepharose-4B (0.3g, Pharmacia Biotech, Cat. No. 17-0430-01) was swollen in 1mM HCl, pH approximately 3, in a 10ml polypropylene tube for 5 min, then washed 4 times by adding 10mls of 1mM HCl at room temperature, resuspending the Sepharose, and rotating the tube on a vertical rotator for 2-3 min, then pelleting the Sepharose at 400g for 1 min with no brake, aspirating the supernatant and repeating the procedure. The Sepharose was then washed, as above, 2 times over 10 min in coupling buffer (0.1M CO_3^- , Na^+ , pH8.5 / 0.5M NaCl) at room temperature. This effectively prehydrolysed some of the reactive groups on the Sepharose, reducing the risk of over coupling and inactivation of the antibody. Immediately after the final wash, 3mg of antibody (in PBS) was added to the 1.5ml Sepharose pellet and the total volume made up to 5ml using coupling buffer. Following rotation of the resuspended Sepharose and antibody mixture, for at least 1 hour at room temperature or overnight at 4°C, the Sepharose was pelleted and the supernatant assayed for the presence of antibody using a spectrophotometer. If no antibody was detected in the supernatant it was aspirated away from the Sepharose and the Sepharose was washed once in coupling buffer. The remaining active groups were quenched by the addition of 10mls of 1M ethanolamine/0.5M NaCl, pH 8.0, and rotation for 2 hours at room temperature. Five alternating washes of 0.1M Acetate/ 0.5M NaCl, pH 4.0 and coupling buffer (approximately 10ml) were used to remove any non-covalently bound antibody. After the final coupling

buffer wash the Sepharose was washed twice in PBS and then stored as a 50% slurry in PBS/0.1% azide at 4°C.

2.2.5 Indirect Immunofluorescence Assay

Target cells were harvested, washed once with medium, and twice with ice cold PBS/BSA/azide (appendix 2, 8.2.1) and resuspended at 1×10^7 cells/ml in PBS/BSA/azide supplemented with 10% normal rabbit serum (NRS, heat-inactivated) (Hunter Antisera) to block any binding to Fc receptors. All subsequent steps were carried out at 0-4°C. Aliquots of 50 μ l of this suspension were dispensed into disposable round-bottomed plastic tubes (Techno-Plas, Cat. No. P-7512). Appropriately diluted antibody, culture supernatant or a saturating level of purified Ab, (50 μ l), was then added to the cell suspension, mixed by vortexing and then incubated for 60 min. The cells were then washed three times in PBS/BSA/azide (2mls/tube). With each wash the cells were pelleted by centrifugation at 200g. After the final wash the supernatant was removed such that approximately 50 μ l of PBS/BSA/azide remained. The tubes were then vortexed and 50 μ l of a 1/50 dilution (in 10% NRS in PBS/BSA/azide) of affinity purified fluorescein isothiocyanate (FITC)-labelled sheep (Fab')₂ anti-mouse Ig (Silenus, Cat. No. DDF) or 1/50 dilution of affinity purified R-phycoerythrin (PE)-labelled goat (Fab')₂ anti-mouse Ig, α -IgG-PE, (Southern Biotechnology Associates, Cat. No. 1030-09) was added to the cells. The cells were incubated for a further 45 min in the dark, and then were washed twice as above and fixed in 0.5ml of 1% paraformaldehyde (MERCK, Cat. No. 29447) in PBS. The samples were stored at 4°C in the dark until analysed. Flow cytometric analysis was performed on a Profile II flow cytometer (Beckman Coulter) or EPICS[®] XL-MCL (Beckman Coulter).

The assay to determine the binding of KIT^S to CHO^{SLF} cells was similar to above with the following modification. Prior to the addition of the anti-c-Kit mAb, cells were incubated with KIT^S for 30 min and then washed.

The inhibition of bSLF γ binding to c-Kit expressing cells was determined using the immunofluorescence assay with the following modifications. KIT^S samples and bSLF γ were incubated in an assay tube, at room temperature for 20 min, prior to the addition of the target

cells. Detection of bSLF_y binding was performed using streptavidin R-PE (SA-PE) (Caltag Laboratories, Cat. No. SA 1004-4).

2.2.6 Fluorescence Activated Cell Sorting

Cells were stained as above (section 2.2.5) in the absence of sodium azide under sterile conditions and resuspended at approximately $1-2 \times 10^7$ /ml in medium. Cells were run on the FACStar^{PLUS} cell sorter (Becton-Dickinson) and those expressing various levels of c-Kit were collected into tubes or individually deposited into wells of 96 well flat-bottomed plates (Nalge Nunc, Cat. No. 167008). Sorted cells were cultured until a suitable cell density was achieved to allow confirmation of expression.

2.2.7 Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) Staining for Intracellular Antigens

Solutions used are listed in appendix 2, section 8.2.3.

Smears of target cells were prepared by harvesting cultured cells, washing once and resuspending at 5×10^5 cells/ml in medium + 50% FBS. Cells, 5×10^4 , were cytocentrifuged on to ethanol-cleaned glass microscope slides in a Cytospin 3 (Shandon Scientific) at 500rpm for 5 min. Cell smears were air dried overnight and stored at 4°C in air tight slide boxes in the presence of self indicating 2-4 mm silca gel (Ajax Chemicals, Cat No. 3681) until ready to use. Slide boxes were allowed to warm to room temperature prior to opening and removal of the slides to be stained. The circumference of the cell smear was marked with a Dakopen (Dako, Cat No. S 2002), leaving a wax ring around the cells to localise applied solutions to the cell smear.

Cell smears were fixed in cold 47.5% acetone, 47.5% methanol, 5% formaldehyde for 30 sec and then immediately rinsed in distilled water and washed in 3 times with agitation in Tris buffered saline (TBS) (see 8.2.3), 5 min between each change. From this point the cells were not allowed to dry to prevent high background staining.

The APAAP technique used for staining of cell smears for the detection of specific antigen expression was a derivation of that described by Erber *et al.* (1984). Cell smears were incubated with the primary mAb (antibody supernatant was diluted 1/2 in 10% NRS in

PBS/BSA/azide and purified antibody was diluted to 5 μ g/ml in 10% NRS in PBS/BSA/azide) for at least 30 min in a humidified chamber at room temperature or overnight at 4°C. All subsequent steps were carried out at room temperature. After washing, as above, in 3 changes of TBS over 5 min, the smears were incubated with the bridging antibody, rabbit anti-mouse Ig, (Dako, Z0259) (diluted 1/50 in 25% normal human serum (NHS) in PBS/BSA/azide) for at least 30 min and then washed as above. The smears were then incubated with the APAAP complex (Dako, D0651) (diluted 1/100 in TBS) for at least 30 min and followed by washing as above. The smears were then incubated with 2 more rounds of bridging Ab and APAAP complex with 10 min incubation times and wash steps between each. After the final wash step the slides were incubated with the substrate, upright in a Coplin jar for 20 min then rinsed in distilled water. The smears were counterstained in haematoxylin for 1 min, rinsed in distilled water, incubated in acid water (0.5% conc HCl in distilled H₂O) for 5 sec, rinsed in H₂O for 10 sec and incubated for 2 min in Scott's gentle alkaline solution (8.2.3). Counterstained slides were rinsed in H₂O and mounted in glycerol-glycine. Cells exhibiting mAb binding appeared bright reddish-pink.

APAAP was also carried out in 96 well plates. Plates seeded with single cells from the FACStar^{PLUS} sorter (Becton Dickinson) were replica plated and allowed to reach confluency. The duplicate plate was then stained for c-Kit expression using the above technique except that to wash the wells 200 μ l of wash buffer was added to each well using a multichannel pipette, and the contents flicked out. This was repeated twice.

2.2.8 Enzyme Linked Immunosorbent Assay (ELISA): Detection of KIT^S

In order to detect KIT^S in solution two ELISAs were established. Both utilised anti-c-Kit mAbs to immobilise the KIT^S. The sandwich ELISA required two antibodies to detect KIT^S, the second was biotinylated and the amount of KIT^S bound to the well was proportional to the signal detected from the labelled antibody. The competitive ELISA involved the competition of test KIT^S with immobilised KIT^S for a known concentration of anti-c-Kit mAb. The amount of bound antibody was determined and compared to standard curves. Standard curves for both ELISAs were generated using serially diluted KIT^S at known concentrations. See figures 3.8 and 5.1 for schematics of the ELISAs.

2.2.8.1 Sandwich ELISA:

The first 4 rows of a round bottom, 96 well plate (Corning Costar, Cat. No. 2595) were coated with 50 μ l of anti-c-Kit mAb, YB5, diluted to 5 μ g/ml in TEN buffer (50mM Tris, 10mM EDTA, 150mM NaCl, pH 7.5) and incubated in humidified container at 4°C for at least 18 hours. The wells were then washed 3 times with 200 μ l/well TEN buffer containing 0.01% Tween 20 (wash buffer). All wells of the plate were then blocked with 200 μ l 1% BSA (Sigma)/ 5% sucrose in TEN buffer for 24 hours at 4°C. All subsequent steps were carried out at room temperature. The well contents were flicked out and duplicate or triplicate 50 μ l aliquots of the samples containing KIT^S, diluted in 10% FBS in TEN buffer, were added to both the YB5 coated wells and the corresponding uncoated wells. After incubation for 2 hours, in the humidified container, the wells were again washed with wash buffer followed by incubation with 50 μ l of biotinylated anti-c-Kit mAb, 1DC3, diluted to 5 μ g/ml in 2% FBS in TEN buffer. The plates were left for 60 min in the humidified container, and then washed again as above and incubated with 50 μ l of streptavidin conjugated to horseradish peroxidase (Amersham, Cat. No. 1051), diluted 1/500 in TEN buffer / 2% FBS. Following a 60 min incubation in the humidified container, the plates were washed as above and incubated with 50 μ l of peroxidase substrate (2mg/ml o-phenylenediamine dihydrochloride (OPD) (Sigma) in 0.1M citrate buffer (Na⁺), pH 6.5, plus 1.5 μ l of H₂O₂ / 5mls) and allowed to stand until colour appeared (approximately 5-15 min). The reaction was terminated with 10 μ l of 1M H₂SO₄ and the plates read on a microplate reader (Bio-Rad, model 3550), at wavelength 490nm.

2.2.8.2 Competitive ELISA:

Wells of a 96 well plate were coated with 50 μ l of purified KIT^S protein (1 μ g/ml in PBS pH 8.0) for 2-3 hours at room temperature, in a humidified container. Plates were then washed 3 times with PBS-T (phosphate buffered saline pH 8.0 with 0.05% Tween 20) (wash buffer) as described in section 2.2.8.1. All wells of the plate were then blocked with 200 μ l 1%BSA (Sigma) in PBS pH 8.0 overnight at 4°C. Samples to be tested for the presence of KIT^S were pre-incubated for 2 hours with the anti-c-Kit mAb Kit4 (60ng/ml) (the binding of Kit4 at 60ng/ml was equivalent to 75% of the maximal signal) in a total volume of 175 μ l. To prevent dilution of the sample to be tested, this was achieved by the addition of 1 μ l of Kit4 at a concentration of 10.5 μ g/ml. Where appropriate, samples were diluted in PBS pH 8.0 + 10%

FBS. Pre-incubated KIT^S samples were incubated with the blocked plate for 60 min at room temperature. The plate was then washed 3 times with wash buffer and incubated with 50µl of anti-mouse IgG-HRP (Silenus, Cat. No. DAH) (diluted 1/250 in PBS pH 8.0 + 1% FBS) for 60 min at room temperature. The plate was then washed as above and incubated with 50µl of 2mg/ml OPD (Sigma) in citrate phosphate buffer (0.1M citric acid, 0.1M Na₂HPO₄ pH 5.0), plus 1.5µl of H₂O₂/5mls and left until an intense colour was observed in positive control wells. The reaction was terminated with 50µl of 2.5M H₂SO₄ and the plates read on microplate reader (Bio-Rad, model 3550), at wavelength 490nm.

2.3 Proliferation Assay: ³H-thymidine incorporation

Factor dependent cells from log-phase cultures greater than 95% viable were washed 3 times in medium + 2% FBS, resuspended in medium + 10% FBS and incubated without growth factors at 37°C / 5% CO₂ for 3 hours. Appropriately diluted SLF and KIT^S or negative controls were added to the wells of a 96 well, flat-bottomed microtiter plate (Nalge Nunc, Cat. No. 167008) in a total volume of 100µl and left at room temperature for 30 min. Starved cells were resuspended to 1x10⁵/ml in medium + 10% FBS and 100µl aliquots were added to the wells of the plate. Cultures were incubated for 2 days at 37°C/5% CO₂, unless otherwise stated, prior to being pulsed with 1µCi ³H-thymidine (specific activity 20 to 40 Ci/mM) (ICN, Cat. No. 2406605) for 16-20 hours. The cells were then harvested on to filters (ICN, Cat. No. 78-115-05) using a cell harvester (Skatron Instruments). The dried filters were immersed in Biodegradable Counting Scintillant (Amersham, Cat. No. NBCS104) and levels of β-particle emission were determined using a United Technology Packard Liquid Scintillation Analyzer 2000CA scintillation counter.

2.4 Adhesion Assay

An *in vitro* adhesion assay was used to investigate whether KIT^S could inhibit the adhesion of c-Kit expressing cells to immobilised SLF or to cells expressing membrane associated SLF.

Wells of a 96-well flat bottomed plate were coated either with 4x10⁴ CHO cells transfected with the membrane anchored form of SLF (kindly provided by Dr A. Zannettino,

Hanson Centre for Cancer Research) (CHO^{SLF}), untransfected CHO cells, purified SLF, G-CSF or 1% BSA, depending upon adhesive interaction to be studied. Plates coated with cells were incubated overnight at 37°C/ 5% CO₂. Cells to be assayed for adhesive properties were washed 3x with serum free medium and incubated for 3 hours in serum free medium at 37°C / 5% CO₂. Plates coated with purified protein were incubated overnight at 4°C. The excess was flicked off and non-specific binding sites were blocked with 2% BSA in HBBS (200µl/well) for 2 hours at 37°C. Blocked plates were washed 3 times with 0.2% BSA in HBBS prior to use.

c-Kit positive cells to be tested for adhesion to cell associated or recombinant SLF immobilised on plates were labelled with Calcein-AM as follows. Cells were washed once and resuspended in 500µl of serum free IMDM. Cells were incubated with 10µM calcein-AM (Molecular Probes) for 30-45 min at 37°C/ 5% CO₂ then washed twice in IMDM containing 0.2% BSA. Labelled cells, 1x10⁵/well, with or without the addition of KIT^S or control solutions were added. The labelled cells were forced to the bottom of the wells by centrifugation for 5 min at 200g at 4°C. The plates were then rapidly warmed to 37°C for 2 min on a heat block and then incubated for 1-2 hours at 37°C/ 5% CO₂. Non-adhered cells were washed from the wells using 0.2% BSA in IMDM until the control wells were clean. Labelled cells were lysed with 150µl of 1% SDS in water and the plates analysed for fluorescence using a Fluorimager with a 570nm filter (Molecular Dynamics).

2.5 Methyl Cellulose Assay

Culturing cells in semi-solid methyl cellulose (appendix 2, section 7.2.2) allowed the isolation of clonal populations of cells. Target cells, in a total of 750µl were mixed with 4.25ml of methyl cellulose, 50µl of 200mM glutamine and 5µl of β-Mercaptoethanol (5x10⁻²M) and plated in 1ml aliquots into triplicate 30mm dishes. Cells were plated at 1000, 100 and 50 cells per dish and incubated in a humidified container for 10-14 days at 37°C/5% CO₂. Plating efficiency was between 30 and 50%. Colonies were picked sterilely and the cells cultured in fresh liquid culture medium.

2.6 Introduction of DNA into Eukaryotic Cells

2.6.1 Transfection by Calcium Phosphate Method

This procedure was based on that described by Graham *et al.* (1973).

Twentyfour hours prior to transfection 60mm tissue culture dishes (Falcon, Cat. No. 3002) were seeded with 2×10^5 cells in a volume of 3ml medium + 10% FBS such that cells were 50-60% confluent at the time of transfection. A co-precipitate of CaCl_2 and DNA was formed as follows (n.b. amounts specified are for 1 dish). To a 10ml polystyrene tube (labelled tube A) 25 μ l of 2M CaCl_2 , 5-20 μ g of circularised or linear purified plasmid DNA (sterilized by filtration through a Spin-X filter; Corning Costar Cat. No. 8160), were added. The volume was made up to 0.5ml using Tris-EDTA (1mM Tris, 0.1mM EDTA, pH 7.9). To a 20ml conical bottom polystyrene tube 495 μ l 2x HEPES Buffered Saline (2xHBS) (280mM NaCl, 50mM HEPES, pH 7.1) and 50 μ l 150mM NaH_2PO_4 were added, (labelled tube B). The pH of the HEPES was checked immediately prior to use and the solution was filter sterilised through a 0.22 μ m filter (Millipore, Cat. No. SLGU025 LS). The contents of tube A were gently mixed and added dropwise, using a plastic pipette, to tube B while bubbling air through the solution in the tube B, using a plastic bulb pipette. The co-precipitate was allowed to stand for 30 min at room temperature. The medium on the cells was replaced with 3.5ml of fresh medium+10% FBS. After 60 min, 1ml of the co-precipitate was added to each dish, attempting to cover all the cells with co-precipitate. The dishes were incubated at 37°C/5% CO_2 .

Twentyfour hours after the addition of the co-precipitate, the supernatant was removed from the dishes and the cells were subjected to glycerol shock by addition of 3ml/dish of medium/10% FBS/15% glycerol and incubation at for 2 min. The glycerol mix was removed, the cells were immediately washed twice with PBS and incubated with fresh medium/10% FBS. Twentyfour hours later the medium was removed, the cells harvested and distributed into four 100mm tissue culture dishes in 10mls of medium/10% FBS supplemented with appropriate selection drug. The selection medium was changed twice weekly until the mock-transfected cells (received no DNA) were all dead. Drug resistant colonies were then screened for the expression of the particular antigen by the methods outlined elsewhere.

2.6.2 Transient Transfection of COS cells

2.6.2.1 Electroporation

COS cells from a flask at not more than 80% confluency were harvested by gentle trypsinisation. The cells were washed three times in cold PBS. Pelleted cells were resuspended in a total volume of 800 μ l in ice cold PBS + 10-20 μ g of target DNA, placed in an ice cold, sterile, 0.4cm cuvette (Bio-Rad, Cat. No. 165-2088) and incubated on ice for 10 min. The contents of the cuvette were resuspended and a single pulse of 300V and 500 μ Fd (determined to provide the highest transfection efficiency) was delivered to the cells using a Bio-Rad Gene Pulser. The cuvettes were returned to the ice for a further 10 min, after which they were mixed with 1ml of RPMI/5% FBS and layered over a 1ml FBS cushion in a 10ml polypropylene tube and centrifuged at 200g for 5 min. The medium was aspirated, the cell pellet resuspended in fresh RPMI/ 5% FBS and transferred to a 75cm² tissue culture flask and incubated for 24 hours at 37°C/5% CO₂. This method was used to produce KIT^S-IGc. To avoid contamination with FBS derived proteins the medium was aspirated and replaced with serum free RPMI and the cells left for a further 72 hours. The culture supernatant was harvested, supplemented with 0.02% azide and stored at 4°C until required.

2.6.2.2 FuGENE 6

This procedure was used to produce KIT^S-IGc. To avoid contamination with FBS derived protein on the Protein A column during purification of KIT^S from COS transfected cells, the FBS used for culture of transfected cells was pre-incubated with Protein A Sepharose overnight at 4°C on a rotator. The Sepharose was pelleted and the depleted FBS removed, added to RPMI-1640 to a final concentration of 10% and filter sterilised.

Twentyfour hours prior to transfection cells were trypsinised and used to seed 60mm dishes (Falcon, Cat. No. 3002) at a density between 4-5x10⁵ cells per dish. Immediately before the addition of the DNA the medium was replaced with 3ml of fresh medium containing 1% FBS that had been depleted of Protein A binding proteins. Avoiding touching the sides of the sterile tube, 6 μ l of FuGENE 6 reagent (Boehringer Mannheim, Cat. No. 1814433) was added to a tube containing 150 μ l of serum free, antibiotic free medium. This was mixed very gently, incubated for 5 min at room temperature and added to another tube

containing 2µg of purified DNA and gently mixed. After 15 min the DNA/FuGENE 6 mix was added dropwise to the dish. Cells were left for 96 hours after which time the supernatant was collected. This was scaled up for the transfection of many dishes of cells at once.

2.6.3 Stable Transfection of CHO Cells

Twentyfour hours prior to transfection cells were trypsinised and reseeded to give 80% confluency on the day of transfection. Cells were harvested, washed twice in cold PBS and resuspended to 6.3×10^5 cells/ml in PBS. 800µl of cells + 10µg DNA were added to ice cold 0.4cm cuvettes. Contents were mixed gently with a pipette and incubated on ice for 10 min. A single pulse of 1300V and 25µFd (determined to provide the highest transfection efficiency) was delivered to the cells using a Bio-Rad Gene Pulser. The cuvettes were left at room temperature for 10 min, the contents mixed with 30mls of medium (F12 + 10% FBS) and transferred to six 60mm dishes (Corning Costar) and swirled for even distribution of cells. Cells were incubated in humidified 37°C incubator with 5% CO₂. After 48 hours the medium was aspirated and replaced with fresh medium supplemented with appropriate selection drug.

2.7 Protein Analysis

2.7.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

This procedure is based on that of Laemmli (1970). Solutions and buffers used are described in appenix 2, (section 8.2.4).

Polyacrylamide gels of the appropriate percentage (7.5-12% as indicated) were cast and layered with a stacking gel. Gels were assembled into Hoefer gel tank apparatus, as per manufacturer's instructions, and immersed in protein electrophoresis buffer. Samples were prepared for electrophoresis by the addition of an equal volume of double strength sample buffer (reducing or non-reducing as indicated) and boiled for 5 min. Samples (15-30µl) were then loaded and subjected to electrophoresis through the stacking gel at 10-15mA (approximately 20 min) and then 20-25mA through the resolving gel (approximately 40 min).

2.7.2 Visualisation of total protein

2.7.2.1 Coomassie Stain

To visualise total protein, electrophoresed gels were stained in Coomassie Brilliant Blue R-250 (Bio-Rad, Cat. No. 161-0400) (0.25% w/v in 5:4:1 H₂O:methanol:acetic acid) for 2 hours and then destained in 5:4:1 H₂O:methanol:acetic acid for at least 2 hours with several changes of destain solution. Gels were then dried between cellophane sheets under vacuum at 80°C for 40 min.

2.7.2.2 Silver Stain

Resolved gels were prepared for silver stain by firstly fixing them in 50:10:40 methanol:acetic acid:H₂O for 30 min at room temperature, followed by 5:7:88 methanol:acetic acid:H₂O for 30 min and then 10% glutaraldehyde in H₂O for 30 min. Gels were washed in a large volume of Milli-Q H₂O overnight, with several changes, and then incubated for 30 min in 5µg/ml dithiothreitol (DTT) followed by 0.1% silver nitrate for 30 min at room temperature. The gels were rinsed in H₂O, twice in developer (50µl of 37% formaldehyde in 100ml 3% sodium carbonate) then soaked in developer over a light box until optimal staining was achieved. The staining reaction was stopped by the addition of 2.3M citric acid for 10 min. Gels were stored in 0.03% sodium carbonate until dried between cellophane sheets, under vacuum at 80°C for 40 min.

2.7.2.3 SYPRO Orange Stain

Resolved gels were stained in 1/5000 dilution of SYPRO Orange protein gel stain (Molecular Probes, Cat. No. S-6650) in 7.5% acetic acid for 2 hours, on a rocking platform, away from light. Gels were incubated in 7.5% acetic acid for 30 min to remove background staining, rinsed in H₂O and fluorescent protein visualised using a FluorImager 595 (Molecular Dynamics), through a 610nm filter.

2.7.3 Transfer of Protein to Nitrocellulose or PVDF membrane

After electrophoresis the gel cassette was removed from the chamber, opened and the stacking gel removed and discarded. A sheet of nitrocellulose membrane (Schleicher and Schuell Cat. No. 401196) or PVDF (Amersham, Cat. No. RPN 303F) (presoaked in 100% methanol for 10 sec) and 4 sheets of Whatman filter paper, all cut to the same size as the gel,

were equilibrated in semi-dry transfer buffer (47mM Tris, 39mM Glycine, 0.037% SDS, 20% Methanol). To assemble the gel for semi-dry transfer, 2 sheets of Whatman paper were placed on the cathode, in the window of a mylor mask (used to prevent shorting of the circuit) followed by the nitrocellulose or PVDF membrane, the gel and then the remaining sheets of Whatman paper. The air bubbles were removed and then the upper section of the transfer apparatus, the anode, was added. The transfer was run at 130mA, approximately 4V, for 1.5 hours.

For submerged transfer, the gel, presoaked membrane and Whatman paper were assembled, as above, within a cassette containing 2 sponges to support the transfer stack. The cassette was loaded into the transfer tank, overlaid with transfer buffer (24mM Tris, 192mM Glycine, 15% Methanol) and the protein transferred at 250mA for 90 min.

2.7.4 Detection of Antigens (Western Blotting)

Two Western Blotting techniques were used. The first utilised the enhanced chemiluminescence (ECL) method the second required the use of a FluorImager 595 (Molecular Dynamics).

2.7.4.1 Enhanced Chemi-Luminescence

To prevent non-specific antibody binding the membranes containing transferred protein were first blocked in 5% non-fat milk powder/0.05% Tween 20 in PBS (blocking buffer) for 2 hours at room temperature on a rocking platform and then stationary at 4°C overnight. After blocking the membrane was washed in 0.1% Tween 20 in PBS, once for 15 min and three times for 5 min each, and then once for 5 min in PBS only then incubated with the primary anti-c-Kit mAb, 1C1.HF (culture supernatant, diluted ½ in blocking buffer) for 3-4 hours. After washing as above, the membrane was incubated with biotinylated F(ab)₂ sheep anti-mouse-Ig (Amersham, Cat. No. RPN 1061) diluted 1/25000 in 3% BSA, for 2 hours, washed again as above and then incubated with streptavidin conjugated to horseradish peroxidase (Amersham, Cat. No. RPN 1051), diluted 1/2000, for 45 min. The membrane was then washed as above however an extra 5 min wash with 0.1% Tween 20 in PBS was included.

Antigens were visualised using enhanced chemiluminescence (ECL) reagents, (Amersham, Cat. No. RPN 2106). Equal volumes of the two ECL reagents were mixed and

poured onto a glass plate. The membrane was removed from the PBS wash, blotted between Whatman filter papers and placed protein side down onto the ECL solution mix. After 1 min the membrane was removed, blotted between Whatman filter paper to remove excess reagent and wrapped in clear plastic film. The blot was then exposed to Hyperfilm ECL (Amersham Corp). First exposure was 10 sec, followed by another at 1 min. These were then used to determine the optimal exposure, which ranged from 2 secs to 30 min.

2.7.4.2 Enhanced Chemi-fluorescence

To prevent non-specific antibody binding, the membranes containing transferred protein were first incubated in 5% membrane blocking solution (non-fat milk) (Amersham, Cat. No. RPN2125)/ TBS-T (20mM Tris-Cl, pH 7.5, 150mM NaCl, 0.1% Tween 20) (blocking buffer) on a rocking platform at 4°C overnight then washed in TBS-T, once for 10 min and four times for 5 min. The membrane was then incubated with the primary anti-c-Kit mAb, 1C1.HF (culture supernatant, diluted ½ in TBS-T) for 3-4 hours. After washing as above the membrane was incubated with biotinylated F(ab)₂ sheep anti-mouse-Ig (Amersham, Cat. No. RPN 1061) diluted 1/5000 in TBS-T, for 2 hours, washed again as above, and then incubated for 45 min with streptavidin conjugated to alkaline phosphatase (Zymed, Cat. No. P50237), diluted 1/2000. The membrane was incubated protein side down on ECF Substrate for Western Blotting (Amersham, Cat. No. RPN 5785) for 1-5 min, placed onto the glass plate of the FluorImager (Molecular Dynamics) and scanned using a 570nm filter.

2.8 Co-immunoprecipitation of KIT^S and bSLF_y from human serum

Normal human serum, 1ml, was incubated with 15µl of washed Kit4-Sepharose or 1D4.5-Sepharose (i.e. anti-c-Kit mAb Kit4 or isotype matched negative control mAb 1D4.5, coupled to Sepharose as per section 2.2.5) with or without 200ng of bSLF_y. Reaction tubes were rotated at 4°C overnight. The Sepharose was pelleted by microcentrifugation and washed three times with PBS, pH 8.0. Pellets were resuspended in 15µl of double strength protein loading buffer (reduced or non-reduced as indicated), boiled for 3 min and the supernatant loaded onto 10% acrylamide gels, electrophoresed and transferred to PVDF membrane (section 2.7.3). Blots were analysed by chemifluorescence (section 2.7.4.2) using 1C1.HF mAb (anti-c-Kit extracellular domain).

2.9 Inhibition of c-Kit Phosphorylation by KIT^S

M07e cells were washed three times and incubated for 3 hours in serum free medium in the absence of growth factors, at 37°C/5% CO₂. Starved cells, 2x10⁶, were pulsed with 80ng/ml of SLF_b with or without KIT^S or control reagent, in a total of 1ml for 2 min at 37°C, pelleted at 16000g for 15 sec and lysed with 1ml of 1% NP40 in 50mM Tris-HCl, 150mM NaCl, 1mM EDTA, pH 8.0 supplemented with protease inhibitors (Boehringer Mannheim), 1mM NaVO₃, and 1mM sodium pyrophosphate. After incubation for 30 min on ice, debris was removed by centrifugation, 16000g/30 min/ 4°C and the supernatant was transferred to new tubes.

c-Kit was immunoprecipitated from lysates by incubation overnight at 4°C on a rotator with 5µg of Kit4 mAb and 20µl Protein A coupled Sepharose. Immunoprecipitates were washed four times with lysis buffer supplemented with 1mM NaVO₃, resuspended with 12µl of double strength protein loading buffer (reduced), boiled for 2 min and loaded onto 8% SDS-polyacrylamide gels. Proteins were separated at 20mA and following electrophoresis transferred to PVDF for 2 hours and then subjected to Western blot analysis (section 2.7.4). Phosphotyrosine was detected using a cocktail of monoclonal antibodies PY20 (Transduction Laboratories) (1/1000) and 4G10 (Upstate Biotechnology) (1/2500), and c-Kit was detected with 1C1.HF mAb supernatant, and scanned using the Fluorimager 595 (Molecular Dynamics).

2.10 Purification of Plasmid DNA from Bacterial Cultures

2.10.1 Small scale plasmid DNA extraction

Bacteria strains used were DH5α, DH10β and MC1061 with or without the P3 plasmid. Reagents can also be found in appendix 2, section 8.2.5.

2.10.1.1 Alkaline Lysis

Bacterial cells, 1ml, from an overnight culture, were transferred to a microcentrifuge tube and pelleted by centrifugation for 1 min at 9000g at 4°C. The pellet was resuspended in 100µl of ice-cold GTE buffer (50mM glucose, 25mM Tris-HCl, 10mM EDTA, pH 8.0). The cells were lysed by the addition of a freshly prepared solution of 0.2M NaOH and 1% w/v SDS, inversion of the tube several times and incubation on ice for 5 min. Chromosomal DNA and

proteins were precipitated by the addition of 150 μ l of ice-cold solution of 3M potassium acetate and 11.5% v/v glacial acetic acid in H₂O and incubated on ice for 5 min. The chromosomal DNA and proteins were pelleted by centrifugation at 9000g for 10 min at 4°C. The supernatant was retained and any residual contaminating chromosomal DNA and proteins were removed by precipitation with 7.5M ammonium acetate on ice for 10 min followed by centrifugation at 9000g for 10 min at 4°C. The plasmid DNA was precipitated by adding 2 volumes of 100% ethanol to the supernatant, incubation on ice for 10 min, followed by centrifugation at 9000g for 10 min at 4°C. The pellets were washed with 70% ethanol, dried under vacuum and resuspended in 50-100 μ l of TE, pH 8.0.

2.10.1.2 Modified Boiling Method

A single colony was grown in 2ml of Luria broth (1% Bactotryptone, 0.5% Bacto Yeast extract, 1% NaCl) overnight. Cultures were chilled on ice and transferred to 1.5ml microcentrifuge tubes. The bacterial cells were pelleted in a microcentrifuge at 9000g for 1 min. The supernatant was aspirated and the pellet resuspended in 250 μ l of fresh lysing buffer (50mM Tris-HCl, pH 7.5, 62.5mM EDTA, pH 8.0, 0.4% Triton X100, 2.5M LiCl), vortexed and incubated on ice for 5 min. To this, 20 μ l of lysozyme (10mg/ml) was added, vortexed for 3 sec, boiled in a waterbath for 1 min and immediately placed on ice for 15 min. Chromosomal DNA and protein was pelleted by microcentrifugation at 9000g for 20 min. The supernatant was collected and the plasmid DNA precipitated by the addition of 500 μ l of 100% ethanol and vortexing. The plasmid DNA was pelleted by microcentrifugation at 9000g for 15 min at 4°C. The pellet was washed in 1ml of 70% ethanol, dried under vacuum and resuspended in 32 μ l of TE.

2.10.2 Large Scale Plasmid DNA Preparation

2.10.2.1 CsCl method

A 2.5L flask containing 500mls of Luria broth with appropriate antibiotic for selection was inoculated with a 10ml overnight culture of cells and incubated, with shaking (200rpm), overnight at 37°C. Cells were harvested by centrifugation at 5500g in a precooled (4°C) rotor (JA14, Beckman), resuspended in 20ml of plasmid buffer (25mM Tris-HCl, pH 8.0, 10mM EDTA, 15% sucrose) and 2ml of 25mg/ml of lysozyme (prepared fresh in plasmid buffer),

and transferred to two 35ml tubes and incubated on ice for 40 min. Cells were lysed by the addition of 12ml/tube of freshly prepared lysis buffer (0.2M NaOH, 1% SDS) and incubation on ice for 10 min. Chromosomal DNA and protein were precipitated by the addition of 7.5ml/tube of 3M sodium acetate, pH 4.6, and careful mixing by repeated inversion, followed by a 40 min incubation on ice. Chromosomal DNA was pelleted by centrifugation at 27000g for 20 min at 4°C (JA-20 rotor, Beckman). The supernatant was carefully removed, avoiding the soft chromosomal DNA pellet, and transferred to 50ml tubes. The supernatant was extracted three times with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) or until the interface between the two phases was clean. Phases were separated by centrifugation at 1000g for 10 min. The upper aqueous phase was pooled into large centrifuge tubes and the plasmid DNA precipitated by the addition of 2.5 volumes of cold 100% ethanol and incubation at -70°C for 30 min. The DNA was pelleted by centrifugation at 8000g for 30 min at 4°C, washed in 70% ethanol and dried. The DNA was resuspended in a total of 5.9ml of TE, pH 8.0, added to 6.44g of solid CsCl and 60µl of ethidium bromide (EtBr) (10mg/ml), placed in two Beckman quick-seal tubes and centrifuged at 300000g for 16 hours at 20°C (Beckman, Optima TLX Ultracentrifuge). After centrifugation the EtBr stained band of plasmid DNA was collected using a syringe and needle, transferred to a 2ml plastic tube and extracted with an equal volume of isopropanol saturated with 5M NaCl (dissolved in TE, pH 7.4, at 1:1 ratio) in the dark. The lower aqueous layer, containing DNA, was re-extracted until the presence of EtBr was no longer evident, and then once more. The extracted plasmid DNA was placed in sterile tubing and dialysed against TE, pH 7.4, with 3 changes over 24 hours and stored at -20°C.

2.10.2.2 BRESApure Midi kit

BRESApure Midi Kits were obtained from Geneworks Australia (Cat No. BT-1210-50). This method allows the purification of plasmid DNA from bacterial lysates by utilising the affinity of the plasmid DNA for a manufactured matrix. The manufacturer's instructions were followed.

Briefly, bacteria from 50-100ml overnight cultures were pelleted at 5000g and resuspended in 4ml BPR buffer containing RNase A. Cells were lysed by the addition of 4ml of BPL buffer, mixed immediately and incubated for 5 min. Chromosomal DNA and protein

were precipitated by the addition of 4ml BPN and gentle inversion 5-6 times. Lysates were then centrifuged at 27000g for 30 min and the supernatants applied to a BRESApure column that had been pre-equilibrated with 10ml of BPQ buffer. Following flow through of the plasmid DNA solution, the columns were washed twice with 10ml Buffer BPW and the DNA eluted with 5ml Buffer BPE. Eluted DNA was precipitated with 0.7 volumes of isopropanol and the DNA was pelleted by centrifugation at 27000g for 30 min at 4°C. The supernatant was discarded and the DNA pellet air-dried and resuspended in 400µl of H₂O and transferred to a 1.5ml tube. The DNA was re-precipitated by the addition of 0.1 volume of 3M sodium acetate, pH 4.6, and 2.5 volumes of 100% ethanol. The tube was inverted 5-10 times and incubated at -20°C for at least 30 min. The plasmid DNA was pelleted by centrifugation at 9000g for 20 min at 4°C, washed with 70% ethanol, air dried and resuspended in 50µl of H₂O.

2.11 Manipulation of DNA Products

2.11.1 DNA quantitation

The concentration of DNA in solution was determined by spectrophotometry $A_{260nm}1=50\mu\text{g/ml}$ (1cm light path). Alternatively, DNA was separated by electrophoresis on agarose minigels and visually compared to the intensities of ethidium bromide stained bands containing known concentrations of DNA.

2.11.2 Electrophoresis of DNA

DNA was separated by electrophoresis on agarose (DNA grade, Progen) gels of appropriate percentage, dependent upon the size of DNA expected, usually 0.8%, 1%, 1.5% or 2%. For analysis of PCR fragments and restriction fragments 0.8-1% agarose gels in 1xTAE (0.04M Tris-Acetate, 0.001M EDTA pH 8.0) were used. Gels were loaded into horizontal electrophoresis tanks containing 1xTAE and DNA samples (10µl) in gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) were loaded into the wells. After electrophoresis at 100V, 70-100mA, until the bromophenol blue front was three quarters along the gel, gels were stained with ethidium bromide (2µg/ml in H₂O) for 10-30 min and destained in H₂O for 10 min. DNA bands were visualised on a shortwave UV transilluminator (254nm) and photographed using Polaroid 600 SE camera with Polaroid 665

or 667 film. Alternatively, ethidium bromide stained gels were scanned and the DNA visualised using the FluorImager 595 (Molecular Dynamics) using a 610nm filter.

2.11.3 Size Determination of DNA Fragments

The sizes of DNA fragments were calculated by comparing their relative mobilities with those of DNA molecules of known size by electrophoresis. The standards used were commercially available (Geneworks) molecular weight markers: *EcoRI* digested fragments of *Bacillus subtilis* bacteriophage SPP1 and *HindIII* digested bacteriophage lambda DNA.

2.11.4 Digestion of DNA with Restriction Endonucleases

Restriction enzyme digests of DNA were carried out using the following general protocol:

DNA (0.1µg-10µg) was digested with 2-20units of restriction endonuclease in the presence of 1x digestion buffer (supplied by the manufacturer as 10 times concentrated stock and often specific for the enzyme) in a total volume of 10-20µl, at 37°C for 1-4 hours. Where a large amount of DNA was to be digested, reactions were scaled up such that the concentrations remained constant. In some cases reactions were allowed to proceed for longer periods of time e.g. overnight. The extent of digestion was determined by visualization of the DNA fragments by electrophoresis as described in section 2.11.2. Following digestion, the enzymes were inactivated by heating at 70°C for 10 min unless otherwise instructed by the manufacturer.

If the DNA was to be digested by more than one enzyme then this could be done simultaneously if the enzymes required the same buffer conditions. If not, the DNA was purified (see section 2.11.5) after digestion with the first enzyme before digestion with the subsequent enzyme(s).

2.11.5 DNA Fragment Purification from solution or agarose

DNA to be manipulated needed to be in the correct buffer conditions for that procedure. This often meant that the DNA had to be 'cleaned', i.e. freed from the contaminating salts etc. This was done by one of the following methods. For purification of target DNA fragments from contaminating DNA fragments the following procedure was undertaken. DNA

fragments to be purified were separated on 1% low melting temperature agarose gels (Sea Plaque GTG Agarose, FMC, Cat. No. 50111) in 1x TAE, as per section 2.11.2. To prevent nicking, preparative samples of the DNA were not exposed to UV light. To accurately predict the mobility of the fragment of interest, a small sample of the DNA was run on the same gel in a lane next to the molecular weight markers. These were then cut away from the rest of the gel, stained with ethidium bromide and viewed under UV light. The fragment of interest in the sample lane was cut out and the rest of the gel placed along side the main part of the gel. The corresponding area in the lane of the main DNA sample was then removed using a scalpel blade. To ensure that the DNA fragment had indeed been removed the entire gel was stained with ethidium bromide and viewed under UV light. The DNA was purified from the agarose using either of the following procedures.

2.11.5.1 BRESA-CLEAN™

This method utilises the affinity of DNA for glass beads to purify the plasmid DNA from the molten agarose. The procedure outlined was a modification of the manufacturer's instructions (Geneworks, Cat. No. BRC-1).

The required DNA band was cut out of the agarose, weighed and BRESA-SALT™ solution corresponding to 3 volumes of gel was added. The agarose was then dissolved at 55°C for 5 min and mixed into solution. Following vigorous vortexing of BRESA-BIND™, 5µl + 1µl/1µg DNA was added to the DNA solution and incubated for 5-20 min at room temperature, with periodic resuspension of the BRESA-BIND™ (longer incubation times were found to improve DNA recovery). The BRESA-BIND™/DNA complex was pelleted by 15 secs of centrifugation and the supernatant removed. The pellet was washed with 3 times with 1ml of BRESA-WASH™ solution. After the third wash the supernatant was removed and the pellet incubated at 55°C for approximately 2 min until dry. The pellet was then resuspended in 10µl of H₂O incubated at 55°C for 5-10 min, with gentle resuspension of the pellet every few minutes. The BRESA-BIND™ was pelleted for 1 min and the supernatant containing the DNA was collected. This elution step was repeated to ensure all of the DNA had been collected.

2.11.5.2 Magic PCR Preps DNA Purification System

This method was carried out essentially as described by the manufacturer (Promega, Cat. No. A7170) and was used to purify DNA fragments of between 500 and 1500bp in size from agarose gels. This method relied on the affinity of the plasmid DNA for a matrix to allow purification. The excised gel slice was weighed in a 1.5ml reaction tube and the agarose was dissolved by heating at 70°C in a heat block. When the agarose was completely dissolved 1 ml of Magic PCR Preps Resin (Cat. No. A7181) was added and the solution vortexed for 20 seconds. A Magic Minicolumn was set up by attaching a minicolumn (Cat. No. A7211) to a 3ml disposable syringe barrel with the plunger removed. The DNA/Resin mix was added to the barrel and the slurry was gently pushed into the minicolumn with the plunger. The minicolumn was detached from the syringe and the plunger removed. The syringe barrel was reattached to the minicolumn and 2ml of 80% isopropanol was washed through the column by pushing it through the minicolumn with the plunger. The syringe was removed and the minicolumn was transferred to a 1.5ml reaction tube and microfuged for 20 secs to dry the resin. To remove any residual moisture from the resin the minicolumn was placed in a Speedvac (Savant) for 2 min. The minicolumn was transferred to a new tube and the DNA eluted from the resin by adding 50µl of TE or H₂O and allowed to stand for 5 min prior to microfuging for 20 sec.

2.11.5.3 Phenol Extraction

For DNA in solution the easiest method for purification was to extract the DNA with phenol (equilibrated, see appendix 2, section 8.2.6.). This was achieved by mixing the DNA containing solution with an equal volume of phenol/chloroform (50% phenol, 50% chloroform), vortexing and leaving on ice for 5 min until the phases separated. The mix was then centrifuged for 3 min and the aqueous phase collected. The procedure was repeated until there was no visible protein band at the interface. If small amounts of DNA were to be extracted then up to 5µl of glycogen (20mg/ml; Boehringer Mannheim) was added as carrier prior to the addition of the phenol. The phase containing DNA was then ethanol precipitated by the addition of 0.1 volumes of 3M sodium acetate and 2.5 volumes of 100% ethanol and incubation on ice or at -20°C for 10-60 min. The DNA was recovered by centrifugation at full speed for 20 min at 4°C. The pelleted DNA was washed in 70% ethanol, dried under vacuum and resuspended in H₂O or TE.

2.11.6 De-phosphorylation of DNA

To prevent religation of the restriction enzyme digested vector DNA during the ligation reaction, the vector DNA was de-phosphorylated using alkaline phosphatase to remove 5' phosphates. Phosphatase treatment was performed in 100 μ l reaction comprising of DNA, 1x OnePhorAll Buffer⁺ (Pharmacia) and 1 unit of calf intestinal alkaline phosphatase (CIP, Pharmacia) per 1 μ g of DNA. Phosphatase reactions were carried out at 37°C for 30 min. The reaction was stopped by heating to 75°C for 10 min and the DNA purified by phenol/chloroform extraction and ethanol precipitation.

2.11.7 DNA Ligation

DNA ligation reactions were carried out using T4 DNA ligase (Pharmacia). Standard reactions were carried out in a total volume of 10 μ l comprising of vector DNA, insert DNA (usually at a ratio of 1:3, approximately 20ng of vector DNA), 1x ligation buffer (50mM Tris-Cl pH 7.6, 10mM MgCl₂, 10mM DTT, 50 μ g/ml BSA, 1mM ATP), 1 Weiss unit of T4 ligase and sterile H₂O at 4°C overnight.

2.12 Preparation of *E. coli* Competent Cells and Transformation

2.12.1 Chemical Competent

Please refer to appendix 2, section 8.2.5 for details of solutions.

Bacterial cells (DH5 α , DH10 β , MC1061, MC1061/P3) from frozen stocks were streaked onto a ψ a agar plate and grown overnight at 37°C. A single colony was used to inoculate 5ml of ψ b medium and grown at 37°C overnight. The culture was then subcultured 1/20 into 100ml of ψ b and grown until O.D._{650nm}=0.4-0.5 (1cm light path). Cells were chilled on ice and pelleted at 5000g for 5 min at 4°C. Cells were resuspended in 2/5 volume of Tfb I buffer and incubated on ice for 5 min, then pelleted as above, resuspended in 1/25 volume of Tfb II buffer and left on ice for 15 min. Cells were aliquoted (50 μ l) into microcentrifuge tubes, snap frozen on dry ice and stored at -70°C until required.

Transformation was carried out by thawing the cells on ice and incubating with the DNA on ice for 20 min. The cells were then subjected to a heat shock at 42°C for 90 secs and returned to ice for 10-15 min. Two volumes of ψ b medium were added and the cells

incubated at 37°C for 30 min with gentle shaking, then plated out onto Luria agar with suitable selection. In the case of vectors encoding genes required for α -complementation, isopropylthio- β -D-galactoside (IPTG) (4 μ l of 1M stock) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) (40 μ l of 20mg/ml stock) (both from Progen), were spread onto the agar prior to plating of the transformants.

2.12.2 Electro-competent Cells

A 10ml culture grown from a single bacterial colony at 37°C overnight in superbroth (Bacto Tryptone 3.2% w/v, Bacto yeast extract 2.0% w/v, NaCl 0.5% w/v) was subcultured into 1L of prewarmed superbroth. This was grown with shaking until an O.D._{600nm} of 0.4-0.6 (light path 1cm) was reached. The cells were then chilled on ice and pelleted at 5000g for 15 min at 4°C. Each pellet was washed three times with 250ml ice-cold water and then resuspended in 5mls of 10% glycerol. The cells were pooled, centrifuged at 5000g for 15 min at 4°C, resuspended in 1ml of 10% glycerol and aliquoted (45 μ l) into tubes on dry ice.

Electro-competent cells were thawed at room temperature and then placed on ice. In a cold 1.5ml polypropylene tube, 40 μ l of cells were mixed with 1-2 μ l of DNA (100ng) or ligation (that had been phenol/chloroform extracted and resuspended in 10 μ l of H₂O), and incubated on ice for 1 min. The cell/DNA mixture was transferred to an ice-cold 0.2cm cuvette and pulsed once at 25 μ F, 200kV and 200 Ω with a Bio-Rad Gene Pulser. Immediately after the pulse, 1ml of SOC medium (section 8.2.5) was added to the cuvette, the cells transferred to 10ml polypropylene tubes and incubated at 37°C for 30 min with gentle shaking. Cells were then plated out onto selective medium and incubated at 37°C overnight.

2.13 Amplification of c-Kit cDNA

2.13.1 Primer Design and Synthesis

Oligonucleotide primers were designed from the published human *c-Kit* sequence (Yarden *et al*, 1987). Details of the sites of binding of the primers and their sequence are detailed in Table 2.1.

Table 2.1: Oligonucleotide Primers

Name	oligonucleotide sequence*	location ^a	orientation
T3	5'- <i>ATTAACCCTCACTAAAGG</i> -3'	-	anti-sense
T7	5'- <i>GTAATACGACTCACTATAGGGC</i> -3'	-	sense
387	5'- <i>GGGGGATCCATGAGAGGCGCTCGCGGC</i> -3'	22-39	sense
476	5'- <i>GGGGGATCCTCATTGCTCTTTGTTGTTACC</i> -3'	1549-1566	anti-sense
764	5'- <i>AGTTCACCTACGTATCTG</i> -3'	1140-1151	anti-sense
765	5'- <i>AGTGAACCTCATCTAACG</i> -3'	1144-1161	sense
862	5'- <i>GGGCTCTCTAGATCTCAACCATCTGTG</i> -3'	94-108	sense
863	5'- <i>GGGCTCAGATCTTCAAGTGTTTATCATGGG</i> -3'	970-984	anti-sense
1070	5'- <i>GCGCTGATGCGGCCGCTCAGTGATGGTGATGGTGATGTTGCTCTTTGTTGTTACC</i> -3'	1549-1566	anti-sense
1244	5'- <i>GGGGGATCCACTTACCTGTTTGCTCTTTGTTGT</i> TAC-3'	1550-1567	anti-sense

* sequences in italics do not correspond to *c-Kit*. The T7 and T3 primers bind to the bacteriophage promoter sequences T7 and T3 respectively, often found in cloning and expression vectors. They are often used as primers to amplify or sequence DNA in a given plasmid. The sequences in italics in the *c-Kit* specific primers encode endonuclease restriction sites and/or a stop codon and/or a splice donor site.

^a location corresponds to the position, in base pairs, of the primer in the *c-Kit* sequence as compared to that published (Yarden *et al.*, 1987).

2.13.1.1 Purification of oligonucleotide primers

Primers were synthesised on an Applied Biosystems 391 DNA synthesiser in the Department of Haematology by Mr. A. Mangos. When obtained, primers were still attached to the synthesis columns and were purified using the following procedure. A 1ml syringe was inserted into one end of the column and into the other end another 1ml syringe containing 500µl of ammonium hydroxide (25% ammonia solution, MERCK, Cat. No. 1.05428) was inserted. The plungers of the syringes were moved backwards and forwards in order to fill the column with ammonium hydroxide and left to stand for 20 min. The ammonium hydroxide was then drawn into one syringe and the contents placed in a 2ml screw-capped tube. The

above procedure was repeated three more times until a volume of 2ml was obtained. The primer/ammonium hydroxide solution was incubated overnight at 56°C. The vials were allowed to cool and the primer dried down by vacuum in a Speedvac. The pellet was dissolved in 100µl of sterile distilled H₂O and the concentration determined by spectrophotometry $A_{260nm} \times 33 = \mu\text{g/ml}$ (1cm light path) or to determine molarity the following formula was used:

$$\text{concentration of oligo(M)} = \frac{X \mu\text{g} \times 10^{-6} \text{g}}{\frac{\text{oligo mwt}}{1 \times 10^{-3}}}$$

molecular weight (mwt) of dNTPs: A=347.2, T=332.2, G=363.2, C=323.2

Alteration to the primer synthesis protocol resulted in an easier method of primer purification. 100µl of oligonucleotide solution was vortexed vigorously with 1ml of butanol and then centrifuged at 9000g for 1 min. The supernatant was removed and the primer pellet air-dried and resuspended in 100µl of sterile H₂O.

2.13.2 Amplification of cDNA by Polymerase Chain Reaction

The amplification of cDNA was carried out by adding 50ng of template DNA to a 0.5ml reaction tube containing 2.5U *Taq* DNA polymerase, (AmpliTaq DNA Polymerase, Perkin Elmer, Cat No. N801-0060), 100ng each of forward and reverse primers, 0.2mM for each deoxynucleotide-triphosphate (dNTP) (dATP, dTTP, dCTP, dGTP (Pharmacia, Cat. No. 27-20(5-8)0-02)), and reaction buffer (50mM KCl, 10mM Tris-Cl pH 8.3, 1.5mM MgCl₂, 0.001% gelatin) in a total volume of 50µl. This was overlaid with mineral oil and amplified in a DNA Thermal Cycler, (Perkin Elmer). The first PCR 'cycle' consisted of denaturation at 94°C for 7 min and subsequent cycles consisted of denaturation at 94°C for 1 min, primer annealing at 50-55°C for 1 min (depending upon primer composition), elongation at 72°C for 1-3 min, for 25-30 cycles. A further elongation step, 72°C for 7 minutes was added at the completion of the 30 cycles to ensure all strands were completely synthesised. Completed reactions were stored at 4°C.

When it became available, *Pfu* polymerase (Stratagene) was employed for amplification as it contained a proof reading activity, greatly reducing the risk of incorrect base

incorporation. Conditions were altered accordingly. Reactions consisted 50ng of template DNA in a 0.5ml PCR reaction tube containing 2.5U *Pfu* DNA polymerase, 100ng each of forward and reverse primers, 0.2mM for each deoxynucleotide-triphosphate (dNTP) (dATP, dTTP, dCTP, dGTP (Pharmacia, Cat. No. 27-20(5-8)0-02)), reaction buffer (10mM KCl, 20mM Tris-Cl pH 8.75, 2mM MgCl₂, 10mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.1mg/ml BSA), supplied as a 10x PCR buffer and sterile Milli-Q water to 50µl. This was overlaid with mineral oil and amplified in a DNA Thermal Cycler, (Perkin Elmer) as above except that the primer annealing temperature was altered to 45-55°C for 1 min (depending upon primer composition).

2.14 Sequencing of double stranded DNA

2.14.1 Manual DNA sequencing

Sequencing of DNA was carried out using a 'Super-Base sequencing reagent kit' (for use with T7 DNA polymerase) (Geneworks, Cat. No. SBK-2) according to the manufacturer's instructions. The principle of the method is based on that explained by Sanger *et al.* (1977). Briefly the method was as follows. The denatured target DNA pellet was resuspended in H₂O, 2µl of 5 times annealing buffer and 100ng of primer to a total volume of 10µl. The contents were mixed well and allowed to anneal at 37°C for 30 min and then room temperature for 20 min. Molar ratios of primer:template between 5:1 and 50:1 were maintained.

The wells of a Terasaki plate (Disposable Products, Cat. No. 239728) were labelled A, G, C, T for each clone to be sequenced. Note the manufacturer's protocol suggests using microfuge tubes but the trays were much easier to use. To each well to be used in the tray, 2.5µl of termination mix was added. To the completed annealing reaction the following were added to initiate the labelling/extension reaction: 2µl Labelling/extension mix diluted 1/10-1/15 in H₂O. (Note that manufacturer supplies as 5x stock and protocol suggests to dilute the mix 1/5. However, it was found that dilution of the mix 1/10-1/15 allowed more of the target DNA to be sequenced. Also added were 1µl 100mM DTT, 1µl H₂O, 1µl (10µCi) ³⁵S-dATP (Geneworks, Cat. No. SBK-2) and 1µl (2 units) T7 DNA Polymerase (diluted in dilution buffer supplied with enzyme). (N.B. enzyme was not supplied with kit due to patent laws and was purchased from Pharmacia, Cat. No. 27-0985-03). Contents were mixed thoroughly by

pipetting and incubated at room temperature for 5 min. Timing began upon the addition of the enzyme. The addition of the enzyme to the different reactions was staggered to allow easier handling of the reaction at later steps. After the 5 min incubation the labelling/extension reactions were terminated by the transfer of 3.5 μ l of the reaction to each of the four corresponding wells of the prewarmed (37°C) Terasaki plate, i.e. A, G, C, T. The contents were mixed by pipetting and incubated for 5 min at 37°C. Timing began at the addition of the labelling/extension mix to the first well. The reactions were stopped by the addition of 4 μ l of stop/loading buffer to each well and the contents mixed thoroughly. Reactions could be stored at -20°C for 1 week or at -80°C for several weeks prior to electrophoresis.

The 6% acrylamide gel (appendix 2, section 8.2.7) was pre-electrophoresed for 30 min. The wells were flushed with buffer to remove unpolymerised acrylamide and urea. Sequencing reactions were heated to 90°C for 5 min prior to loading and 2 μ l of each reaction were loaded onto the gel. Samples were electrophoresed at 1800V and <100mA and <100W. For large DNA fragments that were sequenced e.g. 150 bases, samples were loaded several times, in separate wells, at staggered intervals during the running of the gel. A usual run involved loading 2 μ l of each of the denatured sequence reactions (A,G,C,T) and the gel electrophoresed until the slower dye front was close to the bottom of the gel. The samples were denatured again and another 2 μ l were loaded and allowed to run until the slow dye front was 3/4 of the way down the gel. The third run was allowed to run until the fast dye front was close to the bottom of the gel. After the final run of the samples the gel was removed from the tank, the plates carefully separated with the gel remaining on one of the glass plates. The gel was fixed with 10% methanol/ 10% acetic acid in H₂O for 30 min, overlaid with a piece of Whatman filter paper cut to the same size as the gel and soaked in the methanol/acetic acid solution, and then blotted dry using absorbant paper. The gel was overlaid by an additional piece of Whatman paper, peeled from the glass plate and then dried under vacuum at 80°C for 40 min. The dried gel was exposed to X-ray film (Kodak) overnight or until a suitable signal was detected.

2.14.2 Automated DNA sequencing

The ABIPRISM Dye Terminator Cycle Sequencing Reaction Kit (Perkin Elmer) was used to sequence alkaline lysis purified plasmid DNA. In a sequencing reaction, 8 μ l Terminator Ready Reaction Mix (A, G, C, T-Dye Terminator, dGTP, dATP, dCTP, dTTP, Tris-HCl pH 9.0, MgCl₂, thermostable pyrophosphatase, AmpliTaq polymerase) was added to 500ng template DNA and 100ng of sequencing primer. The volume was made up to 20 μ l with H₂O. The reaction was cycled using a Perkin Elmer GeneAmp PCR System 9600 using the following program: [96°C 10 sec, 50°C 5 sec, 60°C 4 min] x 25 cycles, followed by a 4°C hold step. Following cycle sequencing the DNA was precipitated by the addition of 2 μ l 3M sodium acetate pH 4.6 and 50 μ l 100% ethanol and incubation on ice for 10 min. The DNA was pelleted at 9000g for 15 min at 4°C, washed in 70% ethanol and air dried, and the sequence determined using a Perkin Elmer automated sequencer.

2.15 Cloning of soluble c-Kit cDNA

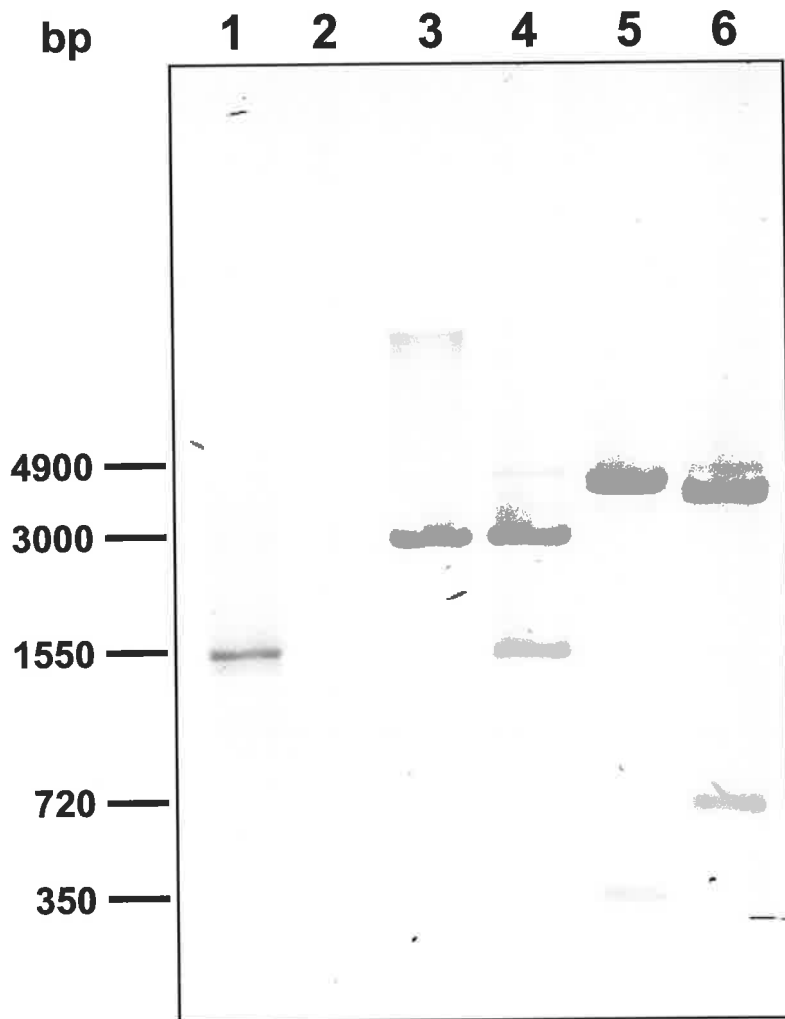
The cDNA encoding for KIT^S was generated by PCR. A clone containing the coding region cDNA of c-Kit in pBluescript (SK) (kindly provided by Dr D. Williams, Immunex Corporation, Seattle, WA, U.S.A.) was used as a template for the PCR. This template had the same open reading frame sequence as that published by Yarden and colleagues (1987). c-Kit specific oligonucleotides 387 and 476 (Table 2.1) were used to initiate strand elongation. Primer 387 contained and bound to the initiating ATG site of c-Kit and primer 476 bound to c-Kit sequence 1549-1566 followed by a stop codon TGA. To facilitate subsequent cloning both contained *Bam*H I restriction endonuclease sites at the 5' ends. PCR conditions are shown in Table 2.2. The 1.5kb amplified fragment, (Figure 2.1, lane 1) was phenol/chloroform extracted and digested with *Bam*H I. The fragment required for cloning was purified using agarose electrophoresis and GeneClean. The PCR product was ligated to the plasmid pBluescript SK⁺ (Stratagene) that had also been digested with *Bam*H I and dephosphorylated using CIP to prevent self-ligation. Ligated DNA was transformed into DH10 β by heat shock. Transformed cells were plated onto LB agar + X-gal/IPTG to allow blue/white selection of positively transformed cells. White colonies were picked and the plasmid isolated for the analysis of KIT^S cDNA insert by restriction endonuclease digestion.

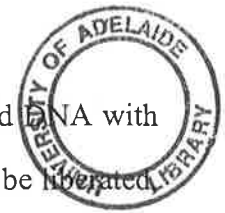
Table 2.2: PCR amplification conditions and expected product size

Clone	5' Primer	3'Primer	Denaturation	Annealing	Elongation	Cycles	Product Size	Enzyme Digestion	Cloned Product Size
pSHR1.6	387	476	94°C/ 1min	64°C/ 30sec	72°C/ 1min	30	1.5kb	<i>Bam</i> HI	1.5kb
pSHR1.26	862	863	94°C/ 1min	41°C/ 90secs	72°C/ 2min	30	900bp	<i>Xba</i> I/ <i>Bgl</i> II	900bp
pSHR1.30His	765	1070	94°C/ 1min	60°C/ 1min	72°C/ 90secs	30	400bp	<i>Not</i> I/ <i>Sph</i> I	360bp
pSHR1.45	387	1244	94°C/ 1min	55°C/ 1min	72°C/ 1min	30	1.5kb	<i>Bam</i> HI	1.5kb

The table outlines the protocols used for the amplification of KIT^S cDNA to be cloned into the various plasmids. Included are the primers (refer to Table 2.1) used to initiate strand elongation, the PCR cycling conditions, the expected size of the PCR product and the restriction endonucleases used to treat the PCR product prior to ligation into the plasmid.

Figure 2.1: Analysis of amplified KIT^S cDNA and restriction digests of pSHR1.6. KIT^S cDNA was amplified using c-Kit specific oligonucleotides 387 and 476. The resultant PCR product was digested with *Bam*H I and ligated into pBluescript SK⁺ that had been similarly digested. The resultant clone, pSHR1.6 was digested with *Bam*H I, *Hind* III and *Eco*R I to verify orientation. Samples were separated on 1% agarose gel, stained with ethidium bromide and visualised using a FluorImager 595 (Molecular Dynamics) and ImageQuant software. **Lane 1:** KIT^S cDNA PCR; **Lane 2:** H₂O PCR control; **Lane 3:** pBluescript SK⁺ digested with *Bam*H I; **Lane 4:** pSHR1.6 digested with *Bam*H I; **Lane 5:** pSHR1.6 digested with *Hind* III; **Lane 6:** pSHR1.6 digested with *Eco*R I.





Initially, clones carrying the KIT^S cDNA were identified by digesting the plasmid cDNA with *BamH* I. As this was the site KIT^S was ligated into the entire KIT^S cDNA should be liberated resulting in a 3.0kbp band and the KIT^S DNA band of 1.5kbp (Figure 2.1, lane 4). Cloning the KIT^S cDNA into the single *BamH* I site meant the cDNA could be in either orientation. To determine the orientation the KIT^S carrying clones were digested separately with *EcoR* I and *Hind* III (Figure 2.1, lanes 5 and 6). Both *Hind* III and *EcoR* I lie to the T7 side of the *BamH* I site in pBluescript SK. In c-Kit, the *Hind* III site is at base pair position 367-372 and *EcoR* I lies at position 704-709. Thus, if the 5' end of the KIT^S cDNA was at the T7 end of pBluescript digestion with *Hind* III should result in two bands, one of approximately 4.2kbp and one of approximately 370bp. In addition, digestion with *EcoR* I would result in two bands one of approximately 3.8kbp and one of approximately 700bp. These sets of bands were seen (Figure 2.1, lanes 5 and 6). One clone was chosen and frozen stocks were made. This clone will be termed pSHR1.6 from here. In subsequent cloning of KIT^S cDNA similar methods were used to identify clones in the correct orientation (see below).

The PCR generated cDNA was sequenced in both directions since the thermostable DNA polymerase, *Taq*, used to amplify the KIT^S cDNA lacks proof-reading capability. To facilitate this process, the 1.5kb KIT^S cDNA from pSHR1.6 was subcloned into smaller fragments by digesting pSHR1.6 with the restriction endonucleases *EcoR* I and *Hind* III and ligation into pBluescript SK⁺.

Sequencing of the KIT^S cDNA was achieved using both cycle sequencing and T7 polymerase sequencing methods (section 2.14). All PCR generated sequence was compared to the sequence published by Yarden *et al* (1987) and no discrepancies were found.

2.16 Cloning KIT^S cDNA into Expression Vectors

The KIT^S cDNA was subcloned into several eukaryotic expression vectors. The pcDNA-1/Neo vector was chosen because it carries the neomycin gene, allowing the selection of cells transfected with this plasmid by their resistance to the cytotoxic drug G418. Continued exposure of the transfected cells allows the selection of clones that have integrated the foreign DNA into their genome allowing the generation of stable, expressing cell lines. Please refer to section 3.4 for further detail. The pRSV009/A⁺ and pEE14 expression plasmid were

chosen because they too contain genes encoding drug resistance. For these plasmids, treatment of transfected cells with increasing concentrations of drug has been shown to select for cell clones with increased copy number of the plasmid and thus increased expression of the drug resistance marker. The target gene is also amplified resulting in increased expression of the target protein. pRSV009/A⁺ was transfected into CHO and SI/SI cells. pEE14 was transfected into CHO cells. For further detail refer to section 3.5.

2.16.1 Cloning KIT^S cDNA into pcDNA-1/Neo

To facilitate the cloning of KIT^S cDNA into the pcDNA-1/Neo expression vector (Figure 2.2) (Invitrogen) both pcDNA-1/Neo and pSHR1.6 were digested with restriction endonucleases *Xba* I and *Xho* I. The KIT^S was gel purified and ligated into pcDNA-1/Neo. The ligated DNA was transformed into chemical competent MC1061/P3 cells. Plasmid DNA isolated from bacterial colonies was screened for KIT^S cDNA inserts by restriction endonuclease digestion. This clone was called pSHR1.7.

2.16.2 Cloning KIT^S cDNA into pRSV009/A⁺

pRSV009/A⁺ (Figure 2.3), a derivative of the vector described by Choo *et al.* (1986) was obtained from Dr J. Wells (Department of Biochemistry, University of Adelaide) and the KIT^S cDNA was subcloned into this expression vector from pSHR1.6. This was achieved by digesting both plasmids with *Bam*H I. The KIT^S cDNA fragment from pSHR1.6 was gel purified away from the rest of the plasmid while the digested pRSV009/A⁺ vector was treated with CIP to prevent self-ligation. The two required pieces of DNA were ligated together and transformed into competent DH10 β cells by heat shock and transformants were selected on ampicillin containing agar plates. Successful introduction of KIT^S cDNA into pRSV009/A⁺ was determined by digestion of isolated plasmid DNA with *Bam*H I and the correct orientation was confirmed by digestion of the plasmid DNA with the enzymes *Hind* III and *Eco*R I. This clone was called pSHR1.15.

Figure 2.2: Diagrammatic representation of pcDNA-1/Neo plasmid.

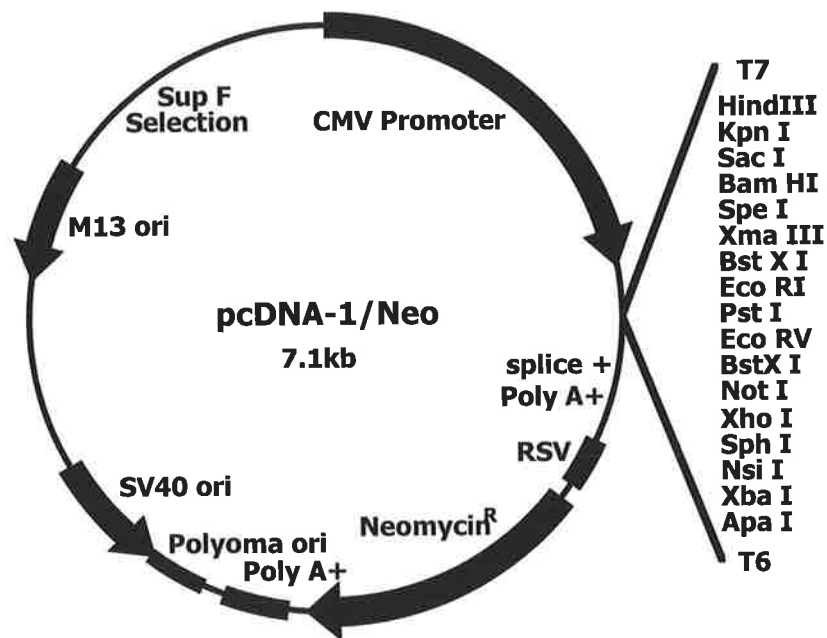
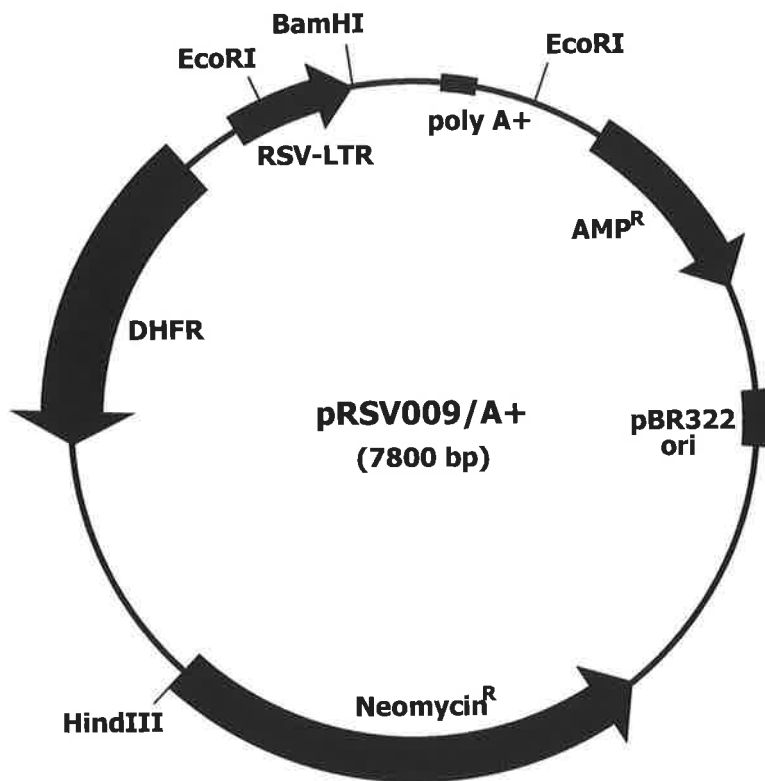


Figure 2.3: Diagrammatic representation of pRSV009/A⁺ plasmid. This plasmid contains a neomycin resistance gene, to allow the selection of transfectants using cellular resistance to geneticin (G418) and the murine dihydrofolate reductase (*dhfr*) gene for methotrexate resistance, allowing the amplification of the target gene under selective pressure. (Derivation described by Choo *et al.* (1986).



2.16.3 Cloning KIT^S cDNA into pEE14

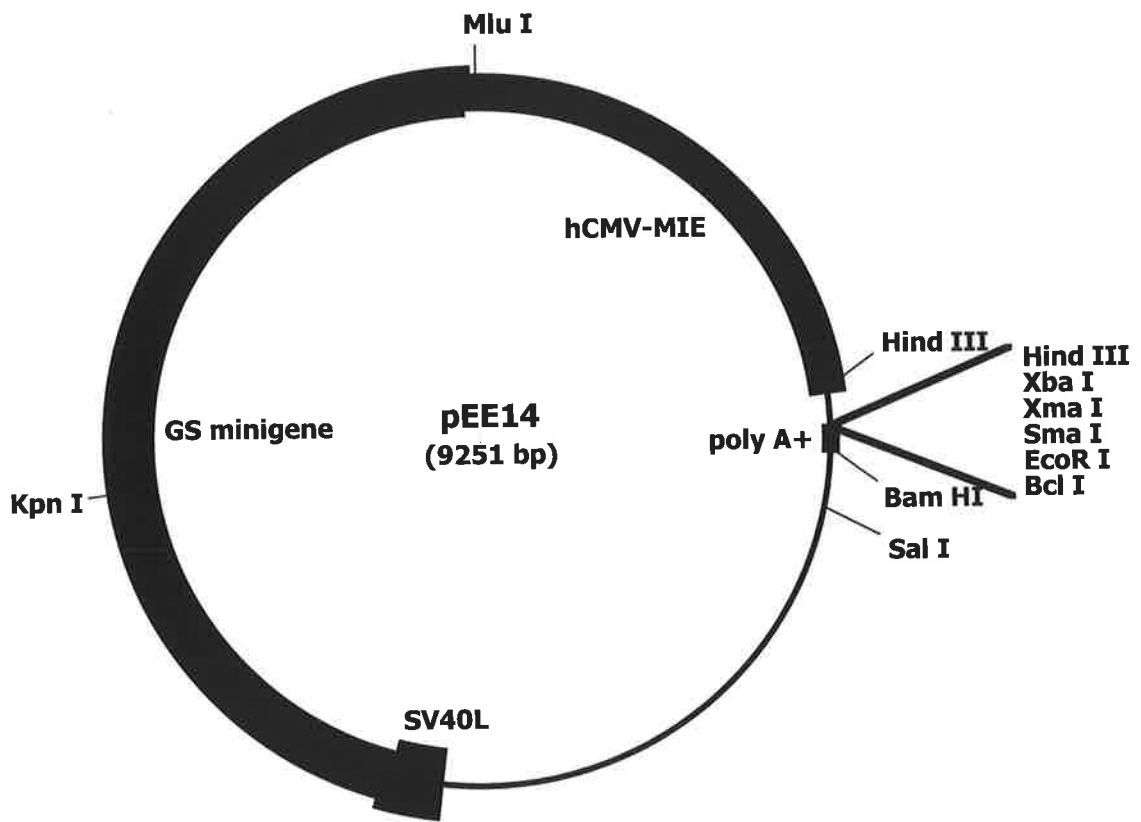
The KIT^S cDNA harboured in pSHR1.6 was excised using restriction endonucleases *Xba*I and *Eco*R V. The *Xba* I end was end filled using the Klenow fragment of DNA polymerase and the purified fragment was ligated into the pEE14 vector (Figure 2.4) which had been linearised with the enzyme *Sma* I and dephosphorylated using CIP. pEE14 was obtained from Genetech, USA. The ligated DNA was transformed into DH10 β by heat shock and transformants selected on ampicillin containing agar plates. Plasmids containing the KIT^S cDNA were identified by digestion of isolated plasmid DNA with *Bam*H I and the insert orientation was determined by digestion with *Eco*R I. The resultant clone was referred to as pSHR1.20.

2.17 Cloning of Tagged KIT^S into Expression Vectors

2.17.1 Cloning KIT^S cDNA into the bacterial expression vector pFLAG

The KIT^S cDNA to be cloned into the pFlag expression vector (Kodak Eastman) (Figure 2.5) was generated by PCR using c-Kit specific oligonucleotides 862 and 863 (see Table 2.1) and the conditions outlined in Table 2.2. Primer 863 binds to the c-Kit sequence at 970-984. This sequence lies at the end of the third immunoglobulin-like loop of c-Kit. Expression of large complex proteins can often prove difficult in bacteria, resulting in the production of insoluble inclusion bodies or small amounts of soluble protein. Therefore, the expression of only the first three domains of c-Kit was tested. The PCR product was digested with restriction endonucleases *Xba* I and *Bgl* II and ligated into pFLAG that had also been digested with *Xba* I and *Bgl* II. The ligated DNA was introduced into electrocompetent *E. coli* cells by electroportation. Transformed colonies were screened for KIT^S cDNA presence by restriction digestion of isolated plasmid DNA with *Xho* I and *Eco*R I. This clone was called pSHR1.26. *E. coli* (DH10 β) were transformed with pSHR 1.26 or empty pFLAG vector, which acted as the negative control. The transformed bacteria were cultured overnight at 37°C in the presence of IPTG at 500nM to induce the production of the KIT^S protein driven by the Lac Z promoter. The bacteria were pelleted and the recombinant protein isolated from the bacterial pellet using a modified method from that published, see section 2.19.2.

Figure 2.4: Diagrammatic representation of pEE14 plasmid. This plasmid contains the glutamate synthase (GS) minigene that allows the amplification of target genes under the selective pressure of methionine sulfoxamine (MSX). The plasmid also encodes resistance to ampicillin, the exact position of the gene is unknown.



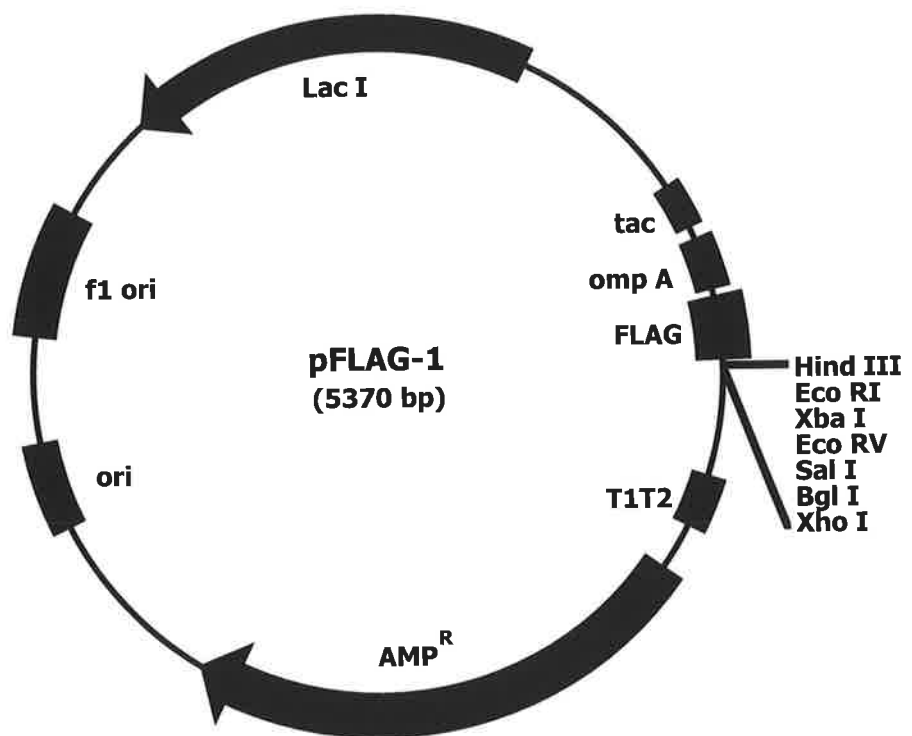


Figure 2.5: Diagrammatic representation of pFLAG-1 plasmid. This plasmid allows the expression of recombinant proteins in bacteria. Expressed proteins are synthesised with an amino-terminal Flag tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) allowing the purification of the protein using anti-Flag Sepharose.

2.17.2 Cloning of KIT^S cDNA into the pIG vector

To clone KIT^S cDNA into the pIG vector (kindly provided by Dr D. Simmons, Imperial Cancer Research Fund, Institute of Molecular Medicine, Oxford, United Kingdom) (Fawcett *et al.*, 1995) (Figure 2.6) a new PCR product had to be generated (see schematic of cloning procedure in Figure 2.7). Expression from this plasmid results in the fusion of the c-Kit mRNA to the mRNA encoding Fc portion of the human IgG protein. Translation from this mRNA results in a fusion protein that is dimeric. The primers used in the PCR reaction were 387 and 1244 (see Table 2.1). In addition to c-Kit sequence primer 1244 also contained the sequence encoding a splice donor site. Both primers contain *Bam*H I restriction sites to allow cloning into the recipient vector. The 1.5kb fragment was amplified using pSHR1.6 as the template and *Pfu* polymerase (Stratagene) as the synthesis enzyme. The amplified cDNA was digested with *Bam*H I and ligated into the vector pIG that had also been digested with *Bam*H I and dephosphorylated, using CIP, to prevent self-ligation. The DNA was introduced into DH10 β /P3 cells by electroporation and the plasmid DNA isolated from individual colonies of transformed cells. Clones carrying the KIT^S cDNA were identified by digestion of the plasmid DNA with *Bam*H I and correct orientation of the insert confirmed by restriction endonuclease digestion with *Hind* III. This clone was termed pSHR1.45. pSHR1.45 was transfected into COS cells and the cells were cultured for four days at 37°C/5% CO₂ in medium supplemented with 1% FBS that had been pre-cleared of Protein A binding proteins. Culture supernatant was collected, cleared of cellular debris by centrifugation, adjusted to pH 8.0 and stored at -20°C.

2.18 Production of KIT^S in Insect Cells Using the Baculovirus Expression System

2.18.1 Generation of a Baculovirus shuttle vector containing KIT^S-His cDNA

KIT^S-His was generated in several steps (see Figure 2.8 for schematic of cloning). Firstly the cDNA encoding KIT^S was excised from pSHR1.6 using restriction endonucleases *Pst* I and *Not* I. The resultant 1.5kb fragment was agarose gel purified and ligated into the Baculovirus transfer vector BacPak8 (Figure 2.9) similarly digested with *Pst* I and *Not* I. This ligated DNA was transformed into DH10 β cells by electroporation and clones carrying KIT^S

Figure 2.6: Diagrammatic representation of pIG 1 plasmid. This plasmid contains the hinge, constant heavy chain domain 2 and constant heavy chain domain 3 of genomic human IgG₁ Fc. The target gene is cloned into this plasmid with a splice donor site at the C-terminus allowing the target mRNA to be fused in frame with the IgG₁ heavy chain. This allows a dimeric protein to be expressed that can be purified on Protein A Sepharose. Plasmid must be propagated in bacterial cells carrying the P3 plasmid, allowing growth in ampicillin and tetracycline containing media.

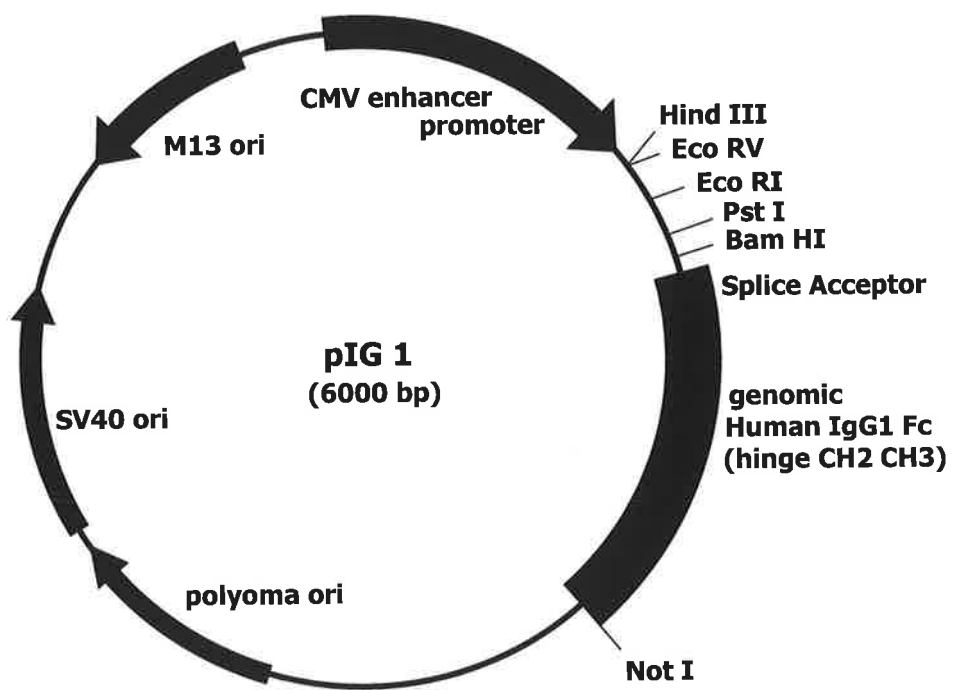


Figure 2.7: Diagrammatic representation of the production of plasmid pSHR1.45. KIT^S cDNA was amplified using c-Kit specific oligonucleotides 387 and 1244. The PCR product was digested with *Bam*H I and ligated into pIG.

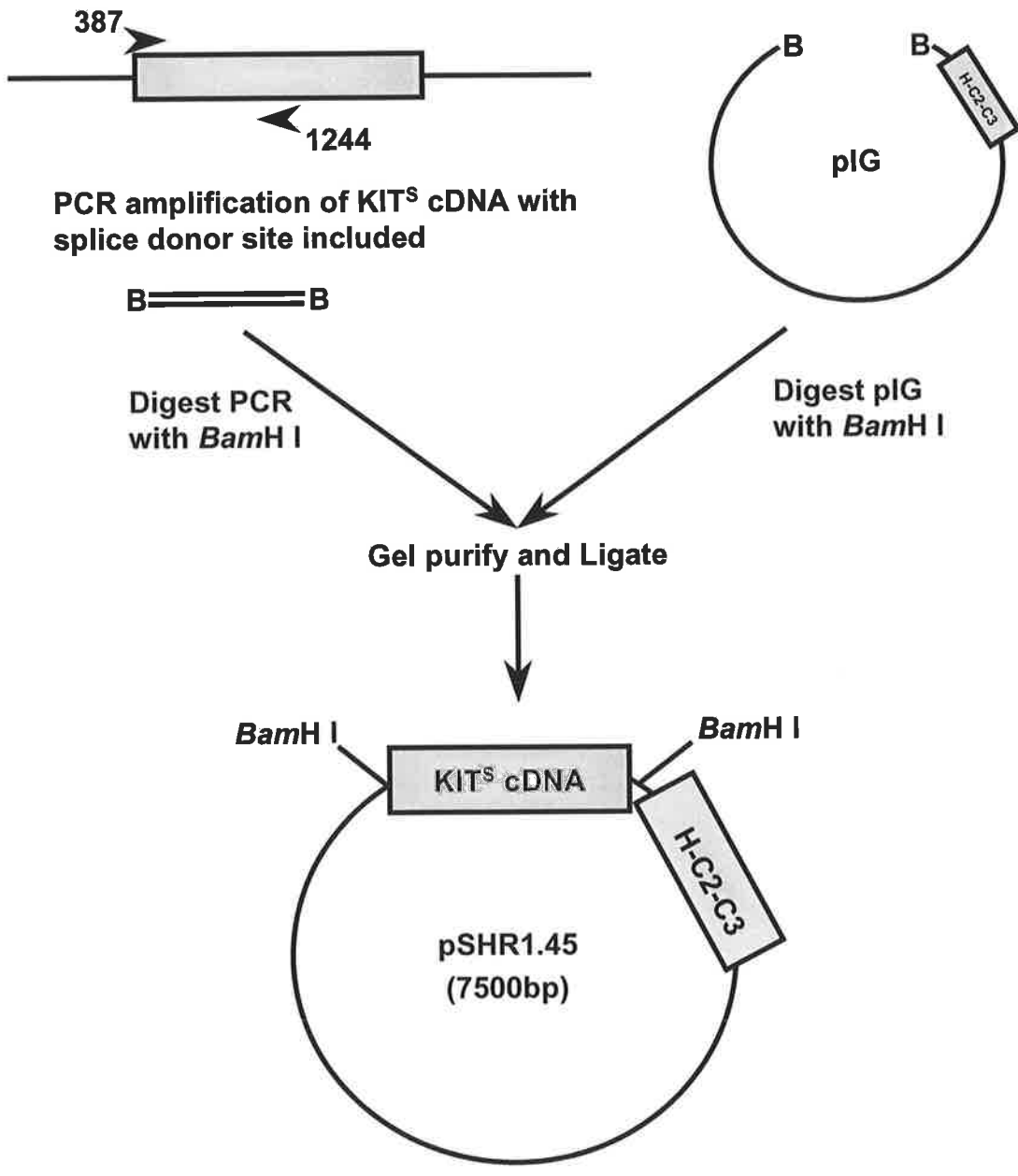


Figure 2.8: Diagrammatic representation of the production of pSHR1.30His. KIT^S cDNA was subcloned from pSHR1.6 into Baculovirus shuttle vector BacPak8 using restriction endonucleases *Pst* I and *Not* I, generating pSHR1.30. To insert a 6xHis tag at the C-terminus c-Kit specific oligonucleotides 765 and 1070 were used to amplify a small fragment of the C-terminus of c-Kit containing a 6xHis sequence. This was ligated into pSHR1.30 that had been cut with both *Sph* I and *Not* I, generating pSHR1.30His.

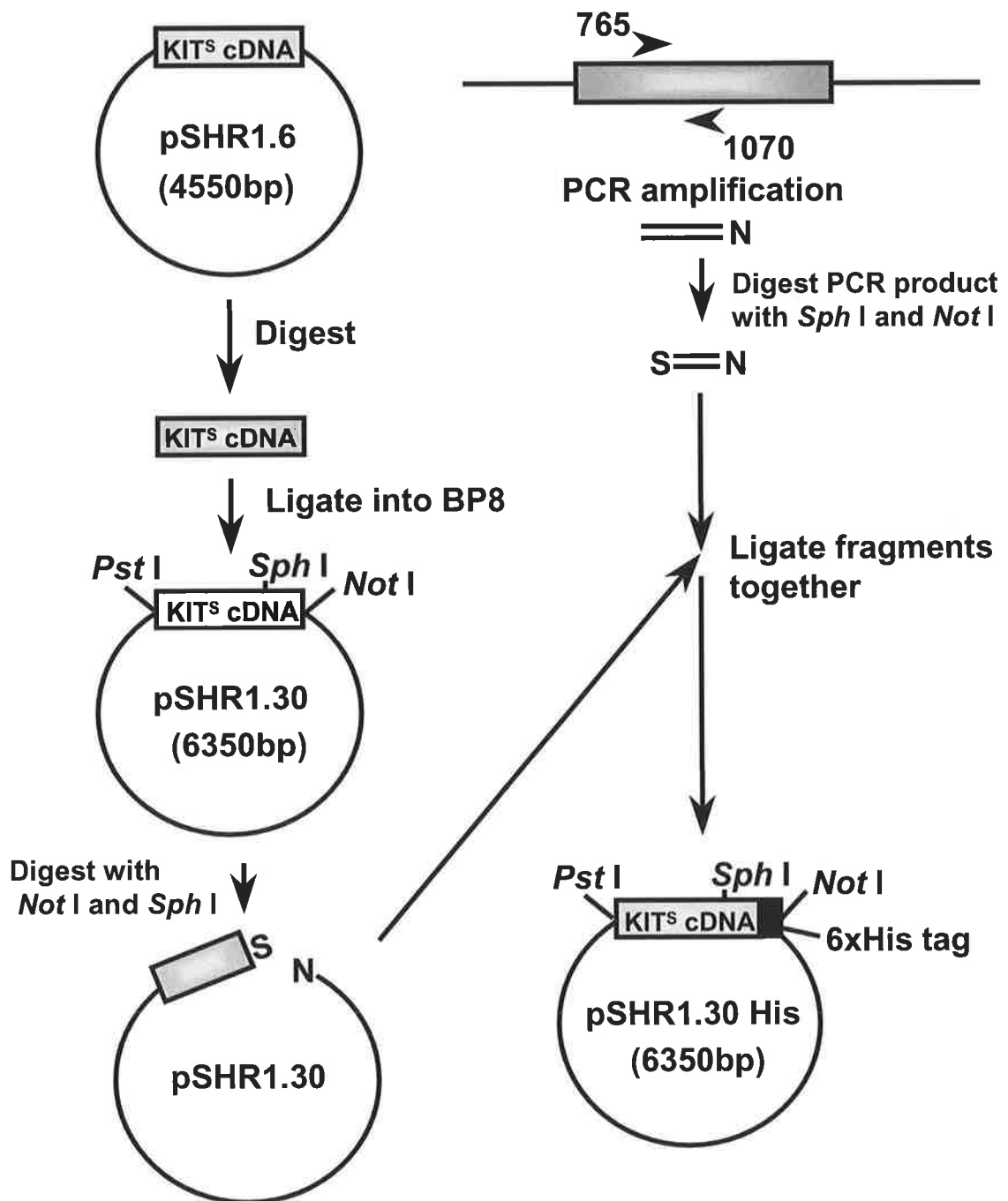
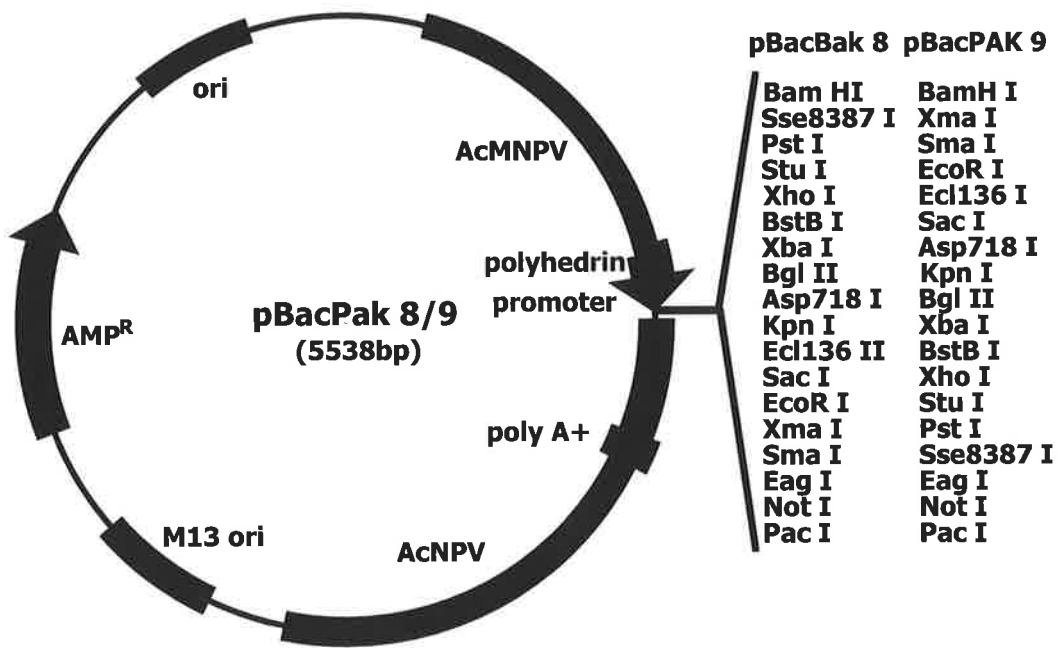
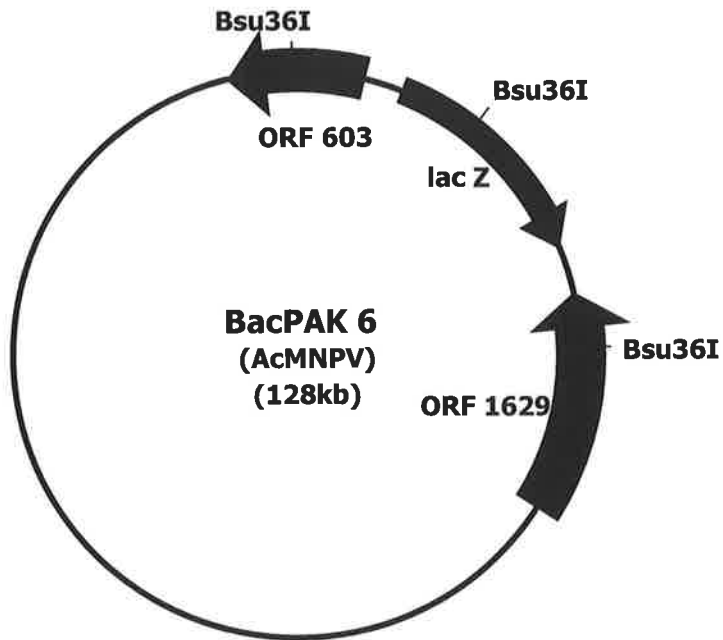


Figure 2.9: Diagrammatic representation of the Baculovirus plasmids BacPak6 (BP6) and pBacPak8/9 (BP8/9). BacPak6 plasmid contains the DNA required to generate infective viral particles. Due to its large size it is not possible to clone directly into this plasmid. Therefore target genes are cloned into one of the shuttle vectors BP8 or BP9. Target genes are transferred from the shuttle vector to BP6 by recombination within transfected insect cells.



cDNA were isolated by restriction endonuclease digestion with *BamH* I. This clone was termed pSHR1.30. The 6xHistidine tag was then inserted into pSHR1.30. This was achieved by PCR using c-Kit specific oligonucleotides 765 and 1070 (see Table 2.1). The primer at the 3' end of the sequence, 1070, encoded 18 bases corresponding to the c-Kit sequence, the 6xhistidine sequence, a stop codon and the *Not* I restriction endonuclease site for cloning. The 6xHistidine tag was incorporated into the C-terminus of KIT^S because of the requirement for the signal sequence at the 5' end of the sequence to allow export of KIT^S protein from the cell. During this process the signal sequence is cleaved and, had the 6xHistidine tag been located before the signal sequence, it too would have been removed thus preventing purification of the recombinant protein using the Ni⁺⁺ resin. Using pSHR1.6 as a template, PCR amplification using oligonucleotides 1070 and 765 generated a 400bp fragment (see Table 2.1). The resultant product was phenol/chloroform extracted and digested with restriction enzymes *Not* I and *Sph* I. The 255bp fragment was gel purified away from the 145bp fragment and ligated into pSHR1.30 that had also been digested with *Not* I and *Sph* I. Ligated DNA was transformed into DH10 β cells by electroporation, selection of transformants on ampicillin containing agar and vectors with a KIT^S cDNA insert were identified by digesting isolated plasmid DNA with *BamH* I. This construct was termed pSHR1.30His.

2.18.2 Generation of a Baculovirus shuttle vector containing KIT^S-IGb cDNA

A Baculovirus shuttle vector encoding dimeric KIT^S was generated by subcloning the cDNA encoding the KIT^S-Ig fusion protein into the Baculovirus vector. This clone was created by excising the relevant fragment from pSHR1.45 using restriction endonucleases *EcoR* V and *Not* I and directionally ligating it into the Baculovirus transfer vector BacPak9 (Figure 2.9) which had been digested with *Not* I and *Sma* I. Ligated DNA was introduced into DH10 β cells by electroporation, selection of transformants on ampicillin containing agar and vectors with a KIT^S cDNA insert were identified by digesting isolated plasmid DNA with *BamH* I and *Hind* III. This clone was referred to as pSHR1.36.

2.18.3 Generation of Recombinant Virus

The BacPAK6 viral DNA (Figure 2.9) is too large to clone the gene of interest into it. Therefore transfer vectors are used. Transfer vector DNA containing the target gene is transfected into Sf21 cells along with *Bsu36I* digested viral DNA (Clontech Laboratories). *In vivo* homologous recombination between the plasmid and viral DNAs rescues the viral DNA and in the process transfers the target gene to the viral genome.

Sf21 cells were transfected using Lipofectin (Clontech) according to the manufacturer's instructions. Briefly, wells of a 6 well plate were seeded with 1×10^6 Sf21 cells 24 hours prior to the transfection. The plate(s) were placed into a humidified box at 27-30°C. The following day the medium was removed and replaced with 2ml of serum free, antibiotic free medium. The plate was gently swirled and the medium removed and replaced with 2ml of serum free, antibiotic free medium. (The lipofection reagent is inhibited by serum). The cells were incubated at room temperature for 30 min while the DNA/lipofectin complexes were formed. The plasmid DNA was diluted to 100ng/ μ l with TE buffer. The following were added in polystyrene tubes:

	Tube1	Tube2 Control
Sterile H ₂ O	40 μ l	45 μ l
Plasmid DNA (100ng/ μ l)	5 μ l	5 μ l
BacPAK6 viral DNA (<i>Bsu36 I</i> digested)	5 μ l	-
Final volume	50 μ l	50 μ l

To the DNA tubes 50 μ l of Lipofectin (supplied with BakPak6 DNA) was added and mixed gently and left at room temperature for 15 min to allow Lipofectin-DNA complexes to form. The medium was removed from the cells and replaced with 1.5ml of serum free, antibiotic free medium and the Lipofectin-DNA mix added dropwise to the medium while gently swirling the plate. The cells were left for 5 hours then 1.5ml of Grace's medium with 10% FBS and antibiotics were added. After 72 hours the medium was harvested and stored sterile at 4°C.

2.18.4 Isolation of Recombinant Viruses

Six well plates were seeded with 1×10^6 Sf21 cells/well in 1.5ml Grace's medium supplemented with 5% FBS. After an overnight incubation the medium was removed and 0.5ml of fresh medium was added. The cells were then incubated with serial dilutions of the

co-transfection supernatant (100µl/well of 10^{-1} , 10^{-2} and 10^{-3} dilutions). The cells and diluted supernatant were incubated at room temperature on a level surface for 1 hour with periodic swirling to prevent the cells from drying. All of the medium and co-transfection supernatant was removed and the cells overlaid with 1% agarose in Grace's medium. Plates were incubated at 27-30°C in humidified containers for 4 days and then stained with 1ml neutral red (1/20 of 0.33% stock in PBS). After 24 hours the wells were examined for viral plaques. Isolated plaques were picked and virus eluted from the agarose plug by incubation in 0.5ml of Grace's medium overnight at 4°C.

2.18.5 Evaluation of Plaque Picks for Target Gene Expression

Sf21 cells seeded at 1×10^6 /well of 6 well plates were incubated with 100µl of plaque pick supernatant in a total of 0.5mls. After 1 hour, 1ml of Grace's medium was added to the wells and the plates incubated at 27-30°C for 3-4 days. After this time the supernatant was harvested. This is Passage One virus stock and is also the source of expressed target protein if excreted from the cells. The cells from the well were also harvested with PBS by gentle pipetting using a pasteur pipette. The titre of the stock was determined by plaque assay, section 2.18.6. The supernatant and cells were assayed for target protein expression as outlined in section 2.7.

2.18.6 Plaque Assay

Plaque assays are designed to produce distinct viral plaques in a monolayer of Sf21 cells. They are used to isolate individual recombinant virus and to determine virus stock titres.

Wells of 6 well plates were seeded with 1×10^6 Sf21 cells in 1.5mls of Grace's medium and incubated overnight at 27-30°C. Virus stock was diluted serially down to 10^{-15} . The medium was aspirated from the cells and replaced with 0.5mls of Grace's medium. To the centre of the well 100µl of virus inoculum was carefully added and the plate gently swirled. The plates were left at room temperature for 60-90 min on a level surface in the dark. During this incubation the agarose overlay was prepared. 5% agarose solution (5% SeaPlaque agarose (FMC) in sterile H₂O) was melted, cooled to 37°C and diluted to 1% in prewarmed Grace's medium. At the end of the infection incubation the virus and medium were removed

by tilting the plate and aspirating from the edge of the well. The cells were overlaid with 2-3mls of the diluted agarose by running the agarose down the side of the well avoiding the disturbance of the cells. Once the agarose had set the plates were transferred to humidified containers and incubated at 27-30°C for 4-5 days. Neutral red (0.33% neutral red in PBS) was diluted 1/20 in PBS and filter sterilised. One ml of this was added to each well and the plates incubated overnight at 27-30°C. The plaques appear as clear circles 0.5-3mm in diameter.

Calculation of virus titre: Plaques were counted in wells that had a reasonable number of plaques (i.e. 10-30) and from this the average number of plaques was calculated from triplicate wells. Since 0.1ml of inoculum was added to the well the titre of the virus stock (pfu/ml) is: average number of plaques per dish x 10 x 1/dilution factor,
e.g. 25 plaques x 10 x (10⁻⁵)⁻¹ = 2.5x10⁷ pfu/ml

2.18.7 Amplification and expansion of virus stocks

Large stocks of recombinant virus were obtained via the following method. Either 50ml suspension culture of Sf21 cells at 5x10⁵ cells/ml or 2-3 150cm² flasks, seeded with 8x10⁶ cells/flask, were infected with Passage One virus stock at 1 pfu/cell. The cells were incubated at 27-30°C for 4-6 days. The supernatant was harvested and centrifuged at 1000g to remove the cells and debris. The supernatant, Passage Two virus stock, was transferred to sterile tubes and aliquoted. Aliquots were stored long term at -70°C and short term at 4°C. The titre of the Passage Two virus stock was determined as described in section 2.18.6. Working stocks were generated by the same method but on a larger scale.

2.18.8 Protein Production

Either 200ml suspension culture of Sf21 cells at 1x10⁶/ml or 10-20 150cm² flasks, seeded at 60% confluency, were infected with working stock virus at a multiplicity of infection, MOI, of 10. After 4-6 days the supernatant was harvested. Azide was added to a concentration of 0.1% and the pH adjusted to 8.2 using 1M NaOH. Increasing the pH resulted in the formation of a precipitate. The cells, debris and precipitate were removed by centrifugation at 200g. The cleared supernatant was transferred to a clean container and stored at 4°C. KIT^S was not detected in the precipitate by SDS-PAGE and Western blot

2.19 KIT^S Protein Purification

2.19.1 Concentration of Culture Supernatant

Filtered culture supernatants were concentrated 10 fold using an Amicon flow cell concentrator fitted with an YM30 membrane with a molecular weight cut off of 30kDa.

2.19.2 Purification of KIT^S from Bacteria

Bacterial pellets (see section 2.17.1) were resuspended in 2ml of 10mM Tris/ 20% sucrose, pH 8.0. Lysis was enhanced by adding 62.5 mM EDTA and 1mg/ml of lysozyme. After a 40 min incubation, the volume was made up to 30mls with ice cold water and left for 30 min. The debris was pelleted and the supernatant retained. The supernatant had a light straw colour. The supernatant was then concentrated and the KIT^S protein purified by passing the concentrated supernatant down an anti-FLAG Sepharose column in the presence of TBS (20mM Tris-Cl, pH 7.5, 150mM NaCl) and 2mM CaCl₂. This is a modification of the protocol since it was found that the addition of PBS and CaCl₂ resulted in a CaPO₄ precipitate that blocked the column flow. The bound KIT^S was eluted with 0.1M glycine pH 3.0. Eluted samples were assayed by SDS-PAGE and Western blot (see section 2.7) using 1C1.HF to detect the presence of KIT^S.

2.19.3 Purification of KIT^S-His using Metal Chelate Affinity Chromatography

Concentrated supernatant was added to 2.5mls of Ni⁺⁺-NTA-Sepharose (previously washed with 7.5ml of deionized water and then 12.5ml of metal chelate affinity chromatography buffer, MCAC; 20mM Tris, 0.5M NaCl, pH7.9), in a 50ml tube and rotated, overnight at 4°C. The suspension was allowed to warm to room temperature and was then poured into a column (Bio-Rad, Glass Econocolumn), at room temperature and the effluent collected, to check for residual KIT^S protein. The Sepharose was extensively washed with MCAC buffer and the bound protein was eluted with the same buffer containing 200mM imidazole, which acts as a competitor for the Ni⁺⁺ attached to the Sepharose. Eluted KIT^S-His protein was dialysed against PBS and stored at 4°C in the presence of 0.1% BSA after the protein concentration had been determined.

2.19.3.1 Regeneration of the NTA-Sepharose

The NTA-Sepharose could be reused after stripping and recharging using the following method: The column of resin was first stripped using stripping solution (0.2M acetic acid/ 6M guanidine HCl) then washed with 5mls of H₂O followed by 2.5mls of 2% SDS and then 2.5mls H₂O. The column was then washed with increasing concentration of ethanol from 2.5mls of 25% ethanol, 2.5ml 50%, 2.5mls 75% and then 12.5mls 100% ethanol. This was followed by decreasing concentrations of ethanol down to 25%. The column was then washed with water, 12.5mls 0.1M EDTA, pH 8.0 and then 10ml water. The NTA-resin was then recharged with Ni⁺⁺ by washing with 12.5ml of 50mM NiSO₄.6H₂O followed by equilibration with 12.5ml of MCAC buffer. If the column was to be stored for more than 24 hours it was washed with 10ml of 20% ethanol and then overlaid with ethanol.

2.19.4 Purification of KIT^S-IGb

Despite being tagged with Fc portion of human immunoglobulin, KIT^S-IGb could not be purified using Protein A coupled to Sepharose, see section 3.10. Therefore, KIT^S-IGb was purified utilising its affinity for the anti-c-Kit mAb 1DC3. Concentrated supernatant was added to 2.5mls of Sepharose that had been coupled to 1DC3 (see section 2.2.5) in a 50ml tube and rotated, vertically overnight at 4°C. The Sepharose and supernatant were poured into a column (Bio-Rad, Glass Econocolumn), at 4°C, and the effluent collected to check for residual KIT^S. Non-specifically bound protein was removed by washing the Sepharose with PBS, pH 8.0 until the O.D._{280nm} was less than 0.05. The bound KIT^S-IGb was eluted with 0.1M glycine, pH 2.7 into a tube containing 1/10 final volume of 1M Tris-HCl, pH 8.6. Eluted KIT^S-IGb protein was dialysed against PBS and stored at 4°C in the presence of 0.1% BSA after the protein concentration had been determined.

2.19.5 Purification of KIT^S-IGc

Concentrated supernatant was added to 2.5mls Protein A Sepharose (Pharmacia, Cat No. 17-0780-01) in a 50ml tube and rotated, vertically overnight at 4°C. The Sepharose and supernatant were poured into a column (Bio-Rad, Glass Econocolumn), at 4°C, and the effluent collected. Non-specifically bound protein was removed by washing the Sepharose with PBS, pH 8.0 until the O.D._{280nm} was less than 0.05. The bound KIT^S-IGc was eluted

with 0.1M glycine, pH 2.7 into a tube containing 1/10 final volume of 1M Tris-HCl, pH 8.6. Eluted KIT^S-IGc protein was dialysed against PBS, concentrated and stored at -20°C in the presence of 0.1% BSA after the protein concentration had been determined.

2.19.6 Determination of KIT^S Protein Concentration

Total protein concentration was determined by measuring the absorption at 280nm on a spectrophotometer, 1cm light path, assuming that O.D₂₈₀ of 1.0 represents 1.12mg/ml of protein. The extinction coefficient for KIT^S was determined using a program kindly provided by Dr C. Bagley, Human Immunology, Hanson Centre for Cancer Research.

The concentration of dilute protein solutions was determined using a commercially available protein assay kit (Bio-Rad). The manufacturer's instructions were followed as described. Briefly: the supplied protein standard, bovine immunoglobulin, (Bio-Rad, Cat. No. 500-0005), was diluted two fold between 1mg/ml and 0.05mg/ml, in water. In triplicate, 10µl of diluted protein standards and protein to be assayed were added to separate wells of a 96 well plate (Corning Costar). To each well 200µl of dye reagent (Bio-Rad, Cat. No. 500-0006), diluted 1/5 in H₂O, was added and the plate incubate at room temperature for 5-60 min. The absorbance was measured at 595nm, using a Bio-Rad microplate reader. Test samples were plotted against the curve generated by the known standard.

Purity of the purified, KIT^S containing solutions was assessed by SDS-PAGE, total protein stain and Western Blot, see section 2.7. The solutions were also tested for KIT^S concentration by ELISA and the data compared to that of the total protein assessments. Together the results from all of the assays were used to determine the KIT^S concentration of the purified samples.

3. Expression, detection and purification of KIT^S

3.1 Introduction

To further the knowledge of the function of the c-Kit protein, experiments utilising the extracellular domain of the c-Kit protein were initiated by Flanagan and Leder (1990). Using a murine c-Kit extracellular domain-alkaline phosphatase fusion protein, expressed in J558L myeloma cells, they showed this fragment of the protein was able to bind to 3T3 fibroblast cells from normal mice but not from mice with the *Sl* mutation, providing evidence that the ligand to c-Kit was encoded by the *Sl* locus. Observations that the binding of EGF to an overexpressed chimeric EGFR/c-Kit protein (extracellular domain of the EGF receptor fused to the cytoplasmic domain of c-Kit) resulted in the transformation of NIH3T3 cells (Lev *et al.*, 1990) raised the question as to whether c-Kit may be involved in malignancies or other hyperproliferative states. To address this Lev *et al.* (1992a) generated a recombinant extracellular domain of c-Kit. Simultaneous addition of SLF and the soluble c-Kit molecule to cells expressing c-Kit resulted in stoichiometric inhibition of SLF binding to these cells and a decrease in tyrosine autophosphorylation of the receptor. In addition, downstream signalling was disrupted, with the association of c-Kit to PI3'K and coupling to the Raf1 protein kinase being inhibited (Lev *et al.*, 1992a). A soluble c-Kit molecule, produced in CHO cells, has been used to establish an enzyme linked immunosorbent assay for naturally occurring soluble c-Kit molecules (Turner *et al.*, 1995; Wypich *et al.*, 1995).

In 1988 Ashman *et al.* observed that high level binding of a monoclonal antibody (YB5.B8) to AML cells correlated with poor prognosis of the patients. The corresponding antigen was later shown to be c-Kit (Lerner *et al.*, 1991). Thus, it appeared the increased, or abnormal expression of this tyrosine kinase receptor may be involved in the transformation of early myeloid cells. It was postulated that inhibition of ligand induced signalling through the c-Kit molecule may prevent the proliferation of the AML cells. The extracellular domain of the c-Kit was considered as a candidate antagonist. This chapter describes the techniques and expression systems analysed to best produce this soluble c-Kit molecule.

3.2 Cloning of soluble c-Kit cDNA

The cDNA coding for KIT^S was generated by polymerase chain reaction (PCR). A clone containing the coding region cDNA of human c-Kit in pBluescript (SK) (kindly provided by Dr D. Williams, Immunex Corporation, Seattle, WA, U.S.A.) was used as a template for the PCR reaction. This template had the same sequence as that published by Yarden and colleagues (1987). c-Kit specific oligonucleotides 387 and 476 (Table 2.1) were used to initiate strand elongation. Both contained *Bam*H I restriction endonuclease sites at their 5' ends and in addition oligo 476 contained bases encoding an in frame stop codon 5' of the restriction site (see Table 2.2 for PCR conditions). cDNA amplification with primers 387 and 476 resulted in a 1566bp product, the last three bases encoding amino acid glutamine 515. This site was chosen so that the c-Kit protein encoded by this cDNA contained all 5 of the immunoglobulin domains but did not contain any of the amino acids involved in the transmembrane domain. By comparison the cDNA used by Yarden and colleagues to generate a soluble c-Kit molecule terminated at the *Dra* I site at a position corresponding to amino acid lysine 509. The 1566bp fragment, (Figure 3.1, lane 1) was ligated into the *Bam*H I site of the plasmid pBluescript SK⁺. This clone will be termed pSHR1.6 from here.

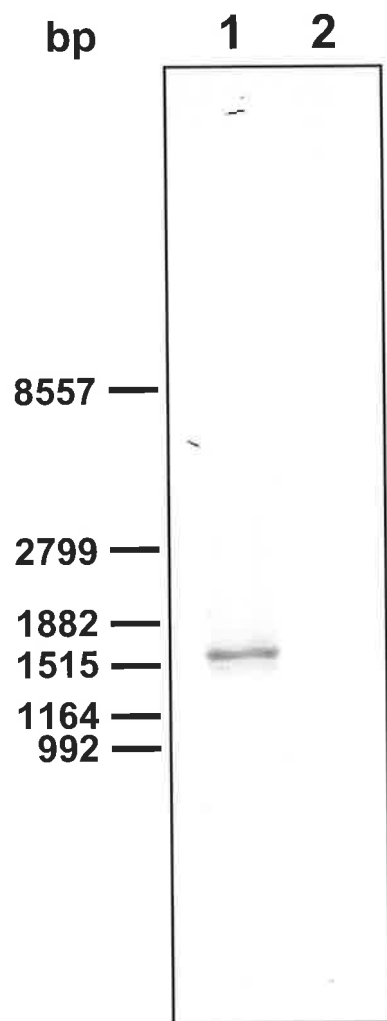
The entire sequence of the PCR generated cDNA was checked for mistakes by sequencing the construct in both DNA strand directions. Errors may have been generated during the amplification process since the thermostable DNA polymerase used to amplify the KIT^S cDNA, *Taq* DNA polymerase, does not contain 3'-5' exonuclease activity and therefore cannot remove mismatched bases. Sequencing of the KIT^S cDNA was achieved using both cycle sequencing and T7 polymerase sequencing methods (see section 2.12). All PCR generated sequence was compared to the sequence published by Yarden *et al.* (1987) and no discrepancies were found.

3.3 Expression of KIT^S using pFLAG and *E. coli* cells

3.3.1 Cloning KIT^S cDNA into pFLAG

While it was the intention to express recombinant soluble c-Kit protein using eukaryotic expression systems, colleagues at the Hanson Centre for Cancer Research had successfully used the pFLAG bacterial expression system (Scientific Imaging Systems (Kodak Eastman))

Figure 3.1: Analysis of amplified KIT^S cDNA. KIT^S cDNA was amplified using c-Kit specific oligonucleotides 387 and 476 (Table 2.1). Samples were separated on 1% agarose gel, stained with ethidium bromide and visualised using a FluorImager 595 (Molecular Dynamics) and Imagequant software. **Lane 1:** KIT^S cDNA PCR; **Lane 2:** H₂O PCR control.



to express small proteins on a small scale. The pFLAG system allows the recombinant protein to be expressed as a fusion protein containing the FLAG peptide at its amino terminal end (see Figure 2.5). Commercially available antibodies recognise the FLAG peptide and allow purification of the fusion protein by affinity chromatography. The pFLAG expression system has successfully been used to produce, for example, FLAG-CNTF, FLAG-IL-13 and FLAG-IL-18 fusion proteins (Rabinovsky *et al.*, 1994; Bost *et al.*, 1996; Elhofy and Bost, 1998). Between the FLAG peptide and the recombinant protein there is an enterokinase site allowing the removal of the FLAG 'tag' should it inhibit protein activity. In addition, an OmpA signal peptide is incorporated at the 5' end to allow the recombinant protein to be exported into the periplasmic space, allowing simple lysis procedures to isolate the protein from majority of host bacterial proteins. To prevent removal of the OmpA and FLAG peptides it was necessary to generate a soluble c-Kit cDNA with the signal sequence removed. Therefore, the KIT^S cDNA to be cloned into pFLAG was generated by PCR using c-Kit specific oligonucleotides 862 and 863 (see Table 2.1) and the conditions outlined in Table 2.2. Ligation of this PCR product into the *Xba* I and *Bgl* II sites of the pFLAG vector resulted in the transcription of mRNA encoding the Omp A and FLAG peptides fused to c-Kit at amino acid 25 (serine). This KIT^S clone was smaller than other KIT^S cDNAs cloned into the eukaryotic vectors, encoding only the first three immunoglobulin domains of the full length c-Kit protein. Large recombinant proteins often form insoluble inclusion bodies within bacteria, requiring the use of denaturing reagents, e.g. 8M urea or 6M guanidine hydrochloride, to isolate and purify the recombinant protein. This clone was called pSHR1.26.

3.3.2 Expression of pSHR1.26

E. coli (DH10 β) were transformed with pSHR1.26 or empty pFLAG vector, pSHR1.27, which acted as the negative control. The transformed bacteria were cultured overnight at 37°C in the presence of IPTG at 500nM to induce the production of the KIT^S protein. The bacteria were pelleted and the recombinant protein isolated from the pellet and purified (see section 2.17.2). Eluted samples from the anti-Flag affinity column were assayed by SDS-PAGE and Western blot, (see section 2.7), using anti-c-Kit mAb 1C1.HF to detect for the

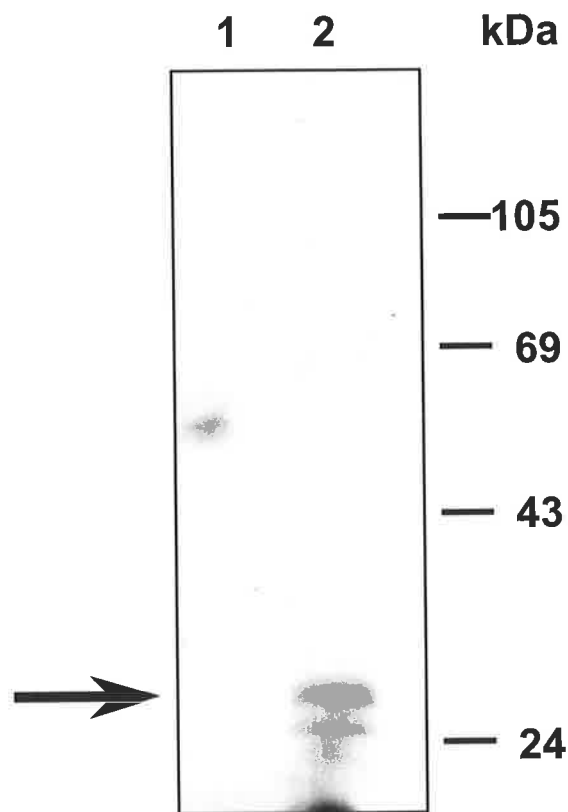
presence of KIT^S protein. A band of molecular weight ~30kDa was detected in the lane of the blot containing purified lysate from pSHR1.26 cells but not of the negative control (Figure 3.2). This weight corresponded with the predicted molecular weight of 32kDa. However, the amount of KIT^S protein produced by one litre of bacterial culture was miniscule, i.e. it could only be detected by Western, and because of this and the expense of the anti-FLAG Sepharose, work with this expression system was not continued. In hindsight, alteration of culture conditions may have improved KIT^S production. Using BL21 cells instead of DH10 β cells, changing the concentration of IPTG used, the culture and induction times or temperature may have improved the amount of KIT^S produced. In addition, more clones should have been screened. While all the cells were carrying the expression plasmid, not all clones produce protein and it is sometimes necessary to screen many clones to find one of high expression.

3.4 Expression of KIT^S using pcDNA-1/Neo and CHO Cells

The expression plasmid pcDNA-1/Neo was chosen to express KIT^S in eukaryotic cells because it contains the SV40 and polyoma origins of replication, allowing a high plasmid copy number in COS cells (African Green Monkey kidney cells). In addition, the target cDNA is under the control of a strong CMV promoter (see Figure 2.2). These characteristics are particularly useful for transient expression of recombinant protein. In other cell lines, e.g. Chinese Hamster Ovary (CHO) cells, integration of the plasmid can occur and stable expressing clones can be selected because the vector carries the neomycin gene, conferring resistance to the antibiotic G418 to transfected cells. The KIT^S cDNA was subcloned from pSHR1.6 into pcDNA-1/Neo via the *Xba* I and *Xho* I sites. This clone was called pSHR1.7.

The pcDNA-1/Neo construct, pSHR1.7, was introduced into CHO cells by electroporation and primary transfectants were selected based on G418 resistance. After selection, viable cells were expanded and assayed for KIT^S expression by immunohistochemistry (APAAP) (see section 2.2.8) using 1DC3 as the primary anti-c-Kit detecting antibody. Although KIT^S is expected to be secreted from the cells intracellular protein could be detected. Brightly staining cells were clearly identifiable but the population was not homogenous for KIT^S expression and approximately 60% of the cells were positive. To isolate clonal populations, KIT^S expressing cells were seeded in a 96 well plate by limiting

Figure 3.2: Western blot of bacterially expressed KIT^S-Flag protein. Bacterially expressed KIT^S-Flag protein was purified using anti-Flag Sepharose (section 2.19.2) and sample loaded onto a 12% polyacrylamide gel, transferred to nitrocellulose and probed with anti-c-Kit mAb 1C1.HF. Detection of 1C1.HF binding protein was performed using biotinylated anti-mouse F(ab)₂ fragments and then streptavidin coupled to horseradish peroxidase. The blot was then incubated with ECL reagents and exposed to film (section 2.7). **Lane 1:** Purified lysate from bacteria transformed with empty pFlag vector; **Lane 2:** Purified lysate from bacteria transformed with pSHR1.26 DNA. Arrow indicates KIT^S band.



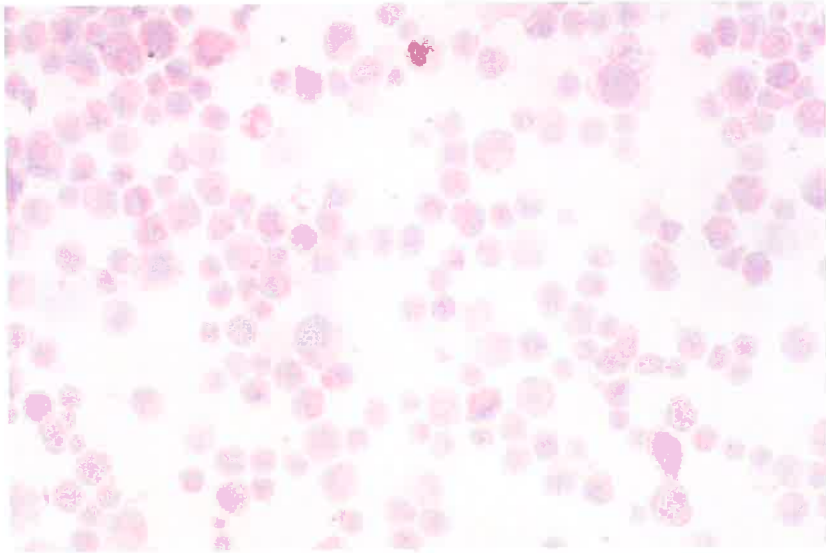
dilution (Oi and Herzenberg, 1980). KIT^S expressing clones were identified by a modification of the APAAP technique. This involved the duplication of the 96 well plate. Only those wells containing colonies initiated by one cell were duplicated. Wells of the second plate were allowed to reach confluency, air dried, fixed and stained *in situ* with anti-c-Kit mAb 1DC3 using the technique described in 2.2.8. Wells of the first plate corresponding to those identified as positive in the second plate were harvested and the clonal cell populations expanded and reassayed for KIT^S expression. The most intensely staining of the 20 clones was chosen for further analysis. This clone, TSHR4.2.C3, was grown in bulk culture and the culture supernatant was harvested and concentrated. The KIT^S protein was purified from the concentrated culture supernatant using its affinity for Wheat Germ Agglutinin-Sepharose as described by Lev *et al.* (1992a). Secretion of KIT^S into the culture medium was confirmed by assaying of the supernatant and purified sample by SDS-PAGE and Western Blot (see section 2.7). However, despite starting as a clonal population (Figure 3.3 (B)), continuous culturing of the TSHR4.2.C3 cells resulted in a decrease in the number of KIT^S positive cells. By 4 weeks of culture and only eight passages the number of cells expressing KIT^S was lower than 1% (Figure 3.3 (C)). A drop in the amount of KIT^S detected in the culture supernatant also occurred. Recloning the KIT^S positive TSHR4.2.C3 cells and culturing of other clones failed to maintain a clone with high expression, so other expression systems were evaluated.

3.5 Increased KIT^S production using amplifiable vector systems

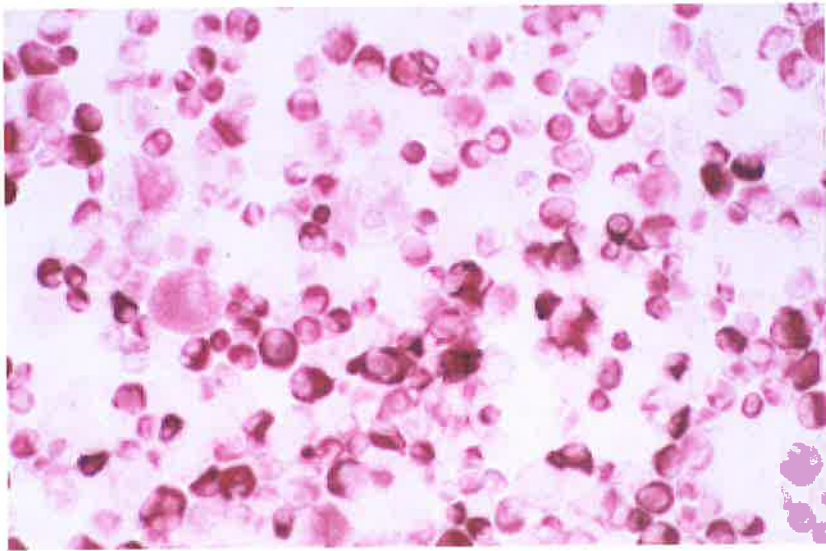
Larger quantities of foreign proteins can be obtained from cell lines carrying amplified chromosomal copies of the gene of interest. To achieve this the target gene is attached to a segment of DNA that carries an essential enzyme, which is inhibited by a particular drug, and this complex is introduced into an appropriate cell line. When the cells carrying such composite structures are exposed to progressively increasing concentrations of the drug, sublines can be selected in which the number of copies of drug resistance marker and the linked foreign gene are greatly amplified. This increases the levels of mRNA and usually the level of protein. Both genomic and cDNAs are amplifiable suggesting the amplification is not due to a particular DNA sequence. The selection protocol serves merely to identify random amplification events occurring with characteristic frequencies in all proliferating cell

Figure 3.3: Immunohistochemical analysis of KIT^S expression by CHO cell transfectant TSHR4.2.C3 (pcDNA-1/Neo vector). Cells were cytocentrifuged onto glass slides, fixed and incubated with the anti-c-Kit mAb 1DC3. Detection of 1DC3 binding to the cells was performed using a bridging rabbit anti-mouse antibody, followed by alkaline phosphatase-mouse anti-alkaline phosphatase complex. Cells were then incubated with substrate, resulting in KIT^S positive cells staining red (section 2.2.7). Cells were visualised using an Olympus microscope and photographed. **A:** TSHR4.2.C3 stained with isotype matched negative control mAb, 3D3; **B:** TSHR4.2.C3 stained with 1DC3 1 week after cloning; **C:** TSHR4.2.C3 stained with 1DC3 after 4 weeks of culture. All photographs were taken at 20x magnification.

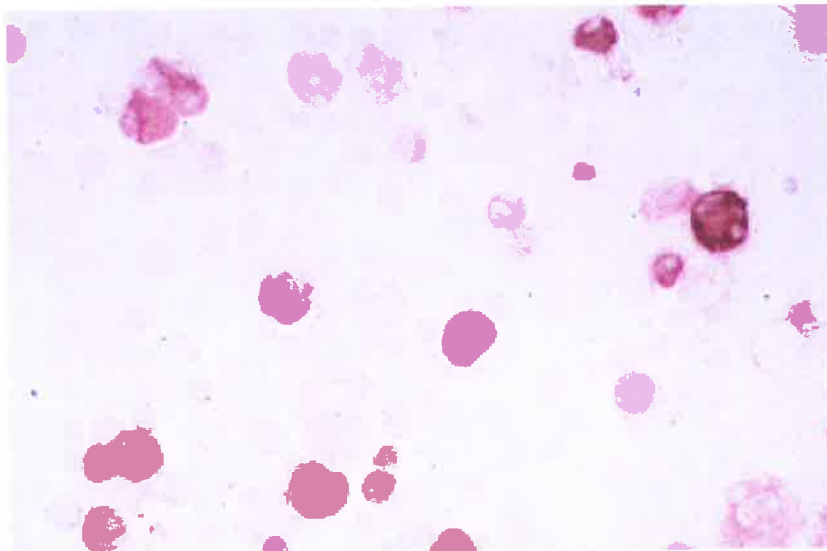
A



B



C



populations, possibly due to DNA replication errors. The region of chromosomal DNA amplified is much greater than the enzymes coding sequence, up to 100kb, resulting in the co-amplification of other sequences and corresponding protein production (Bebington and Hentschel, 1987).

3.5.1 Expression of KIT^S using pRSV009/A⁺ and CHO and SI/SI cells

The most extensively used amplification system employs the dihydrofolate reductase gene, *dhfr*. Methotrexate, Mtx, an analogue of folate inhibits dihydrofolate reductase. Culturing cells in the presence of increasing concentrations of Mtx selects clones with increased copy number of the *dhfr* gene, with concomitant amplification of extensive regions of DNA that flank the *dhfr* sequence (Schimke *et al.*, 1982).

The pRSV009/A⁺ vector (Figure 2.3), similar to the one described by Choo *et al.* (1986), had successfully been used in the lab to gain clones of NIH3T3 cells expressing different levels of full length c-Kit (Caruana *et al.*, 1998). The KIT^S cDNA was subcloned into the *Bam*H I site of this expression vector from pSHR1.6. This clone was called pSHR1.15.

pSHR1.15 was transfected into *dhfr*- CHO cells by the calcium phosphate technique (see 2.5.1) and SI/SI cells (Fujita *et al.*, 1989) by electroporation (see 2.5.3). SI/SI cells were chosen because they do not express the ligand for c-Kit, SLF, preventing possible co-purification of SLF along with the KIT^S protein. Pools of transfected cells were subjected to 50nM Mtx. After a week, stocks of the surviving cells were made and the rest were subjected to 100nM Mtx. Again after about one week and at least one change of medium the cells were stably resistant to 100nM Mtx. Analysis of the surviving cells for expression of KIT^S revealed that none of the cells resistant to Mtx stained positively for KIT^S as measured by APAAP (see section 2.2.8). Repeated transfection of *dhfr*- CHO and SI/SI cells with pSHR1.15 failed to achieve stable expression of KIT^S. Transient transfection of COS cells with pSHR1.15 did result in some cells staining positively, by APAAP, for KIT^S, indicating that the construct was functional, but the efficiency was so poor that this method was not pursued.

3.5.2 Expression of KIT^S using pEE14 and CHO cells

Glutamine is a key metabolite required in a number of biosynthetic and catabolic pathways and must be provided or synthesized from glutamate and ammonia by glutamine

synthetase. Glutamine synthetase can be inhibited by L-methionine sulphoximine, Msx. In glutamate deficient media glutamine synthetase is an essential enzyme for cell survival and inhibition by Msx is lethal. Similar to the action of Mtx, treatment of cells transfected with a plasmid carrying the glutamine synthetase gene with Msx results in the selection of clones that have an increased copy number of the glutamine synthetase gene and surrounding sequences. Unlike the *dhfr* gene, the use of glutamine synthetase gene for amplification has the advantage that levels of expression achieved in the initial treatment are higher than those achieved with *dhfr*, thus requiring only one round of selection (Bebbington, 1991). Using a vector containing the glutamine synthetase gene, pEE14, Bebbington (1991) achieved production levels of monoclonal antibodies up to 200µg/ml. The pEE14 vector was derived from the pEE6 vector described by Stephens and Cocket (1989). pEE14 has also been successfully used to express human thyrotropin receptor and soluble tumour necrosis factor (Harfst and Johnstone, 1992; Mohler *et al.*, 1993). Since pEE14 was designed for immunoglobulin expression it seemed ideal for KIT^S expression since KIT^S contains five immunoglobulin like domains.

The KIT^S cDNA harboured in pSHR1.6 was subcloned into the *Sma* I site of pEE14 (refer to Figure 2.4 and section 2.15.4). The resultant clone was referred to as pSHR1.20. pSHR1.20 DNA, linearised with *Sal* I, was co-transfected into CHO cells by electroporation, with pSV2Neo at a ratio of 10:1. Cells were subjected to 400µg/ml of G418 until all mock-transfected cells, i.e. those cells not transfected with DNA, were dead. Expression of soluble c-Kit by viable transfected cells was determined by APAAP (see section 2.2.8). The majority of the cells were c-Kit positive. A clonal population was generated by plating the cells in methylcellulose at 500 cells/dish (section 2.4). Colonies were picked and assayed for KIT^S expression. Five clones were selected for further analysis. These clones were treated with increasing concentrations of methionine sulfoximine. Unlike the *dhfr* system, the cells were not exposed to gradually increasing concentrations of Msx but were incubated with either 30, 50, 100, 250, 500 or 1000µM of Msx from the start. Although this selection could have been used to select transfected cells, the cells were co-transfected with a neomycin resistance carrying vector, pSV2Neo, to allow selection of transfected cells with G418, hopefully improving the ability to obtain c-Kit expressing clones resistant to Msx. Initially all of the

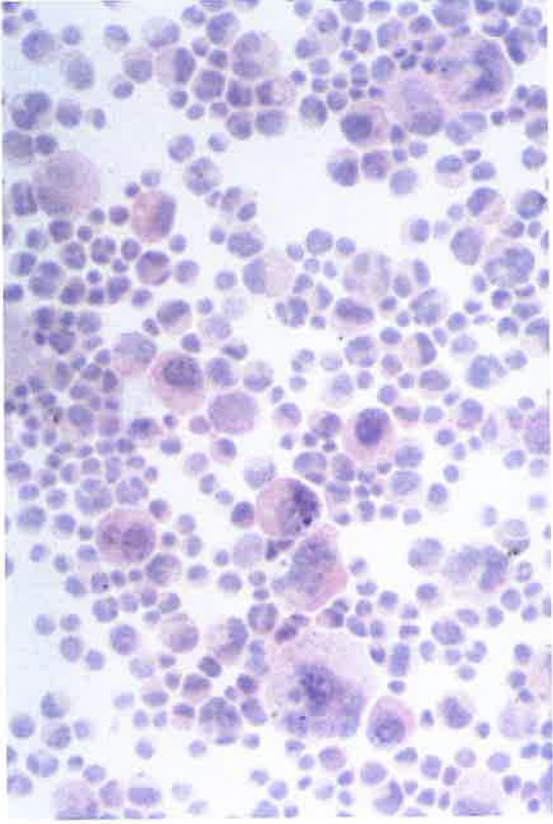
cells were expressing KIT^S protein. As the concentration of Msx was increased the percentage of positive cells decreased and the amount of KIT^S protein produced by the remaining positive cells was less than the starting population. At 500 μ M of Msx none of the transfected cells of a particular clone were expressing KIT^S despite the fact that these cells still contained the vector sequences since they were still resistant to the Msx. These cells proliferated more quickly than other cells subjected to Msx selection. Cells expressing KIT^S and resistant to 50 μ M of Msx were cloned out by limit dilution as described in section 3.4.2 (Figure 3.4). Over time the percentage of 1DC3 immunoreactive cells declined to zero (Figure 3.4 (C)). Many attempts were made to generate transfected cells that stably expressed KIT^S but all attempts were unsuccessful.

3.6 Expression of a dimeric KIT^S protein using pIG and COS cells

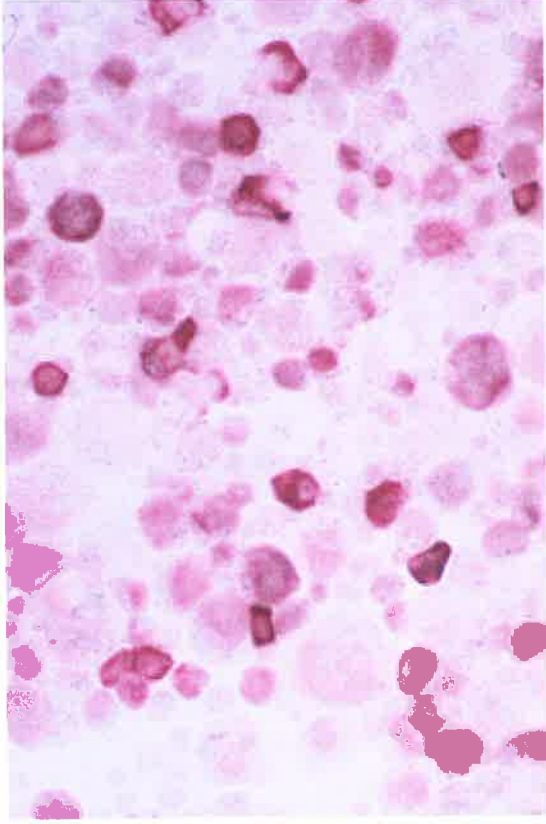
3.6.1 Cloning of KIT^S cDNA into the pIG vector

The pIG vector (Simmons, 1993; Fawcett *et al.*, 1995) (Figure 2.6) has been used by several groups to express soluble proteins fused to the Fc portion of immunoglobulin. Proteins expressed using this vector include CD31 (Fawcett *et al.*, 1995), a member of the immunoglobulin superfamily, like c-Kit, and CD44 (Fox *et al.*, 1994). Using a similarly structured vector Brown *et al.* (1998) expressed EpoR, TNFR, IL-4R and IL-6 as IgG1 fusion proteins and showed all of these soluble molecules had affinity constants similar to that of full-length receptor. Construction of this vector utilised sequences from CDM8 (Seed and Aruffo, 1987) as its backbone and included the CMV promoter sequence. pIG contains sequences encoding the hinge-constant 2-constant 3 domains of the Fc portion of the human IgG₁ molecule. It also has a splice acceptor site at the 5' end of the IgG sequence. Upon expression of this vector in the host cell, the mRNA encoding the target protein, in this case KIT^S, is spliced onto the mRNA of the human IgG. Translation of this spliced mRNA results in the expression of a fusion protein of KIT^S and the Fc domain of the human IgG molecule capable of dimerising through the disulphide bond in the hinge domain of the Fc arm of the IgG. Since ligand binding to native c-Kit results in receptor dimerisation, it was envisaged that a dimeric soluble receptor might have a greater affinity for soluble ligand than a soluble

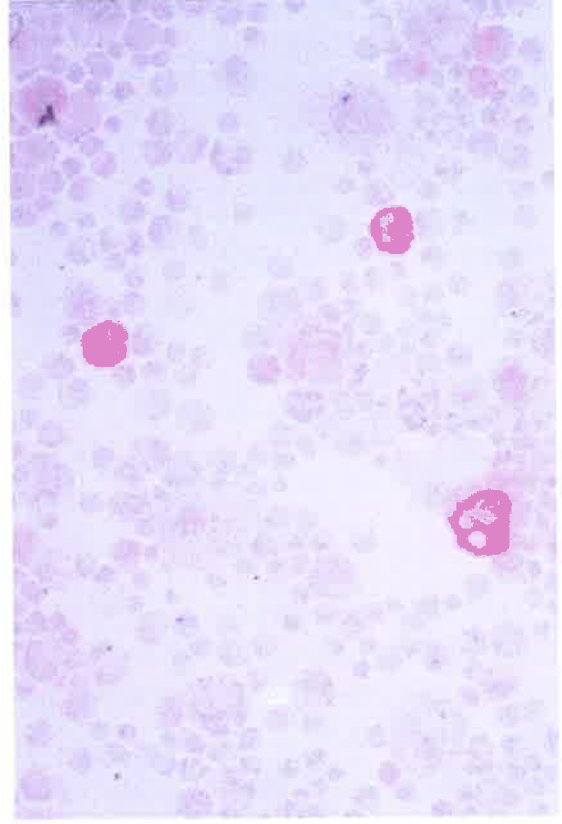
Figure 3.4: Immunohistochemical analysis of KIT^S expression by CHO cell transfectant TSHR5.2.A4C4 (pEE14 vector). Cells were cytocentrifuged onto glass slides, fixed and incubated with the anti-c-Kit mAb 1DC3. Detection of 1DC3 binding to the cells was performed using a bridging rabbit anti-mouse antibody, followed by alkaline phosphatase-mouse anti-alkaline phosphatase complex. Cells were then incubated with substrate, resulting in KIT^S expressing cells staining red (section 2.2.7). Cells were visualised using an Olympus microscope and photographed. **A:** TSHR5.2.A4C4 stained with isotype matched negative control mAb, 3D3; **B:** TSHR5.2.A4C4 stained with 1DC3 1 week after cloning; **C:** TSHR5.2.A4C4 stained with 1DC3 after 4 weeks of culture. All photographs were taken at 20x magnification.



A



B



C

monomeric counterpart. The IgG molecule also allows purification of the fusion protein via the affinity of the human Fc arm for Protein A coupled to Sepharose.

To clone KIT^S cDNA into pIG a new PCR product had to be generated (see schematic of cloning procedure in Figure 2.7). The primers used in the PCR reaction were 387 and 1244 (see Table 2.1). Primer 1244 contained the sequence encoding a splice donor site. Both primers contain *Bam*H I restriction sites to allow cloning into the pIG vector. This clone was termed pSHR1.45.

3.6.2 Expression of pSHR1.45 in COS cells

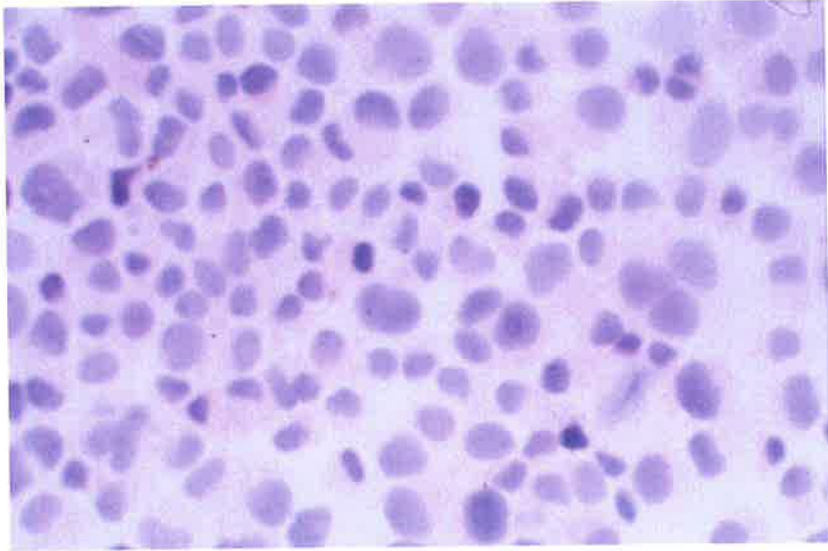
Transient transfections of COS cells were used to produce KIT^S from this vector expression system (Figure 3.5). Electroporated cells were incubated for 24 hours after transfection in medium containing 5% FBS, and then changed to medium supplemented with 1% FBS that had been depleted of Protein A reacting proteins, for a further 4 days. Supernatants were collected and stored at -20°C until sufficient supernatant had been collected.

3.7 Expression of KIT^S using the Baculovirus expression system in Sf21 cells

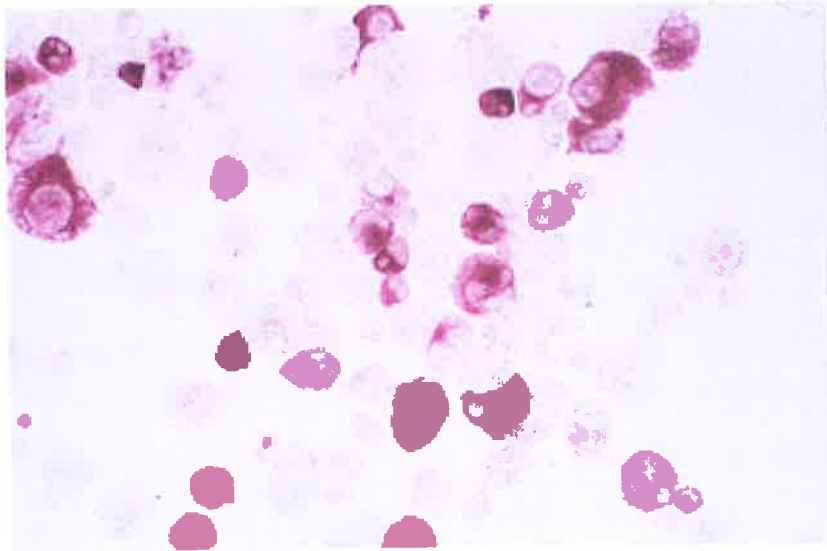
The major attraction of the Baculovirus expression system was the reported high protein expression of many different recombinant proteins. Reported protein production levels vary from 1mg/L to 500mg/L (King and Possee, 1992). In the very late stage of viral replication two genes are transcribed at very high rates. The product of one of these, the polyhedrin protein, a structural protein, can account for over 30% of the total cellular protein. When the virus is propagated in the budded form, the polyhedrin protein is not required so replacement of this gene, under the control of the very strong polyhedrin gene promoter, with the target gene allows large quantities of target protein production to be achieved. Other advantages of the Baculovirus expression system include expression in eukaryotic cells, ensuring faithful processing of foreign genes, the virus can accommodate large amounts of foreign DNA without affecting normal replication and the system is amenable to scale-up to large volume production in fermenter systems. Disadvantages include discontinuous production since the viral cycle is lytic, and variability of protein glycosylation in insect cells. Another advantage

Figure 3.5: Immunohistochemical analysis of KIT^S expression by COS cells transfected with pSHR1.45 (pIG vector) by electroporation. Cells were cytocentrifuged onto glass slides, fixed and incubated with the anti-c-Kit mAb 1DC3. Detection of 1DC3 binding to the cells was performed using a bridging rabbit anti-mouse antibody, followed by alkaline phosphatase-mouse anti-alkaline phosphatase complex. Cells were then incubated with substrate, resulting in KIT^S positive cells staining red (section 2.2.7). Cells were visualised using an Olympus microscope and photographed. **A:** pSHR1.45 transfected cells stained with isotype matched negative control mAb, 1B5; **B:** pSHR1.45 transfected cells stained with 1DC3 24 hours after transfection. Photographs were taken at 20x magnification.

A



B



is that the system is available commercially allowing easy establishment of the system in the laboratory. Many proteins have been produced by the Baculovirus system in either membrane targeted or secreted forms. These include CD4 receptor (Webb *et al.*, 1989), human immunoglobulin (Hasemann and Capra, 1990), transferrin receptor (Domingo and Trowbridge, 1988), insulin receptor (Herrera *et al.*, 1988) and the EGF receptor (Greenfield *et al.*, 1988) and importantly murine c-Kit (Liu, 1993).

As a consequence of its large size, 128kb, the AcMNPV baculovirus genome is difficult to manipulate directly, so recombinant vectors were constructed in two steps. KIT^S encoding cDNA was ligated into one of the shuttle vectors BacPak8 or BacPak9 (Figure 2.9) (Clontech Laboratories). These relatively small vectors, <10kb, contain the promoter and polyadenylation sequences of the polyhedrin gene (other transfer vectors contain different promoters), origin of replication and antibiotic resistance for propagation in bacteria but are unable to replicate in insect cells. The target gene is inserted between the polyhedrin promoter and polyadenylation sequence. The second step involves co-transfection of the modified transfer vector and AcMNPV DNA (BacPak6 (Figure 2.9)) that has been linearised with *Bsu36* I. Digestion of AcMNPV with *Bsu36* I results in the deletion of an essential gene required for viral replication (King and Possee, 1992 and references within). However, recombination between the AcMNPV DNA and the shuttle vector results in the circularised DNA containing all the necessary genes for viral propagation. The target gene is also transferred across to the viral DNA fragment during the recombination event.

3.7.1 Generation of a Baculovirus shuttle vector containing KIT^S-His cDNA

Two different constructs were expressed in Baculovirus. The first was KIT^S fused to a 6xHistidine tag at the 3' end of the KIT^S cDNA (KIT^S-His). The 6xHis tail was fused to KIT^S to allow purification utilising an immobilised Ni⁺⁺-NTA Sepharose matrix. The 6xHistidine structure has high affinity for the Ni⁺⁺ (Hochuli *et al.*, 1987; Janknecht *et al.*, 1991). Incubating the target protein with the Ni⁺⁺-charged resin results in the binding of the target protein to the resin. After washing the column with buffer the bound protein can then be eluted with a competitive molecule e.g. imidazole. The higher the affinity for the metal the higher the concentration of imidazole required to elute the protein.

KIT^S-His was generated in several steps (see Figure 2.8 for schematic of cloning). Firstly the cDNA encoding KIT^S was excised from pSHR1.6 using restriction endonucleases *Pst* I and *Not* I. The resultant 1.5kb fragment was ligated into the Baculovirus transfer vector BacPak8 similarly digested with *Pst* I and *Not* I. This clone was termed pSHR1.30. The 6xhistidine tag was then inserted into pSHR1.30. This was achieved by polymerase chain reaction (PCR) using c-Kit specific oligonucleotides 765 and 1070 (see Table 2.1). The primer at the 3' end of the sequence, 1070 encoded 18 bases corresponding to the c-Kit sequence, the 6xhistidine sequence, a stop codon and the *Not* I restriction endonuclease site for cloning. The 6xhistidine tag was incorporated into the C-terminus of KIT^S because of the requirement for the cleavable signal sequence at the 5' end of the sequence to allow export of KIT^S protein from the cell. Using pSHR1.6 as a template, PCR amplification using oligonucleotides 1070 and 765 generated a 400bp fragment (see Table 2.2). The resultant product was digested with restriction enzymes *Not* I and *Sph* I. The 255bp fragment was gel purified away from the 145bp fragment and ligated into pSHR1.30 that had also been digested with *Not* I and *Sph* I. This construct was termed pSHR1.30His.

3.7.2 Generation of a Baculovirus shuttle vector containing KIT^S-IGb cDNA

It has been documented that the Baculovirus insect cell host does not always efficiently glycosylate the target recombinant protein (Jarvis and Finn, 1995). To address the importance of glycosylation of c-Kit in the interaction of c-Kit with SLF, a comparison of the activity of dimeric KIT^S produced by the pIG expression system, (section 3.6), and that produced by Baculovirus was implemented. A Baculovirus shuttle vector encoding dimeric KIT^S was generated by subcloning the cDNA encoding the sequence which generates the dimeric KIT^S molecule from the pIG vector into the Baculovirus vector. This clone was created by excising the KIT^S-IG encoding cDNA domain from pSHR1.45 using restriction endonucleases *EcoR* V and *Not* I and directionally ligating it into the Baculovirus transfer vector BacPak9 which had been digested with *Not* I and *Sma* I. This clone was referred to as pSHR1.36.

3.7.3 Generation of Baculovirus expressing KIT^S-His or KIT^S-IGb

Production of a recombinant baculovirus expressing either KIT^S-His or KIT^S-IGb (see Figure 3.6 for schematic) was achieved by co-transfection of either pSHR1.30His or pSHR1.36 and commercially acquired, *Bsu*36 I linearised, BacPak6 DNA into the insect cell line Sf21. Several infections were required to obtain clones of recombinant virus and increase the viral titre to working concentration. This was achieved by using supernatant from the transfected cells to infect cells in a plaque assay (see section 2.16.6). Individual plaques were picked and eluted into medium overnight. This solution was then used to infect individual wells of a 6 well plate coated with Sf21 cells. After 4 days this medium was harvested and stored. The harvested medium was used to infect a 150cm² flask of 60% confluent Sf21 cells. Small aliquots of culture supernatant were also analysed for KIT^S protein expression by SDS-PAGE and Western Blot (Figure 3.7). The lanes of the gel loaded with supernatant from infected cells were positive for KIT^S protein. The KIT^S protein expressed by the pSHR1.30His and pSHR1.36 Baculovirus constructs were termed KIT^S-His and KIT^S-IGb respectively. KIT^S-IGb had a molecular weight of approximately 75kDa on a reduced gel and 145kDa on a non-reduced gel (Figure 3.7), although larger aggregates and monomer were also detected in the latter case. KIT^S-His had a molecular weight of approximately 63kDa on either reduced or non-reduced gels (Figure 3.7). The Ig fusion was expected to contribute 25kDa to the size of the KIT^S-IGb protein, accounting for the size difference between KIT^S-His and KIT^S-IGb on the reduced gel. The calculated molecular weight of the native KIT^S protein is 58kDa (determined with the help of Dr Chris Bagley, (Human Immunology, Hanson Centre for Cancer Research)). If the KIT^S protein was fully glycosylated it was predicted to be approximately 98kDa in size. It therefore appeared that some glycosylation of the protein was occurring but not to the extent that the manufacturers suggest. The difference in molecular weight of KIT^S-IGb between the reduced and non-reduced gels was assumed to be due to the reduction of the disulphide bond between the two Ig domains of the molecule.

Having established that the transfection and production of recombinant proteins had been successful, further cells were infected with the viral supernatant. The supernatant harvested from these cells was termed the primary viral stock. The titre of this stock was determined by plaque assay and it was used to infect a large batch of cells to generate a secondary stock, the

Figure 3.6: Diagrammatic representation of cloning and expression of recombinant KIT^S Baculovirus constructs. KIT^S constructs were cloned into shuttle vectors BacPak8 or BacPak9. These were co-transfected into SF21 cell with *Bsu36* I linearised BacPak6 vector by lipofection. Recombinant virus was harvested from culture supernatants and incubated with cells immobilised within agarose to generate plaques. Virus particles isolated from plaques were screened for KIT^S expression by re-infection of Sf21 cells and testing culture supernatants for c-Kit immunoreactivity by Western blot. Positive clones were expanded for recombinant virus and protein production.

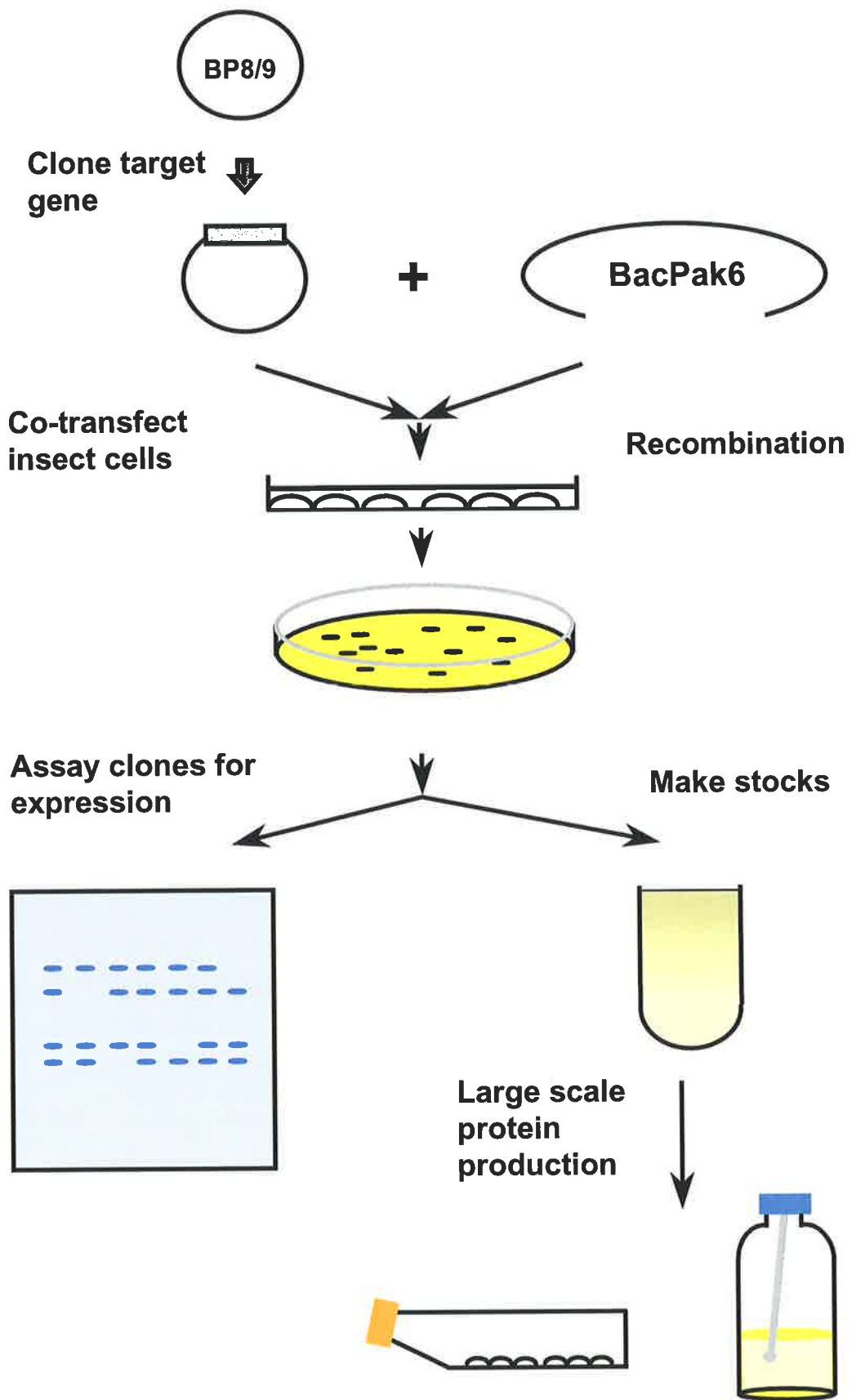
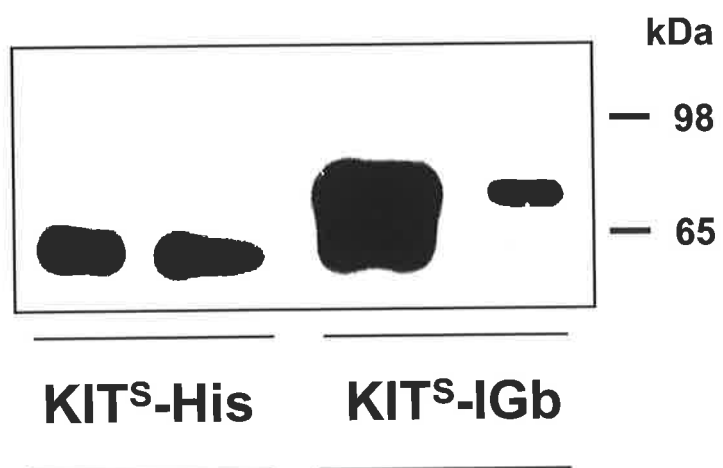


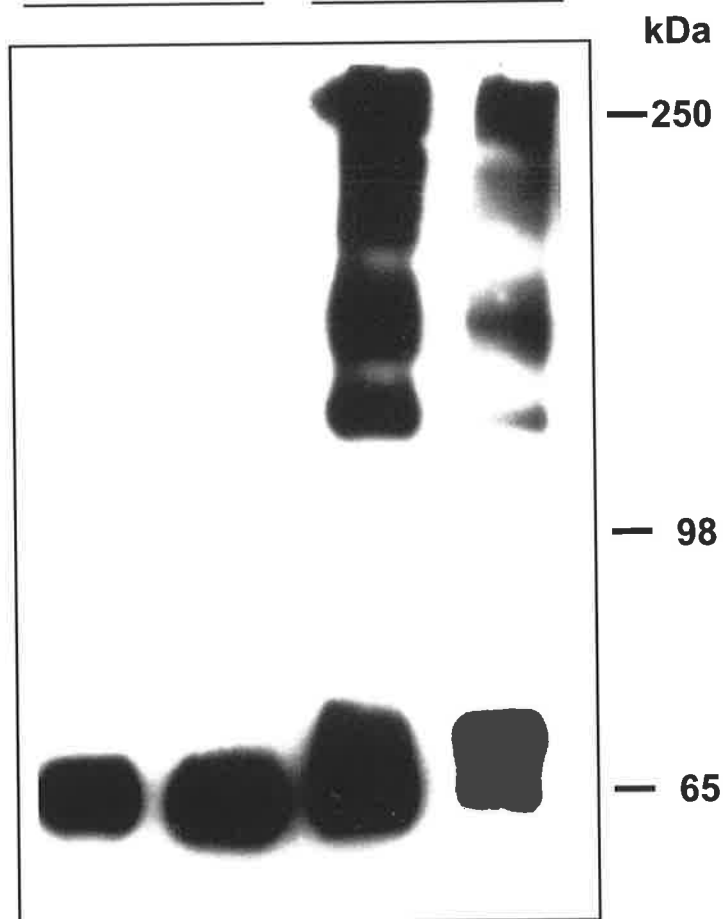
Figure 3.7: Western blot of Baculovirus expressed KIT^S-His and KIT^S-IGb protein.

Samples of infected Sf21 cell culture supernatants were separated on 8% polyacrylamide gels, transferred to nitrocellulose and immunoblotted with anti-c-Kit mAb 1C1.HF. Presence of 1C1.HF binding was detected with biotinylated anti-mouse F(ab)₂ fragments followed by streptavidin coupled to horseradish peroxidase. Immunoreactive proteins were detected with ECL and exposed to film (section 2.7). **A:** Reduced samples; **B:** Non-reduced samples. Culture supernatants from two clones of both KIT^S-His and KIT^S-IGb were analysed.

A



B



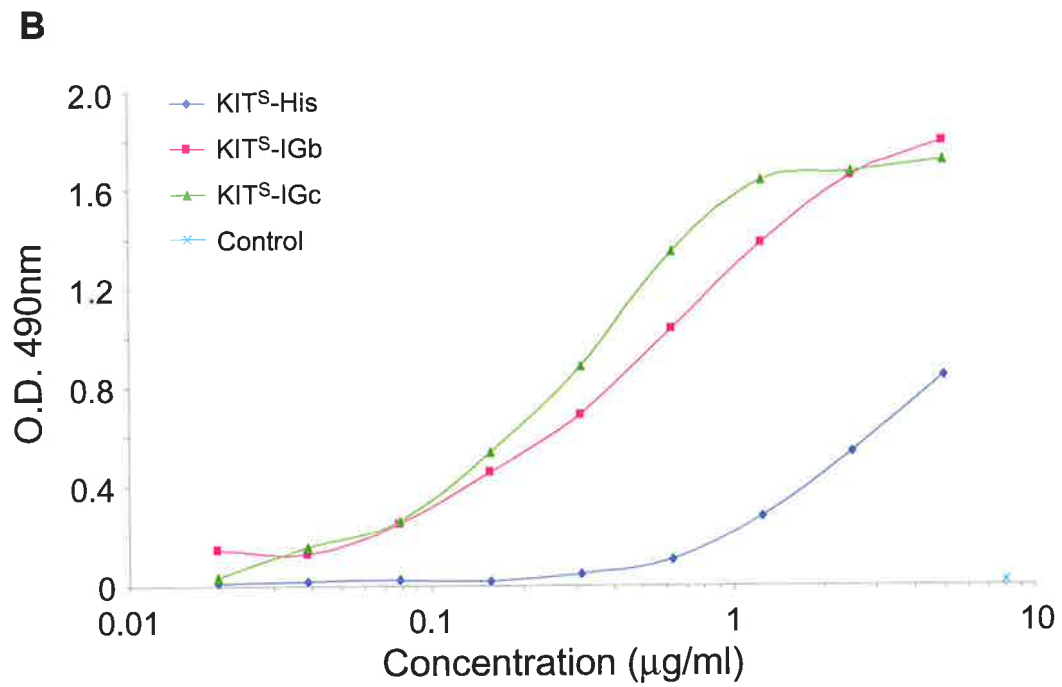
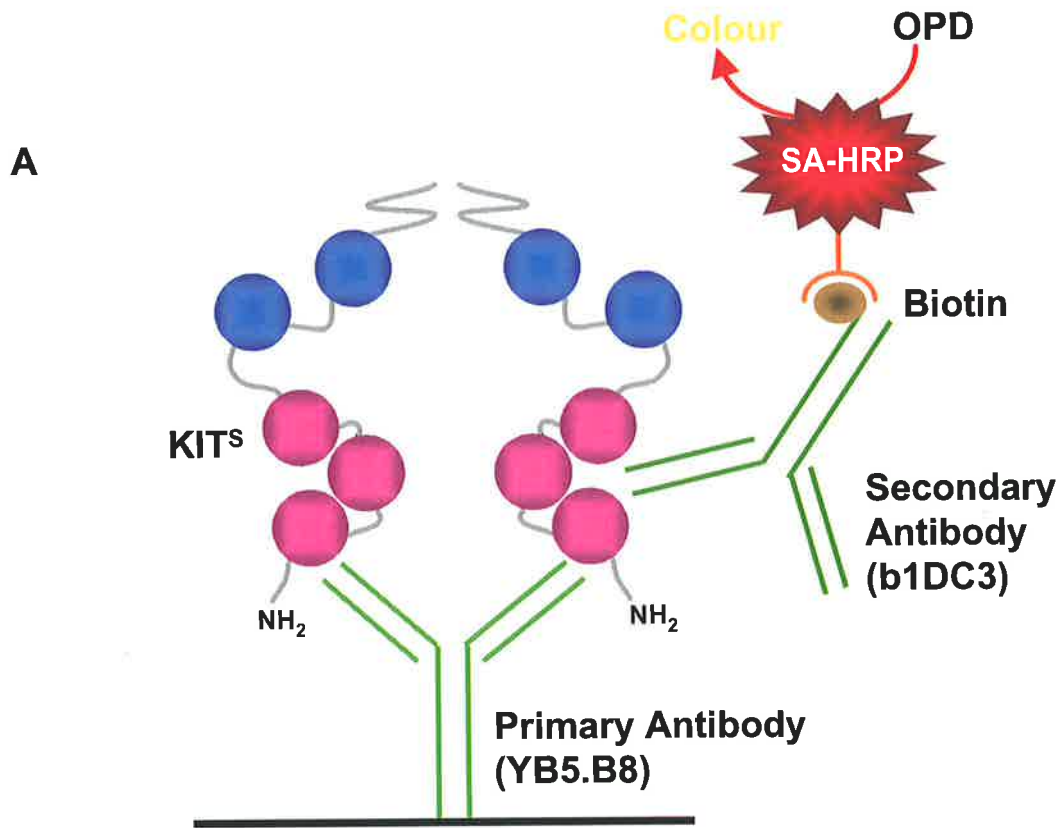
titre of which was also determined by plaque assay. The bulk of the secondary stock was stored at -70°C and the rest used to infect cells to generate a large batch of working stock. The titre of the working stock was also determined by plaque assay and this was stored at -20°C , or when thawed, at 4°C . The titre was approximately 10^8 - 10^{10} in all cases. Since the escape of the Baculovirus from the host cell is a lytic one it was not possible to generate stably infected cell lines. Therefore large stocks of viral supernatant had to be generated to allow continual infection of fresh Sf21 cell cultures.

Of all the systems studied only the eukaryotic pIG expression system and the Baculovirus expression system appeared to produce KIT^{S} protein at levels that may be of use. Having decided upon these systems, further refinement of the respective expression procedures was carried out. This included determination of the best method for purifying the KIT^{S} protein, evaluation of the purity and determination of its ability to recognise and bind to SLF. To aid in this process a sandwich style enzyme linked immunosorbent assay (ELISA) was established. The ELISA permitted the monitoring of the KIT^{S} protein during purification steps, confirmed purified KIT^{S} protein had retained immunoactivity and allowed the determination of KIT^{S} protein concentration.

3.8 Development of a Sandwich Enzyme Linked Immunosorbent Assay (ELISA)

The development of an ELISA able to detect the presence of KIT^{S} protein within a solution was essential for the further characterisation of the KIT^{S} protein. A sandwich style ELISA was developed (Figure 3.8A). For a sandwich type ELISA it is important that the antibodies used do not recognise the same or closely associated epitopes. The antibodies used in this assay, YB5.B8, the first anti-c-Kit mAb (Gadd and Ashman, 1985) and 1DC3, were known not to cross compete (G.W. Aylett and L.K. Ashman, unpublished). YB5.B8 binds to Ig domain 2 and 1DC3 binds to Ig domain 1 of c-Kit (Y. Yarden, personal communication). Both are of the IgG_1 isotype. YB5.B8 was used to coat the wells of 96 well plates at $5\mu\text{g/ml}$. After blocking with BSA, coated wells were incubated with the solution containing the KIT^{S} protein, washed and incubated with the second antibody, 1DC3. This antibody had previously

Figure 3.8: A: Diagrammatic representation of the Sandwich ELISA technique. Wells of a 96 well plate were coated with YB5.B8, blocked and then incubated with the solution containing KIT^S. KIT^S binding was detected with biotinylated 1DC3 and the presence of this secondary mAb detected using streptavidin coupled to horseradish peroxidase. Altered substrate colour was quantitated using Bio-Rad microplate reader. **B:** Representative standard curves generated by the titration of KIT^S-His, KIT^S-IGb and KIT^S-IGc using the ELISA protocol.



been biotinylated and its presence within a well was detected by incubation with streptavidin conjugated to horseradish peroxidase, followed by the substrate OPD. While the ELISA was being established both Turner *et al.* (1995) and Wypich *et al.* (1995) published sensitive ELISA protocols for the detection of KIT^S protein. Their systems were also sandwich style ELISAs but they used a rabbit polyclonal antibody to coat the wells and the high affinity anti-c-Kit mAb SR-1 conjugated to horseradish peroxidase as the secondary antibody. Adoption of the blocking and diluent solutions (1%BSA/ 5% sucrose in TEN buffer (50mM Tris, 10mM EDTA, 150mM NaCl, pH 7.5) and 0.01% Tween 20 in TEN buffer, respectively), from the procedures of Wypich *et al.* (1995) improved the sensitivity of the ELISA and removed problems with protein binding in control wells. Figure 3.8B depicts typical standard curves generated in the sandwich ELISA by monomeric and dimeric KIT^S. The ELISA was not as sensitive as that published (Turner *et al.*, 1995; Wypich *et al.*, 1995). The limit of detection for monomeric KIT^S was 310ng/ml and for dimeric KIT^S 40ng/ml (see Figure 4.1 and Table 4.1 for more detailed analysis). By comparison the ELISA used by Turner *et al.* (1995) had a limit of detection of 10pg/ml and the limit of the linear range of the ELISA used by Wypich *et al.* (1995) was 0.5ng/ml. The decreased sensitivity of the ELISA used in this study was attributed to the lack of a polyclonal antibody. All samples of KIT^S used in the assays described above and below had concentrations determined by ELISA using the same standards. For example the concentrations of KIT^S-IGc batches were determined using a KIT^S-IGc standard. The concentrations of the standards had been determined by spectrophotometry, the extinction coefficient calculated based on the amino acid composition. The purity of all samples was determined by SDS-PAGE and total protein stain. Adjustments were made to the calculated concentration based on the purity. At worst the samples were 50% pure, resulting in only a two fold difference in the values detailed in the text. The initial establishment of the ELISA was carried out using KIT^S protein obtained from the concentrated supernatants of stably transfected CHO cells (TSHR4.2.C3, see section 3.4.2) and later using the KIT^S protein produced by either the pIG or Baculovirus expression systems.

3.9 Refinement of KIT^S production

3.9.1 pIG Expression System

Initially pSHR1.45 DNA was introduced into COS cells by electroporation. The efficiency of transfection by electroporation was compared to that using a new transfection reagent, FuGENE 6 (Roche Biochemicals). A higher percentage of cells immunoreactive with the anti-c-Kit mAb 1DC3 were obtained using the FuGENE 6 procedure. This correlated with an increase in the amount of KIT^S detected in the supernatant of these cells as measured by ELISA. Figure 3.9 depicts the amount of KIT^S present in the transfected cell supernatant over a 5 day period. Based on this, the supernatant was collected after 120 hours. Cells were cultured in media supplemented with 1% FBS that had been depleted of Protein A reacting contaminants. The absence of high concentrations of serum did decrease the amount of KIT^S detected in the culture supernatant slightly but this was overshadowed by the benefits of low serum media during the purification procedure (see section 3.10). Supernatants containing KIT^S protein were collected and stored at -20°C until sufficient supernatant had been collected to warrant purification of the KIT^S (see section 2.17.5 and 3.10). Refer to section 3.10 for a further discussion of the production and purification of KIT^S using this expression system.

3.9.2 Baculovirus expression system

Improvement of the amount of KIT^S protein produced by Baculovirus infected Sf21 cells was achieved by the manipulation of the infection process and the way the cells were cultured. The titre of the recombinant virus stocks had been determined by plaque assay (see section 2.16.6 and 3.7.3). This represents the number of infectious viral particles per millilitre of culture supernatant. The methods manual provided with the Baculovirus kit recommended infecting 1 cell with 1 viral particle (Clontech Laboratories). However, it was conceivable and had been reported by others, that infection of the Sf21 cells with more than one viral particle would increase the amount of KIT^S protein production and reduce the time required to produce this amount of protein. Infecting adherent monolayers of Sf21 cells with varying amounts of viral particles per cell resulted in quantitative differences in the amount of KIT^S protein produced over a 120-hour period. Figure 3.10 shows the amount of KIT^S produced by

Figure 3.9: Determination of the optimal culture time of COS cells transfected with pSHR1.45 DNA. Supernatant from cultured COS cells, transfected using the FuGene6 method (section 2.6.2.2), was collected every 24 hours for 5 days. KIT^S production at each time point was determined by ELISA.

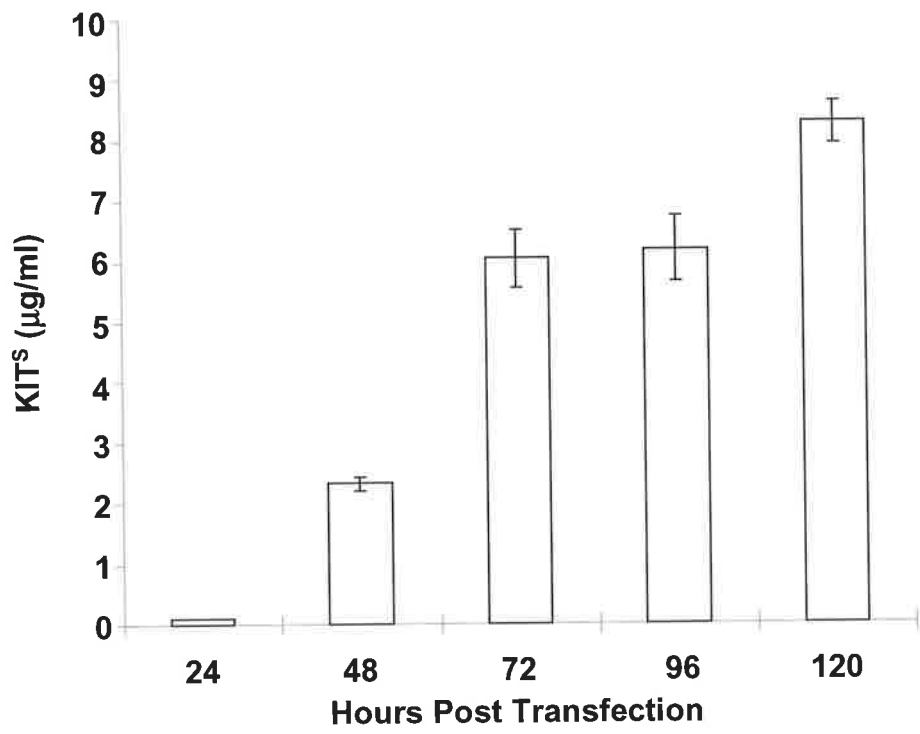
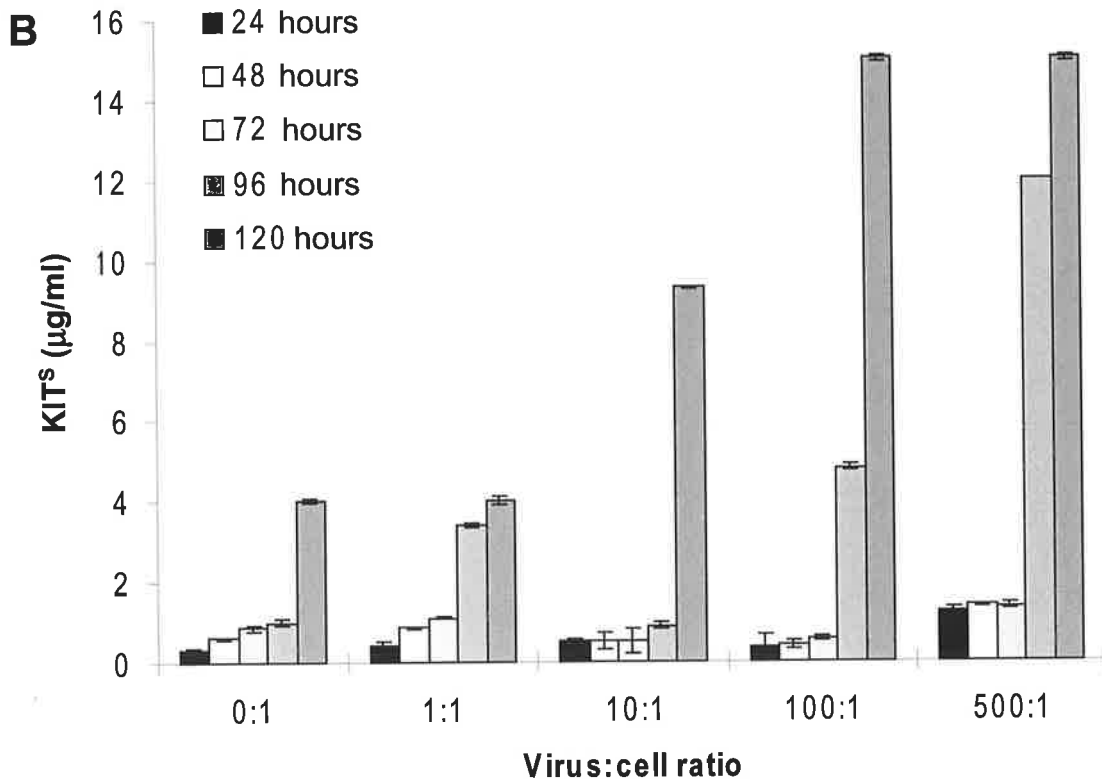
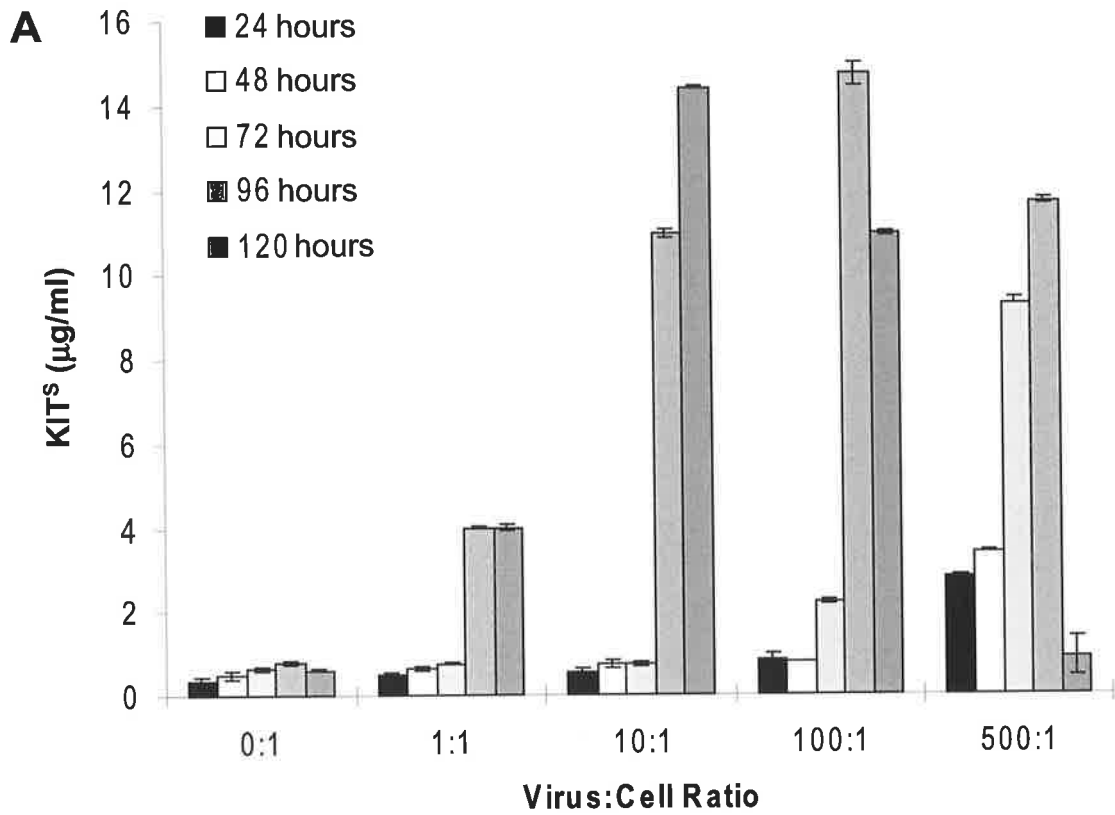


Figure 3.10: Determination of the optimal multiplicity of infection of Sf21 cells with KIT^S expressing Baculovirus constructs. Supernatant from cultured Sf21 cells infected at various virus to cell ratios was collected every 24 hours for 5 days. KIT^S production at each time point was determined by ELISA (section 2.2.8). **A:** Cells infected with KIT^S-His construct; **B:** Cells infected with KIT^S-IGb construct. Mean \pm SEM of three samples.



Sf21 cells infected with virus encoding either KIT^S-His (A) or KIT^S-IGb (B) as determined using the ELISA technique. As the number of infectious particles increased, so did the amount of protein produced. For the KIT^S-His producing virus, maximal production occurred between 72 and 96 hours at either a virus to cell ratio of 10:1 or 100:1, i.e. multiplicity of infection (MOI) of 10 or 100. After 96 hours at a ratio of either 100:1 or 500:1 the amount of protein produced started to decrease. Since similar levels of protein were produced using either a MOI of 10 or 100, a MOI of 10 was chosen as this would result in good KIT^S protein yields while reducing the rate of viral stock consumption. The production of KIT^S-IGb protein did not plateau until 120 hours with a virus to cell ratio of 500 (Figure 3.10 (B)). However, reasonable KIT^S levels were obtained after 120 hours of infection using a MOI of 10.

Sf21 cells can be cultured in either in culture flasks as an adherent monolayer or in bottles as a non-adherent culture. Both systems were used to culture Sf21 cells when infected by recombinant Baculovirus and little difference was seen in the amount of KIT^S produced (data not shown). Thereafter culture flasks were used for the KIT^S production.

3.10 Purification of KIT^S from culture supernatant

Serum free culture supernatant from transfected COS cells expressing KIT^S-IGc or Sf21 cells infected with KIT^S-His or KIT^S-IGb encoding virus was collected. After the removal of whole cells and cellular debris by centrifugation and filtration, the supernatant was concentrated 20 fold by membrane filtration using a 30kDa exclusion membrane. Different methods of purification were utilised for the three different KIT^S protein constructs.

To purify KIT^S-His, the concentrated supernatant was incubated overnight with 2-3ml of Ni⁺⁺-NTA-resin (Qiagen), in a 50ml tube on a rotator at room temperature. The resin was then loaded onto a column, washed with buffer without imidazole and the KIT^S eluted using 200mM of imidazole. The manufacturer's instructions suggested washing the resin with buffer containing low concentrations of imidazole to reduce non specific binding. However, some of the bound KIT^S was eluted from the column at low concentrations of imidazole e.g. 20mM. Complete elution was only achieved using a buffer containing 200mM imidazole. This was determined by assaying samples taken from fractions eluted with imidazole

concentrations ranging from 10mM to 1000mM by SDS-PAGE and Western blot using an anti-c-Kit mAb, data not shown. Using serum free culture conditions minimised the extent of contaminating proteins.

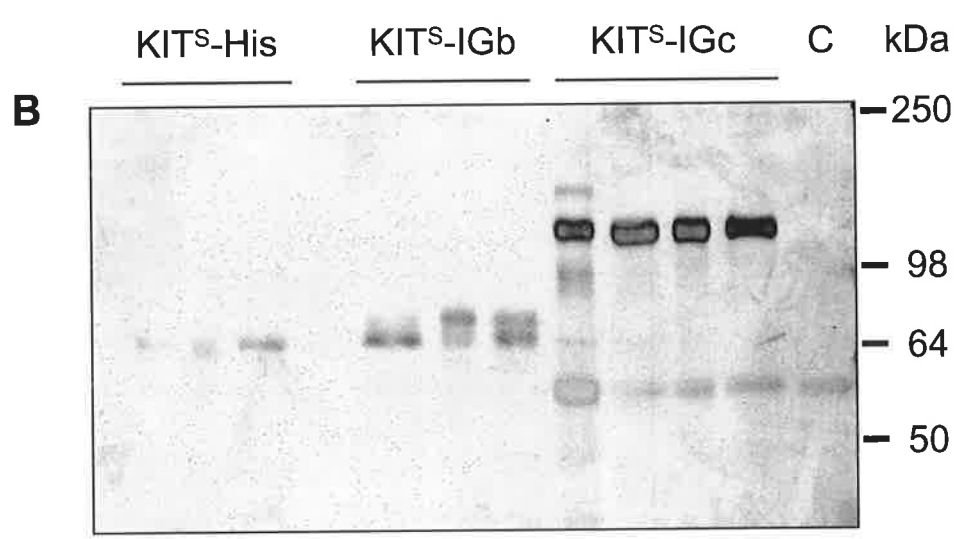
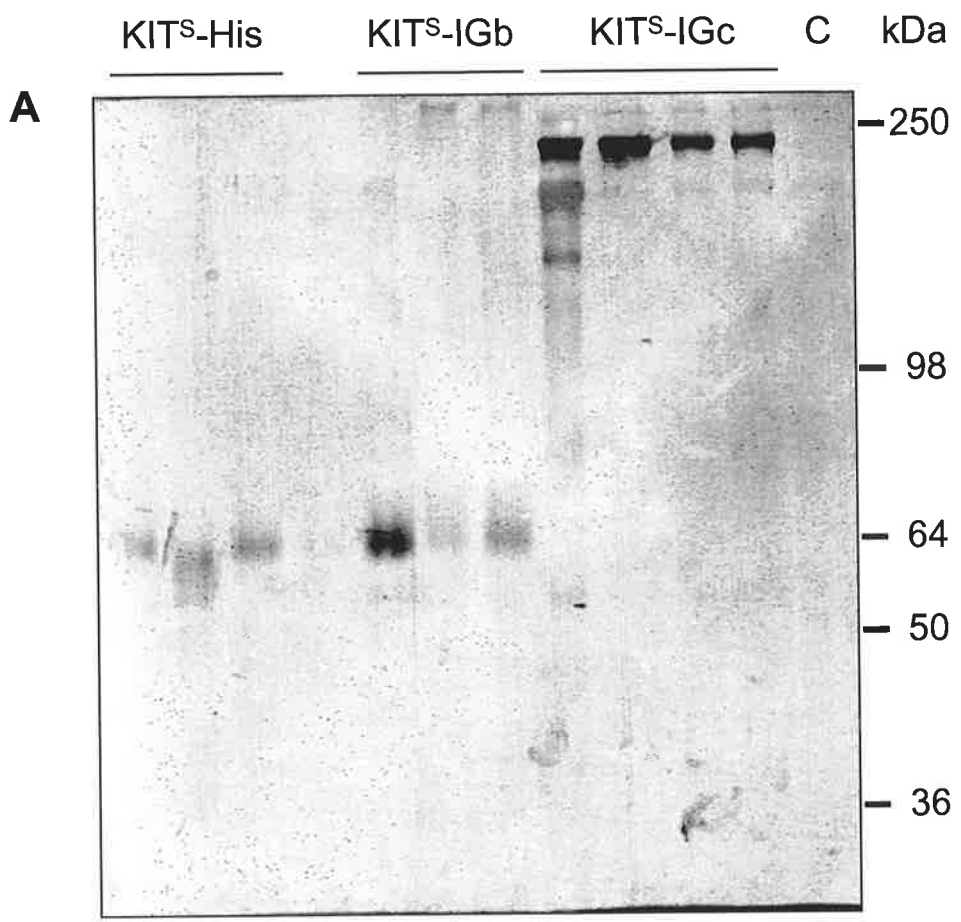
Purification of KIT^S-IGc from transfected COS cell supernatant was achieved with Protein A coupled to Sepharose. The binding of KIT^S-IGc to Protein A was assumed to be via the Ig tag of the fusion protein, based on the principles of IgG purification using Protein A or Protein G Sepharose (Forsgren and Sjqvist, 1966; Ey *et al.*, 1978). 50mls of concentrated supernatant from pSHR1.45 transfected COS cells was incubated with 2ml of packed Protein A Sepharose, rotating overnight at 4°C. The Sepharose was then loaded onto a column, at 4°C, washed with PBS, pH8.0, until the O.D._{280nm} was at background levels. The KIT^S was eluted using 0.1M glycine, pH 2.7 and immediately neutralised with 1M Tris-HCl, pH 8.6.

Unlike KIT^S-IGc, attempts to purify KIT^S-IGb protein using Protein A-Sepharose were unsuccessful. KIT^S-IGb was therefore purified as above except anti-c-Kit antibody, 1DC3, coupled Sepharose was used instead of Protein A-Sepharose. The KIT^S was eluted using 0.1M glycine, pH 2.7, and neutralised as above. In all cases, eluted KIT^S protein was dialysed against PBS, pH 7.4, concentrated by membrane filtration, a sample removed for assessment of purity and the remainder was stored at -20°C in the presence of 0.1% BSA.

Purity of the KIT^S protein samples was verified by SDS-PAGE and silver stain or SYPRO orange stain. Both non-reduced and reduced gels were run. The calculated molecular weight of monomeric KIT^S-IGc fusion protein was 120kDa, 95kDa of KIT^S and 25kDa of the Ig tag. The non-reduced blot (Figure 3.11(A)) shows the effect of the Ig tag of KIT^S-IGc resulting in a protein band at twice the monomeric molecular weight, approximately 250kDa, indicating dimerisation via the Ig constant region. Dimer formation was confirmed by comparison to the protein molecular weights detected on the reduced blot (Figure 3.11(B)). While dimeric KIT^S-IGb protein had been detected by Western blot (see Figure 3.7) it was not detectable by the less sensitive total protein stain and the monomeric KIT^S-IGb protein appeared as a doublet. Contamination of the KIT^S protein produced by the insect cell system with other proteins was not detected. Purification of KIT^S-IGc from culture supernatant using Protein A Sepharose resulted in some contamination with a protein of approximately 60kDa, (clearly seen in Figure 3.11 (B)) which may be BSA from the residual FBS in the supernatant.

Figure 3.11: Total protein stain of column purified KIT^S-His, KIT^S-IGb and KIT^S-IGc.

Approximately 750ng of protein was separated on 8% SDS polyacrylamide gels and total protein detected using Silver stain technique (section 2.7). Lane labelled C contains column purified supernatant from untransfected COS cells. Three different batches of purified KIT^S-His and KIT^S-IGb and four batches of purified KIT^S-IGc were loaded to show similarity of purity of each of the batches. **A:** Non-reduced samples, **B:** Reduced samples.



To show the proteins detected by total protein stain were indeed KIT^S, identical SDS polyacrylamide gels were run with approximately 200ng of protein, transferred to nylon membrane and probed with the anti-c-Kit mAb 1C1.HF (Figure 3.12). Western blotting of the non-reduced gel (Figure 3.12 (A)), revealed some monomeric KIT^S-IGc (approximately 120kDa) in one preparation but the majority of the protein was dimeric. This blot also shows a band of approximately 140kDa in the lanes containing KIT^S-IGb protein, indicating this protein may be able to dimerise but the majority of the KIT^S-IGb protein appears as a monomer. The lack of this band in the total protein stain suggests that although this protein is immunoreactive there is not much present in the preparation. There is also protein with a molecular weight greater than 250kDa, which may be aggregates of KIT^S-IGb since this protein is immunoreactive with the anti-c-Kit mAb. In both the total protein stain and the Western blot two bands are seen in the reduced lanes containing KIT^S-IGb. The lower of these bands has the same molecular weight as that of KIT^S-His suggesting almost half of the KIT^S-IGb protein does not contain the Ig tag. Probing these proteins with anti-human Fc antibody (Figure 3.12 (C)) failed to detect any protein in the KIT^S-His or KIT^S-IGb lanes but did detect the KIT^S-IGc protein. This suggests that the splicing of the Ig tag mRNA onto the KIT^S mRNA was inefficient in insect cells and accounts for why the KIT^S-IGb protein could not be purified using Protein A coupled to Sepharose (see above). By both total protein stain and Western blot the KIT^S-His protein appeared as expected. The majority of the functional studies (see chapter 4) were conducted using KIT^S-IGc protein purified from the supernatant of transfected COS cells containing 1% Protein A-depleted FBS. Figure 3.13 shows a total protein stain of two batches of KIT^S-IGc used. Some contaminating protein, probably BSA, is seen in both preparations (especially in preparation number 1, Figure 3.13, lanes 1 and 4) corresponding to the only protein seen in control lanes (lanes 3 and 6). As in Figure 3.12 a small amount of monomeric protein can be seen in non-reduced lanes. The altered size of the contaminating protein in non-reduced lanes is assumed to be due to oligomerisation of the protein, as is known to occur with BSA.

Table 3.1 summarises the purification of some of the batches of KIT^S from either the Baculovirus or pIG expression systems. Data from this table suggest that the amount of KIT^S obtained from the large scale production methods was 5-10 fold lower than in the optimisation

Figure 3.12: Western blot of purified KIT^S-His, KIT^S-IGb and KIT^S-IGc. Approximately 200ng of protein were separated on 8% polyacrylamide gels, transferred to PVDF and immunoblotted with anti-c-Kit mAb 1C1.HF. Presence of 1C1.HF binding was detected with biotinylated anti-mouse F(ab)₂ fragments followed by streptavidin coupled to alkaline phosphatase. Immunoreactive proteins were detected with ECF substrate and scanned using a Fluorimager 595 (Molecular Dynamics) (section 2.7). Lane labelled C contains column purified supernatant from untransfected COS cells. Three different batches of purified KIT^S-His, KIT^S-IGb and KIT^S-IGc were loaded to show uniformity of purity of within each of the batches. **A:** Non-reduced samples; **B:** Reduced samples. **C:** Partially reduced samples probed with anti-human Ig mAb instead of anti-c-Kit mAb.

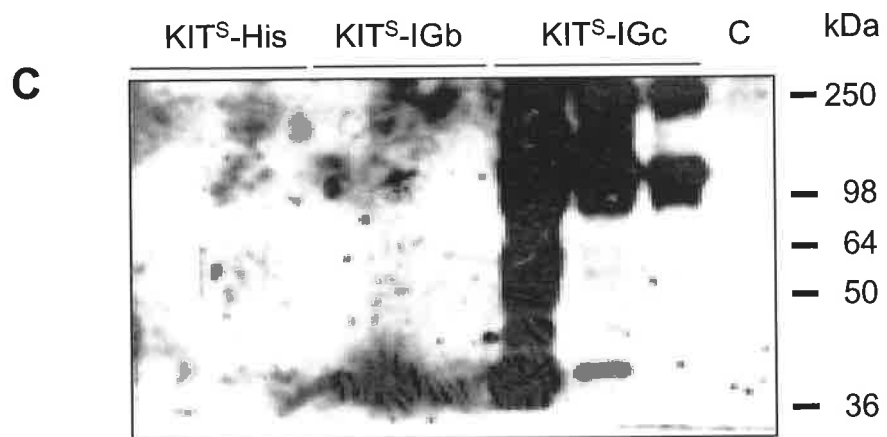
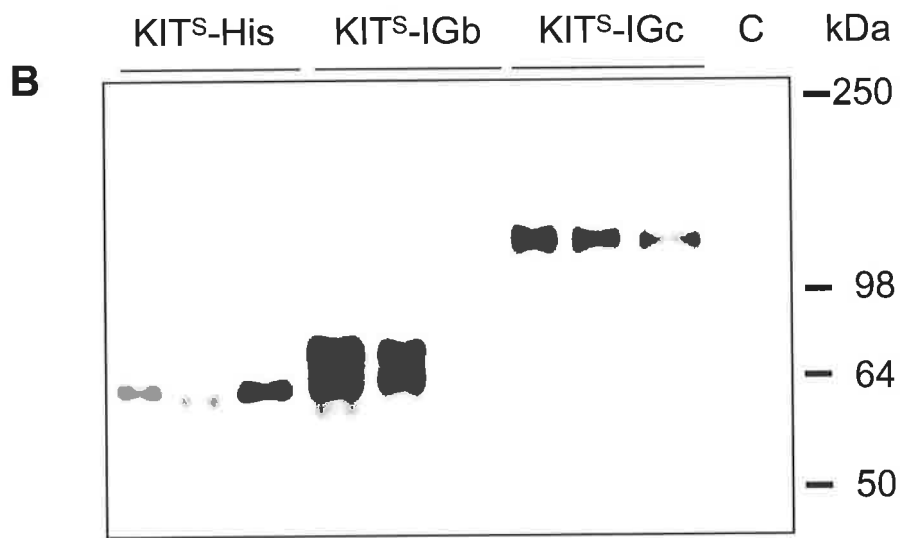
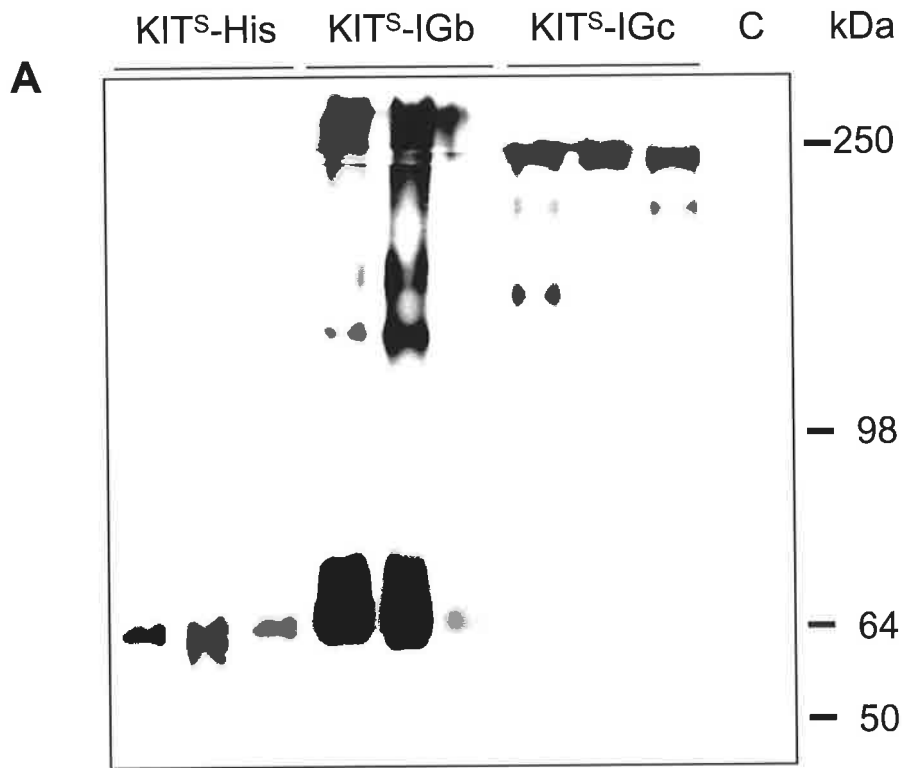


Figure 3.13: Total protein stain of purified KIT^S-IGc. 2µg of purified KIT^S-IGc (two different batches) used in the majority of functional studies were separated on 8% SDS polyacrylamide gels under reduced and non-reduced conditions as indicated (section 2.7). Samples of each KIT^S-IGc batch have been loaded into lanes 1 and 4, 2 and 5 respectively and control purified supernatant from untransfected cells loaded into lanes 3 and 6. The polyacrylamide gels were stained with SYPRO Orange in 7.5% acetic acid and scanned using Fluorimager 595 (Molecular Dynamics) using a 610nm filter.

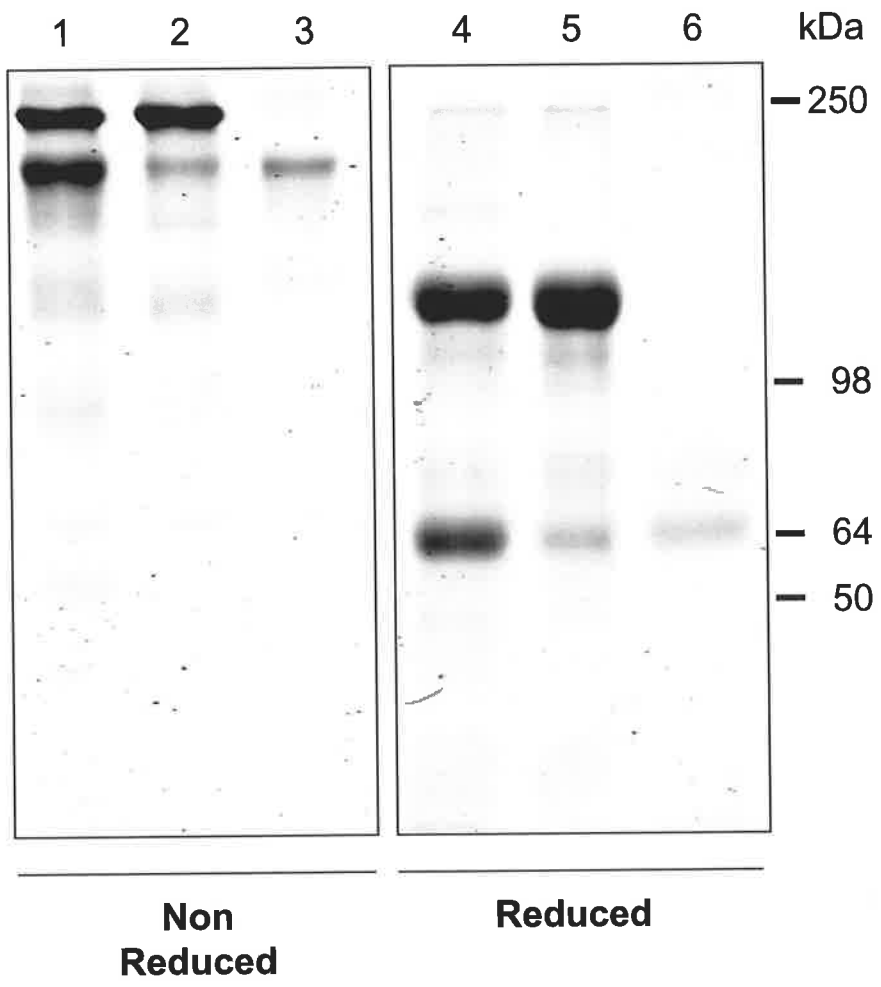


Table 3.1: Details of the purification of different KIT^S batches. Total protein was determined by spectrophotometry and KIT^S concentrations determined by ELISA (section 2.2.8) using KIT^S samples of known concentration (determined by Bradford assay and purity checked by SDS-PAGE and total protein stain) (section 2.19.6) as standards.

	Starting Volume (ml)	Total Protein (mg)	Total KIT ^S (μg)	KIT ^S Yield (μg)	Percent Recovery	Fold Purification
KIT ^S -His	1000	210	1300	439	34	129
KIT ^S -IGb #1	850	150	300	328	109	450
KIT ^S -IGb #2	500	126	900	600	66	126
KIT ^S -IGb #3	500	95	760	760	100	114
KIT ^S -IGc #1	970	16	368	236	64	26
KIT ^S -IGc #2	600	462	840	600	86	495
KIT ^S -IGc #3	550	200	500	380	93	372

experiments (see above). This may be a scale up phenomenon, i.e. scaling up to larger production volumes results in less than optimal cellular culturing conditions. The percent recovery was quite variable. While the anti-c-Kit affinity column appeared to isolate all of the KIT^S, in 2 of 3 cases, the Ni⁺⁺-NTA resin and Protein A Sepharose purification systems resulted in KIT^S loss. This loss was possibly due to a lack of affinity of some of the KIT^S molecules, due to errors in folding, for the respective column matrices. In addition, some loss may have occurred during the concentration step, with some KIT^S protein aggregating or precipitating.

3.11 Discussion

The establishment of an efficient expression system producing large quantities of soluble c-Kit protein was far more difficult than envisaged. This chapter discusses many different expression systems evaluated including systems that utilise bacterial, mammalian and insect cells as factories. Only two systems, the pIG expression vector transfected into COS cells and Baculovirus, infecting insect Sf21 cells, were able to produce enough KIT^S protein to allow functional studies to be carried out.

Both the pRSV009/A⁺ and pEE14 expression vectors were considered because they had been shown to allow the production of large quantities of protein due to their ability to increase in copy number in response to cytotoxic drugs (Bebbington, 1991; Harfst and Johnstone, 1992; Mohler *et al.*, 1993; Caruana *et al.*, 1998) and pEE14 was reported to allow the production of up to 200µg/ml of immunoglobulin (Bebbington, 1991). These vectors also had the advantage that cell lines stably expressing the target gene could be generated such that continuous culture of these cells would allow large quantities of conditioned supernatant to be collected with little manual labour. Unlike these systems, the pIG expression system could not produce stably transfected cell lines requiring frequent, labour intensive, transient transfections of COS cells to generate conditioned medium containing KIT^S protein. However, the former expression systems, while easily generating stable transfectant clones, as measured by their resistance to the cytotoxic drugs Mtx and Msx respectively, did not provide homogeneous cell lines expressing KIT^S and eventually these cell lines lost KIT^S expression completely.

It appeared that high expression of KIT^S by transfected cells was detrimental to the cell and that loss of expression was required for the cell to survive. KIT^S expressing cells grew more slowly than their non-expressing counterparts, measured informally by cell counts, using a haemocytometer, so it is possible that the KIT^S producing cells were also outgrown by non-expressors. This is contrary to findings of other researchers. Murine KIT^S was stably expressed by J558L myeloma cells (Flanagan and Leder, 1990) and stably transfected CHO cells were used to express human KIT^S using plasmids pSV-DHFR and pDSR α (Lev *et al.*, 1992; Turner *et al.*, 1995). The former contained the *dhfr* gene to allow amplification of the vector with increasing concentrations of methotrexate (up to 1 μ M) and the latter contained the cDNA encoding the 12bp insert isoform of c-Kit, the same sequence used in the experiments described here. This suggested that forced increased expression of KIT^S was not toxic to cells nor did the 12bp insert/deletion alter KIT^S expression levels greatly.

Loss of expression of target protein by transfected cells is not unique to KIT^S. It appears that the expression of many proteins from vectors which encode both the target gene and the selectable marker is downregulated. Plasmids and retroviral vectors displayed a 5-10 fold decrease in expression of reporter genes when they also harboured neomycin resistance genes (Artelt *et al.*, 1991). This effect was not dependent on the insertion site or the orientation of the neomycin gene fragment. The gene conferring resistance to puromycin did not influence adjacent promoters and may have been a better choice. Thus, the decreased KIT^S expression levels but unimpaired resistance to cytotoxic drugs maybe associated with negative *cis* acting elements, including the neomycin gene. However, this is not the case for expression from the pEE14 vector and the loss of KIT^S expression in pEE14 transfected cells was possibly due to toxicity of overexpression.

The smaller than expected size of the Baculovirus/insect cell produced KIT^S was likely to be due to a lack of complex-type carbohydrate since sequencing of cDNA clones did not reveal any errors. Lack of N-linked carbohydrates on many glycoproteins produced in insect cells has been reported (Jarvis and Finn, 1995 and references within). Little information about insect cell N-linked oligosaccharide processing is available. Jarvis and Finn (1995) demonstrated a Baculovirus protein gp64 lacked detectable complex N-linked oligosaccharide side chains containing outer chain galactose or sialic acid, when expressed in any of three

insect cell lines. However, gp64 contained these side chains when expressed in COS cells. Others have reported the expression of recombinant proteins in insect cells with complex-type carbohydrate chains. It is possible the insect cells can produce complex side chains on glycoproteins but this may be followed by the rapid removal of outer chain sugars by exoglycosidases in these cells. Lepidopteran cell lines have been shown to express glycotransferases involved in complex-type N-glycosylation but upon Baculovirus infection the activity of these enzymes decreases within 48 hours (Van Die *et al.*, 1996). Additionally, considerable β -N-acetylgalactosaminidase and β -N-acetylglucosaminidase activity was observed in insect cells whether they were infected with Baculovirus or not and these enzymes were also detected in the culture supernatant. These findings provide evidence that recombinant proteins expressed in insect cells may not contain complex-type N-linked carbohydrates and a possible explanation for the altered KIT^S protein size detected.

To facilitate the purification of KIT^S and demonstrate the purified protein retained characteristic epitopes of the membrane bound counterpart, a sandwich style ELISA was developed, using two anti-c-Kit mAbs generated in this laboratory, YB5.B8 and 1DC3. Incorporating some of the methodology of Turner *et al.* (1995) and Wypich *et al.* (1995) improved the sensitivity of the assay. This assay was used to optimise KIT^S production and monitor purification.

Numerous alterations to the Baculovirus and pIG expression systems finally resulted in methods allowing usable quantities of KIT^S to be produced. For the Baculovirus expression system this included alteration of the multiplicity of infection and culture times. Adoption of the FuGENE 6 transfection reagent and method, in place of electroporation, greatly increased the efficiency of transient transfection and production of KIT^S from transfected COS cells. Improved purity of the KIT^S protein was achieved by culturing insect cells in specially modified serum free medium and culturing transfected COS cells in reduced serum levels (1%) and using serum that had been depleted of Protein A binding proteins.

The smaller than expected molecular weight of KIT^S-IGb and the minimal amount of KIT^S-IGb dimeric protein detected was of some concern. Liu *et al.* (1993) had shown that their KIT^S protein containing an Ig tag, produced by insect cells, was dimeric and that this could be reduced to the monomeric form in the presence of DTT. However, the KIT^S-IG

fusion protein generated from this construct was expressed from a cDNA clone containing the c-Kit cDNA directly fused, in frame, to the Fc cDNA of human IgG₁. It is thus possible that the lack of dimeric protein expressed by the insect cells described here maybe due to the inability of the insect cell machinery to efficiently splice the KIT^S-IG mRNA transcript to allow the translation of a single fused protein, although insect cells do have the ability to splice mRNA transcripts since many insect genomic sequences contain introns. Probing the different batches of KIT^S protein with an anti-human Ig antibody confirmed that the KIT^S-IGb protein lacked the Ig tag and this explains why the protein could not be purified using Protein A immobilised to Sepharose.

4. Analysis of KIT^S activity

4.1 Introduction

In chapter three, many expression systems were evaluated for their ability to allow large-scale production of KIT^S protein. Of all the systems studied only the eukaryotic pIG expression system and the Baculovirus expression system appeared to produce KIT^S protein at levels that may be of use.

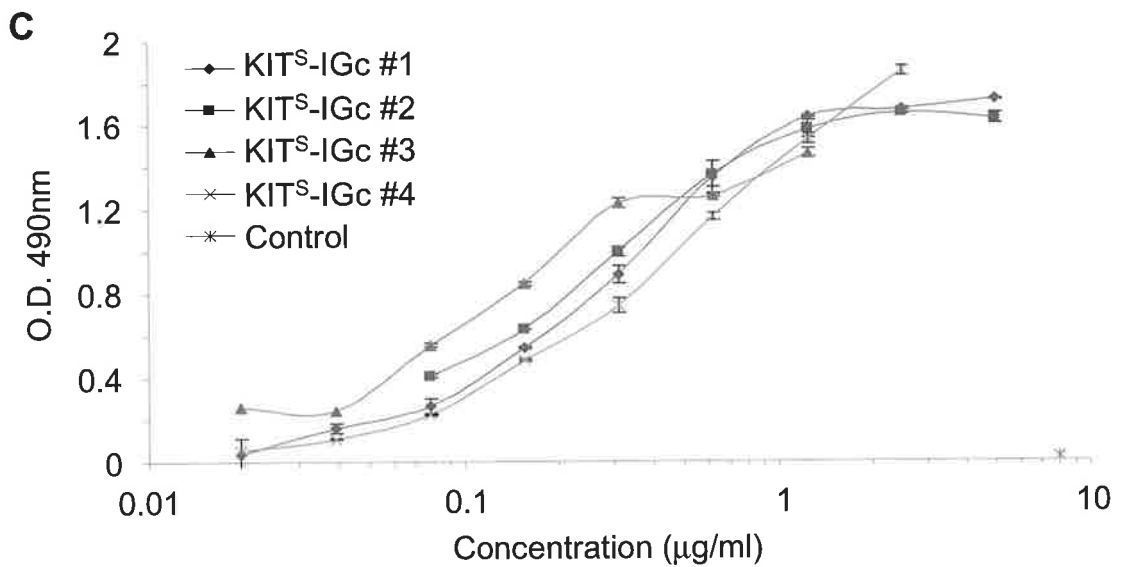
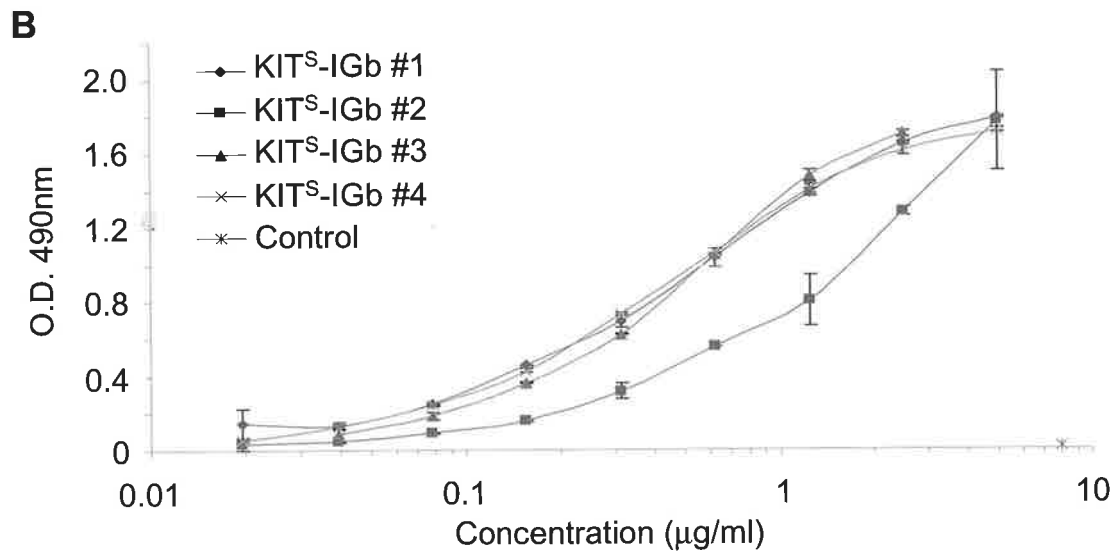
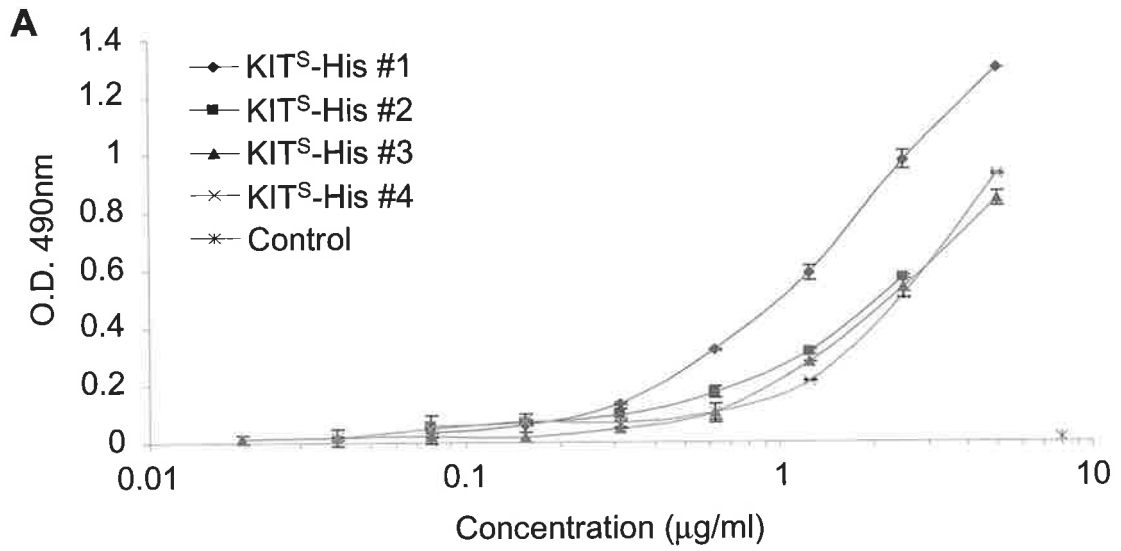
Initially the functional activity of purified KIT^S protein was assayed by its ability to bind cell associated SLF and to inhibit the binding of SLF to c-Kit expressing cell lines measured by indirect immunofluorescence assay. Preventing factor dependent, c-Kit expressing cell lines from proliferating in response to ligand was considered the best way of verifying functional KIT^S activity. To this end, c-Kit expressing human cell lines M07e and TF-1 as well as murine FDC-P1 cells transfected with cDNA encoding human c-Kit were cultured in the presence of human SLF and KIT^S and their proliferation was measured by ³H-Thymidine incorporation. In addition, the effect of KIT^S on c-Kit tyrosine phosphorylation was examined and its ability to prevent c-Kit/SLF mediated adhesion of c-Kit expressing cells to immobilised SLF was assayed.

4.2 Activity of KIT^S samples

4.2.1 ELISA

Initial analysis of KIT^S activity was determined using the sandwich ELISA (see section 3.8). Various batches of purified KIT^S-His, KIT^S-IGb and KIT^S-IGc were titrated from 5µg/ml down to 0.02µg/ml and their binding characteristics compared. Figure 4.1 depicts the comparison of four different batches of each of the different KIT^S proteins. In each case, minor batch-to-batch variation was found. However, the different KIT^S proteins varied considerably in their ability to bind the anti-c-Kit mAbs. The maximum signal was achieved between 1.25 and 2.5µg/ml of KIT^S-IGc with approximately 0.2µg/ml giving half maximal signal. In comparison, greater than 5µg/ml of KIT^S-His was required to give maximal signal and at least 1.25µg/ml of protein was required to generate a half maximal signal. KIT^S-IGb signals fell in between at approximately 5µg/ml and 0.7µg/ml respectively. Taking into account the different molecular weights of the KIT^S constructs, 20nM KIT^S-His, 8.7nM

Figure 4.1: Titration of KIT^S batches using the sandwich ELISA. A KIT^S-His, B KIT^S-IGb and C KIT^S-IGc. Control in each graph represents the signal generated by the negative control that does not contain KIT^S. Sample concentration had been determined by Bradford assay and sample purity determined by SDS-PAGE and total protein stain (section 2.7 and 2.19.6).

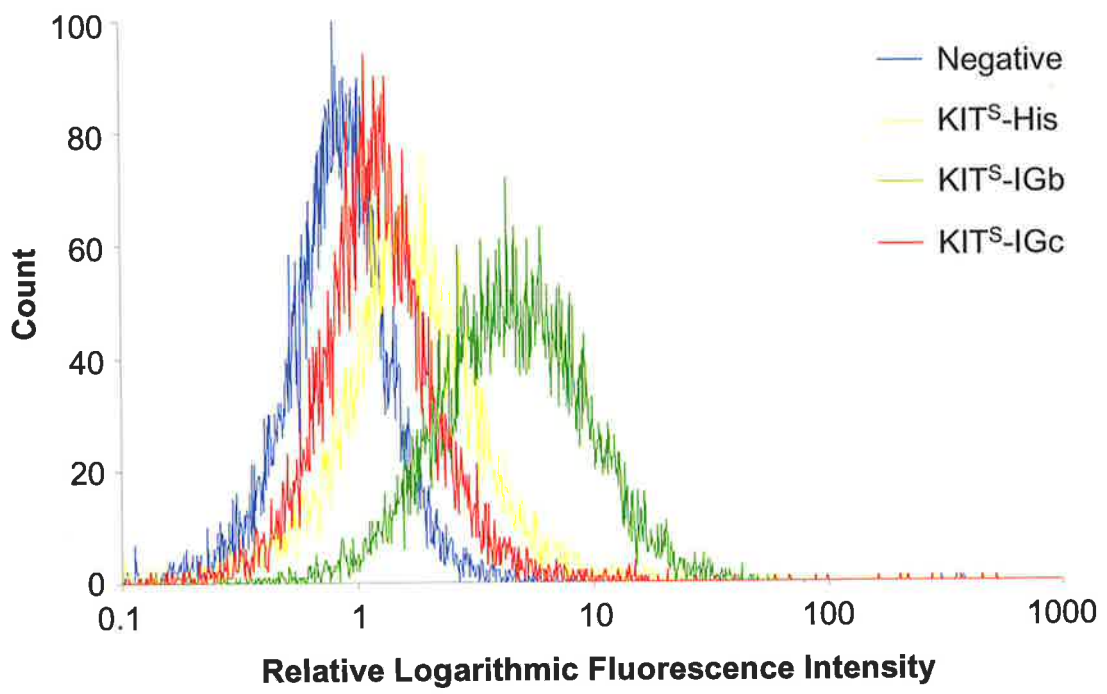


KIT^S-IGb and 0.8nM KIT^S-IGc were required to generate half the maximal signal detected. The differences in binding in the ELISA may be due to the monomeric or dimeric nature of the protein, with dimeric KIT^S-IGc allowing more b1DC3 to bind therefore generating a greater signal with less protein. It was expected that the KIT^S-IGb protein would generate signals similar to that of KIT^S-IGc since both should have been dimeric. However, KIT^S-IGb binding in the ELISA was weaker than KIT^S-IGc although it was stronger than KIT^S-His. In section 3.10, total protein stains and Western blots of KIT^S-IGb protein indicated that approximately half of this protein did not contain the Ig tag. Therefore, much of the KIT^S-IGb protein was unable to dimerise, resulting in a lower potency than the dimeric KIT^S-IGc protein. The difference in binding in the ELISA was not due to errors in the determination of sample concentration. The purity of all samples was determined by SDS-PAGE and total protein stain. Adjustments to the calculated concentration were made based on the purity. At worst the samples were only 50% pure, resulting in only a two-fold difference in the values detailed here.

4.2.2 Binding of KIT^S to CHO^{SLF} cells

While the ELISA data detailed in section 4.2.1 indicated the KIT^S protein retained the correct conformation, at least with respect to the epitopes recognised by the two antibodies used in the assay, it did not verify that the KIT^S protein could bind SLF. To determine this, KIT^S was assayed for its ability to bind to CHO cells transfected with the cDNA encoding the membrane bound isoform of human SLF, CHO^{SLF}, (Zannettino *et al.*, 1997). Figure 4.2 shows the binding of KIT^S-His, KIT^S-IGb and KIT^S-IGc to CHO^{SLF}, detected using an anti-c-Kit mAb, H3K, known not to compete with the SLF binding site (S. Read, unpublished). These data confirmed the ability of the different KIT^S constructs to bind to SLF. The weak binding of KIT^S-His and KIT^S-IGc was of some concern but other assays indicated they were capable of binding to soluble SLF (see below). The concentration of KIT^S may not have been saturating and use of a higher protein level may have increased binding. However, the concentrations used were the highest available, 550µg/ml of KIT^S-His and 700µg/ml of KIT^S-IGb or 8.8mM and 4.9mM respectively. Up to 420µg/ml (1.7mM) of

Figure 4.2: Binding of KIT^S-His, KIT^S-IGb and KIT^S-IGc to CHO^{SLF} cells. Cells were incubated with 1.25µg of KIT^S-His, KIT^S-IGb or KIT^S-IGc followed by the anti-c-Kit mAb H3K. Binding of mAb to the cells was detected using a second stage PE-labelled α-Ig reagent and flow cytometric analysis (see section 2.2.6). The figure illustrates flow cytometry histograms. To generate the negative control histogram the cells were not incubated with KIT^S.



KIT^S-IGc was used in this assay but the binding detected did not increase above that depicted in Figure 4.2.

4.2.3 Inhibition of SLF Binding to Cell Lines expressing c-Kit

Having established that the KIT^S constructs could indeed bind to SLF, their ability to prevent the binding of SLF to cell lines expressing c-Kit was determined by indirect immunofluorescence assay. Briefly, c-Kit expressing human cell lines M07e, HEL-DR⁺ or murine FDC-P1 cells transfected with cDNA encoding the GNNK⁺ isoform of human c-Kit (FD^{GNNK⁺}) (Caruana *et al.*, 1999) were incubated with biotinylated SLF (bSLF_y) alone, as a control, or with bSLF_y preincubated with the KIT^S protein constructs at various concentrations. bSLF_y was used at a concentration of 60ng/ml as this gave 75% of the maximum bSLF_y binding to the cells in the absence of added KIT^S. Cells were then washed and the level of bSLF_y binding was determined using a streptavidin-R-phycoerythrin conjugate. While concentrations within the range of 2.5- 40µg/ml of KIT^S-IGc were able to inhibit the binding of bSLF_y to M07e, HEL-DR⁺ and FD^{GNNK⁺} (Figure 4.3), the proteins produced in insect cells were less effective. Concentrations of at least 80µg/ml of KIT^S-IGb and 30µg/ml of KIT^S-His proteins were required to inhibit the binding of bSLF_y to M07e cells by 50% (Figure 4.4).

These results provided evidence that the KIT^S produced and purified by the different systems was indeed functional. However, the limited functional activity of KIT^S-IGb demonstrates that while correct conformation and ability to bind SLF had been determined in previous assays, see above, this did not translate into an efficient ability to inhibit the binding of bSLF_y to c-Kit expressed on the surface membrane of eukaryotic cell lines. This assay also confirmed that the dimeric KIT^S-IGc was more efficient in preventing bSLF_y from binding to cell surface c-Kit. This enhanced activity compared to its monomeric counterpart may be due to a greater affinity of the dimeric KIT^S protein for bSLF_y, providing an increased ability to compete with cell surface c-Kit molecules that form stable dimers upon SLF binding. This phenomenon was seen with several preparations of KIT^S-IGc and KIT^S-His suggesting that the difference in ability to inhibit SLF binding to c-Kit expressing cell lines was not due to batch-to-batch variation.

Figure 4.3: Inhibition of bSLF_y binding to c-Kit expressing cells by KIT^S-IGc. bSLF_y (60ng/ml) was incubated with various concentrations of KIT^S-IGc for 30 mins prior to the addition of the cells. Binding of bSLF_y to the cells was determined using SA-PE and flow cytometric analysis (section 2.2.6). Negative histogram was generated by cells incubated in the presence of SA-PE only and represents the background signal. bSLF_y histogram was generated by incubating the cells with bSLF_y in the absence of KIT^S and represents maximal bSLF_y binding. Control represents the histograms generated by incubating the cells with supernatant processed from COS cells that had not been transfected with KIT^S-IGc. **A:** M07e, **B:** HEL-DR⁺, **C:** murine FDC-P1 cells expressing human c-Kit, FD^{GNNK+}.

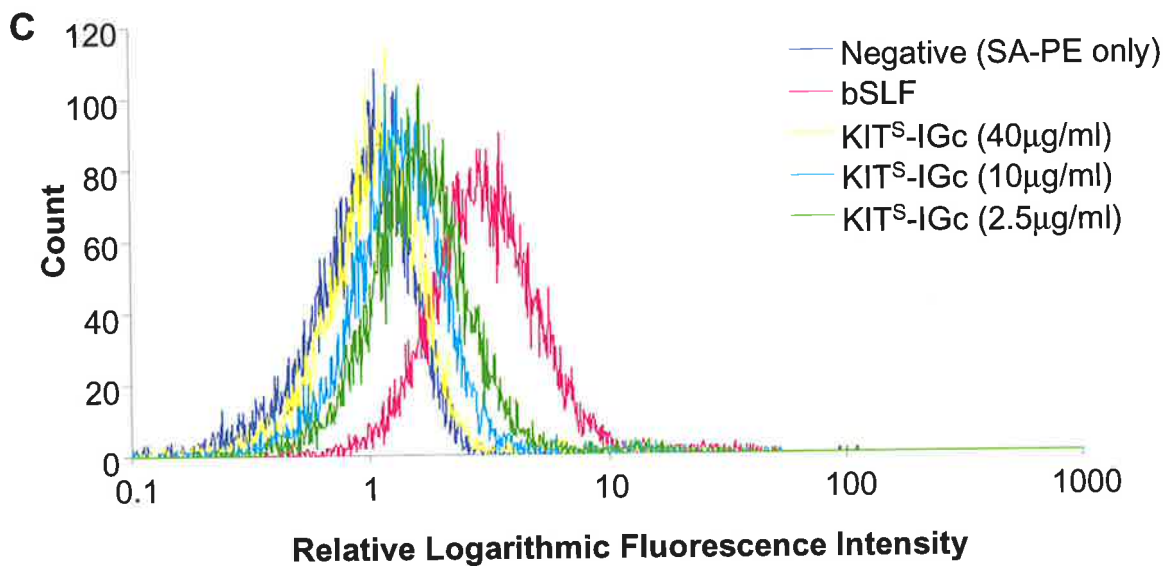
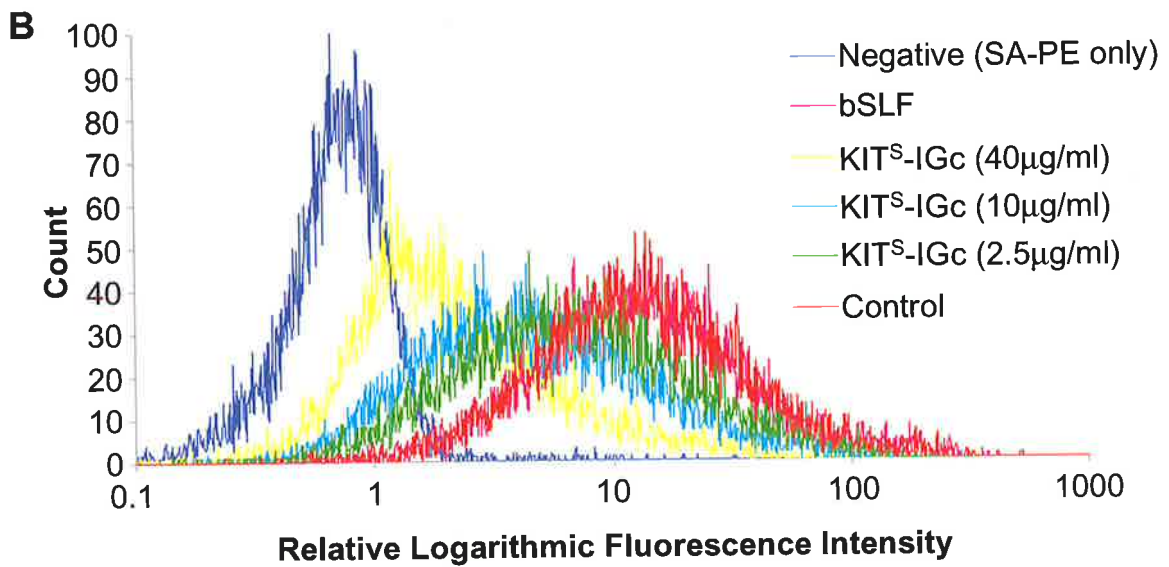
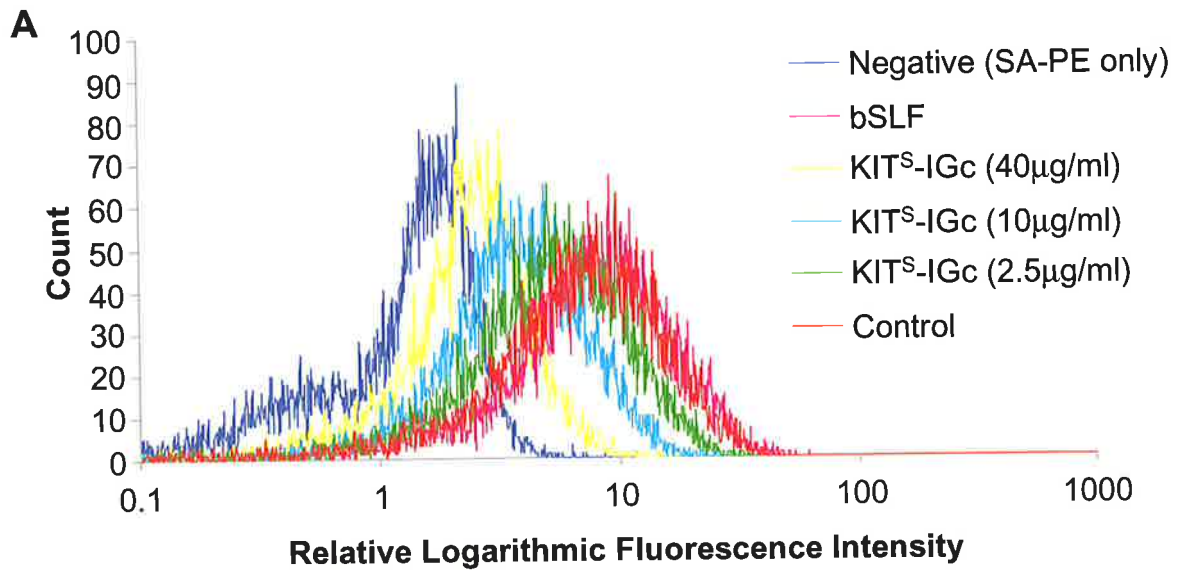
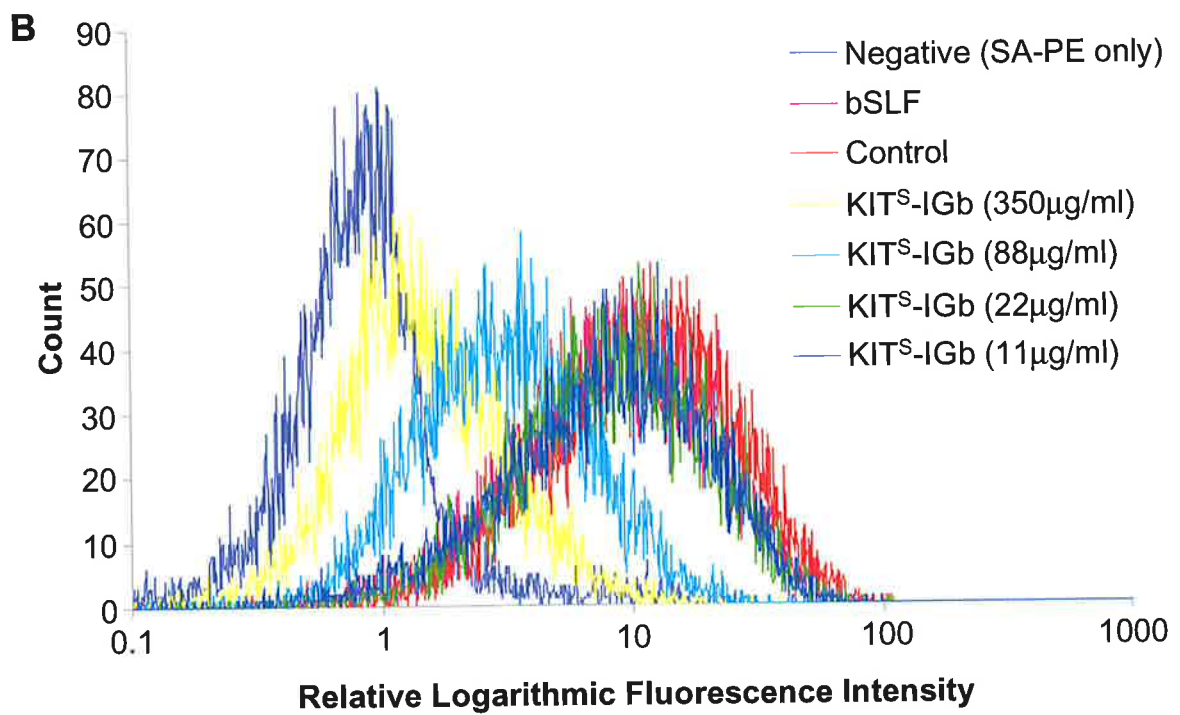
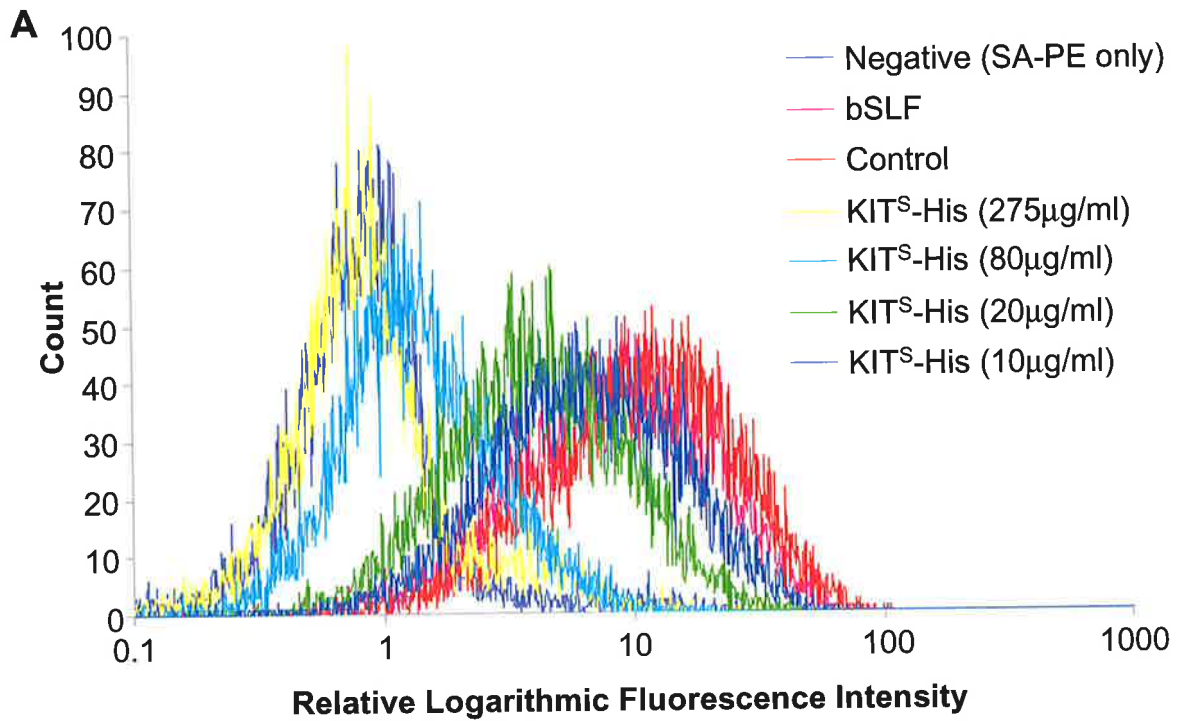


Figure 4.4: Inhibition of bSLF_y binding to M07e cells by KIT^S produced in insect cells.

bSLF_y (60ng/ml) was incubated with various concentrations of KIT^S for 30 mins prior to the addition to M07e cells. Binding of bSLF_y to the cells was determined using a SA-PE and flow cytometric analysis (section 2.2.6). Negative histogram was generated by cells incubated in the presence of SA-PE only and represents the background signal. bSLF_y histogram was generated by incubating the cells with bSLF_y in the absence of KIT^S and represents maximal bSLF_y binding. Control represents the histograms generated by incubating the cells with supernatant purified from Sf21 cells that had not been infected with KIT^S. **A:** KIT^S-His, **B:** KIT^S-IGb.



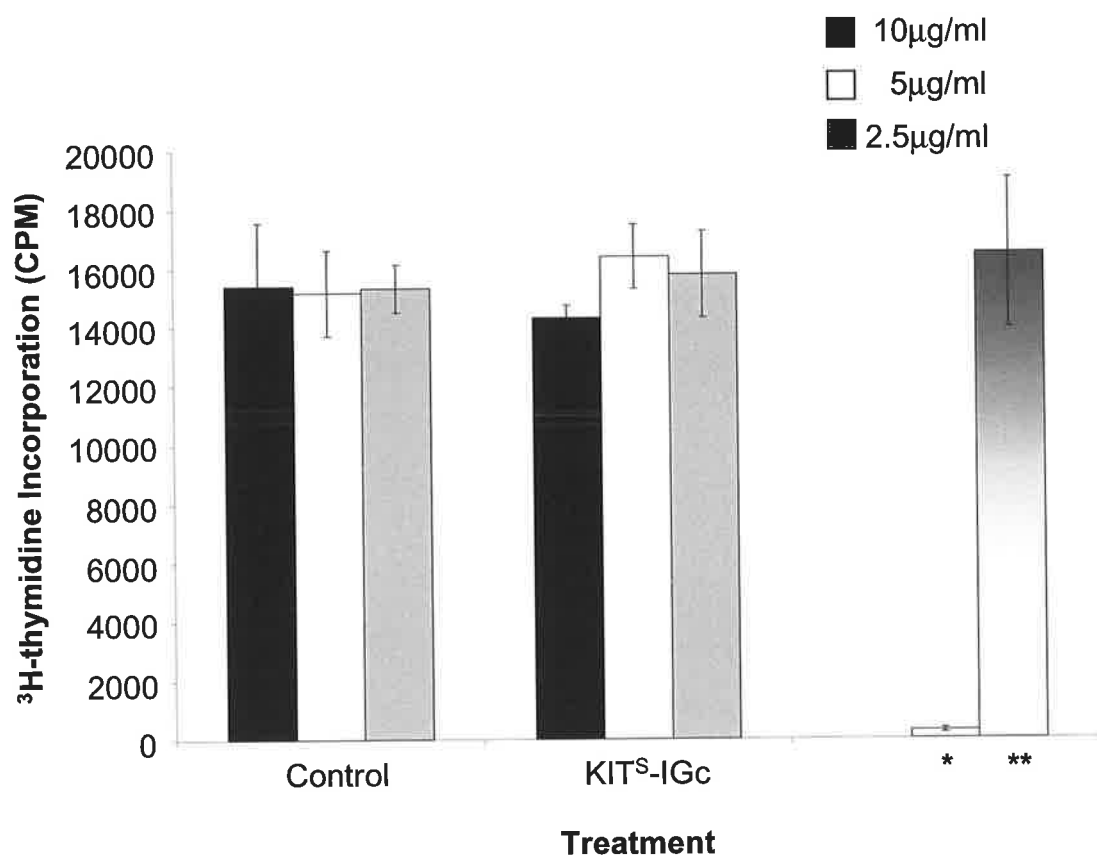
4.3 Inhibition of Factor Dependent Cell Line Proliferation

While the above assays demonstrated an ability of KIT^S protein to prevent SLF binding to c-Kit expressing cell lines, these static assays did not provide evidence that the ultimate goal of this project, to block SLF function, was achievable. To address this question, factor dependent cell lines expressing c-Kit were assayed for their ability to proliferate in the presence of SLF and KIT^S protein. The human megakaryocytic cell line M07e, and the human erythroleukaemia cell line TF-1, are normally cultured in the presence of human IL-3 or GM-CSF. Withdrawal of the growth factors results in a rapid decline in cell viability. However, if GM-CSF or IL-3 is substituted with SLF these cell lines are able to survive and proliferate (Kitamura *et al.*, 1989; Kiss *et al.*, 1993). Murine early myeloid cells, FDC-P1, are also factor dependent requiring murine GM-CSF for survival. FDC-P1 cells stably transfected with cDNA encoding human c-Kit are able to survive and proliferate in the presence of human SLF (Caruana *et al.*, 1999).

Initial experiments were conducted using the cell line M07e. M07e cells were seeded into wells of a 96 well plate at 1×10^4 cells/well in the presence of SLF at 25ng/ml (previously shown to cause 70% of maximal proliferation, data not shown) with or without the addition of KIT^S. After 48 hours the wells were pulsed with ³H-thymidine and cultured for a further 16 hours. The cells were then harvested and analysed for ³H-thymidine incorporation using a β -scintillation counter. In the experiment described in section 4.2.3 (Figure 4.4), a concentration of KIT^S, produced by insect cells, of at least 80 μ g/ml for KIT^S-His and 350 μ g/ml for KIT^S-IGb was required to block the binding of bSLF_y to M07e cells. This result implied that neither of these KIT^S proteins would be effective inhibitors of factor dependent cell line proliferation. In addition, the insect cell expression system could not efficiently produce the amount of KIT^S protein required in the proliferation assay to effectively test the ability of KIT^S-His and KIT^S-IGb to block cellular proliferation. It was therefore decided to concentrate on the effects of KIT^S-IGc on the proliferation of c-Kit expressing cell lines. Compared to the controls, KIT^S-IGc was unable to inhibit the proliferation of M07e (Figure 4.5). The level of KIT^S-IGc used in this assay was based on the result from Figure 4.3 that indicated KIT^S-IGc, at 10 μ g/ml, was able to prevent SLF binding to M07e cells by approximately 20-50%. Subsequent experiments, see below, suggested that the concentrations of KIT^S used in this assay were

Figure 4.5: Proliferative capacity of M07e cells cultured in the presence of KIT^S-IGc.

M07e cells were washed and starved of growth factors for 3 hours and then cultured for 3 days in the presence of SLF_b at 25ng/ml with or without KIT^S at the indicated concentrations. Proliferation was determined by ³H-thymidine incorporation in the last 16 hours of culture (section 2.3). Data are presented as the mean ± S.E.M. of quadruplicate wells. Control represents cells incubated with supernatant purified from untransfected COS cells. * cells incubated in the absence of SLF_b, ** cells incubated in the presence of SLF_b only.



insufficient to inhibit cellular proliferation. Higher concentrations of KIT^S may have inhibited SLF induced proliferation of M07e.

The amount of c-Kit on the cell surface of M07e is very high, 3.6×10^5 receptors per cell, compared with approximately 2×10^4 receptors per cell on CD34⁺ cells isolated from normal bone marrow (Cole *et al.*, 1996). Data from previous experiments suggested that the KIT^S samples might not be very efficient at preventing SLF from binding to c-Kit expressed on the surface of cells. Since the c-Kit expression level is so high on M07e it is possible that a low level of receptor occupancy is sufficient to generate a proliferative signal. It was therefore decided to examine the inhibitory effects of KIT^S on cell lines expressing lower levels of c-Kit on their surface membrane. Figure 4.6 depicts a fluorescence histogram of anti-c-Kit mAb (1DC3) binding to the cell lines M07e, HEL-DR⁺, TF-1 and FDC-P1 cells transfected with the GNNK⁺ isoform of human c-Kit (FD^{GNNK+}). This histogram overlay clearly shows M07e cells express more c-Kit molecules on their surface than the other cell lines with a mean fluorescence intensity of 66 compared to 47 for HEL-DR⁺, 24 for FD^{GNNK+} and 6.9 for TF-1 cells.

Using the procedure outlined above, ³H-thymidine incorporation was measured for TF-1 cells cultured in the presence of SLF at various concentrations and KIT^S-IGc at 20µg/ml. To control for non-specific inhibition, the cells were incubated with SLF in the presence of an equivalent amount of processed supernatant from untransfected COS cells or CD14-Ig (soluble CD14 protein fused to the constant domain of human IgG, kindly provided by Drs P. Simmons and A. Zannettino, Department of Haematology, Hanson Centre for Cancer Research). The CD14-Ig protein was used to control for possible effects of the Ig Fc part of KIT^S-IGc binding to Fc receptors on the cells in this and subsequent experiments. While neither of the controls appreciably retarded the proliferation of TF-1 cells in response to SLF, treatment of these cells with 20µg/ml of KIT^S-IGc resulted in 52%, 58% and 58% inhibition of cells treated with 25ng/ml, 12.5ng/ml or 6.25ng/ml of SLF respectively (Figure 4.7 (A & B)). Curiously an increase in inhibitory action by KIT^S-IGc was not seen at lower SLF concentrations. This point is addressed further in section 4.4. Titration of the KIT^S-IGc on these cells while maintaining a constant SLF concentration of 3ng/ml resulted in increased proliferation of the TF-1 cells at lower KIT^S levels, suggesting that the inhibitory effects were

Figure 4.6: Expression levels of c-Kit surface protein on FD^{GNNK+}, HEL-DR⁺, M07e and TF-1 cells. Cells were labelled with the anti-c-Kit mAb 1DC3 or an isotype-matched negative control mAb 1B5. Binding of the mAb to the cells was detected using a second stage PE-labelled α -Ig reagent and flow cytometric analysis (section 2.2.6). The negative control histograms superimposed each other and a representative histogram is depicted.

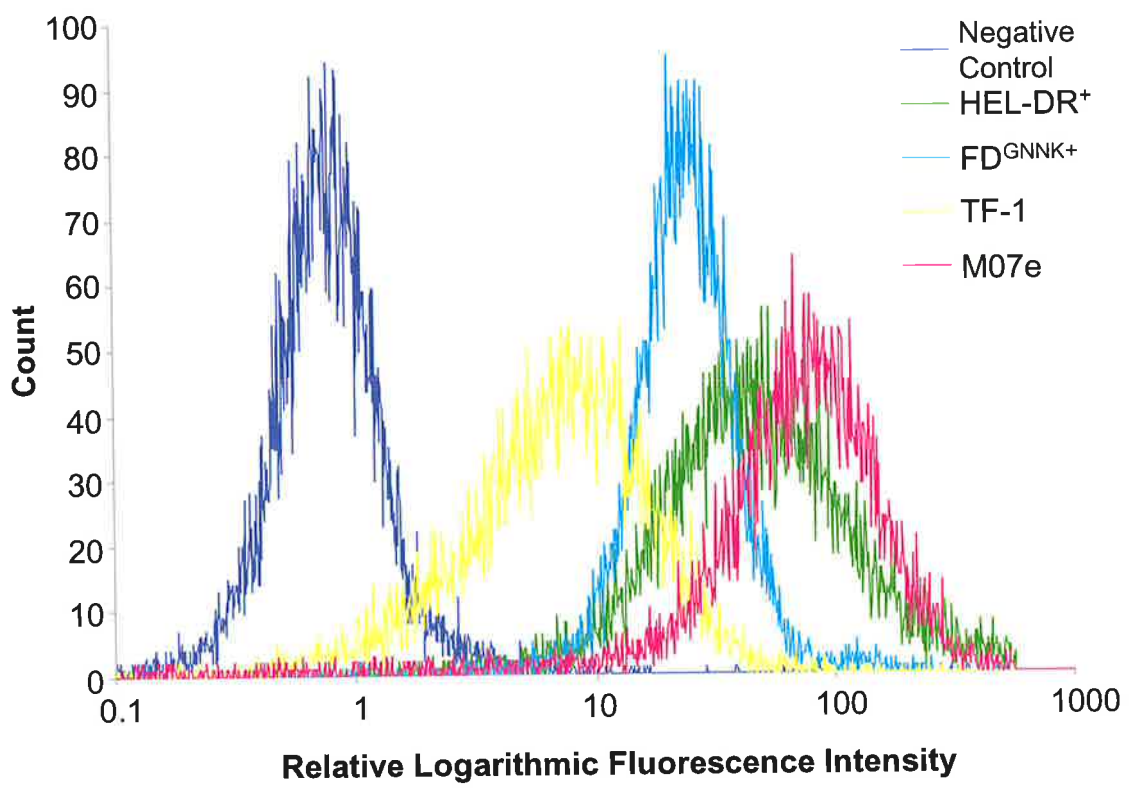
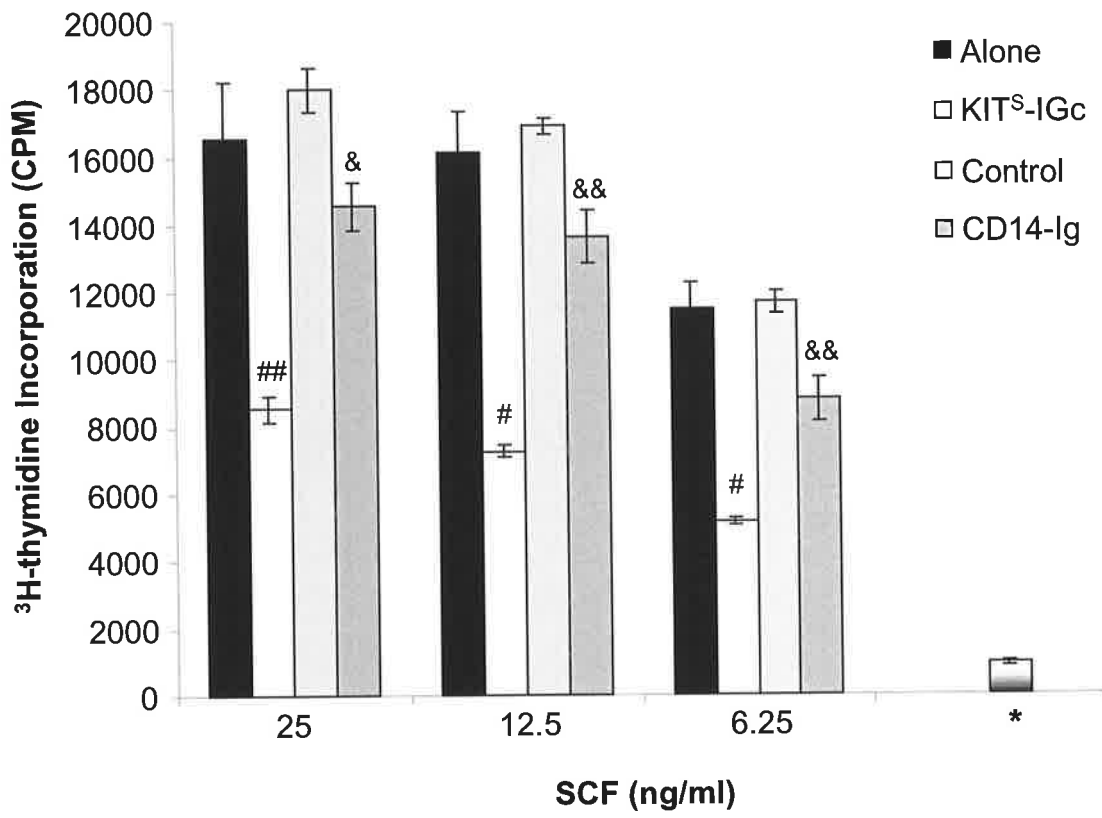
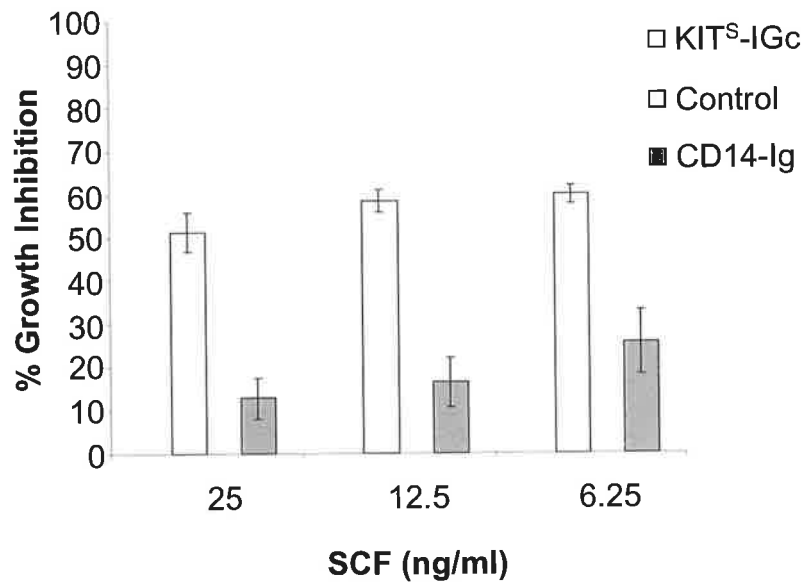


Figure 4.7: Proliferative capacity of TF-1 cells cultured in the presence of SLF_b at various concentrations with or without KIT^S-IGc. TF-1 cells were washed and starved of growth factors for 3 hours and then cultured in the presence of SLF_b at 25, 12.5 or 6.25ng/ml with or without KIT^S or control samples at 40μg/ml. Proliferation was determined by ³H-thymidine incorporation in the last 16 hours of culture (section 2.3). Data are presented as the mean ± S.E.M. of quadruplicate wells. Control represents cells incubated with an equivalent amount of supernatant processed from untransfected COS cells. * cells incubated in the absence of SLF_b.

A: ³H-thymidine incorporation. #: p<0.0001, ##: p<0.001, &: p>0.05, &&: p<0.05, relative to “alone”, 1-tailed Student t-test, 2 sample equal variance.

p<0.0005, comparison of KIT^S-IGc to CD14-Ig at each concentration of SLF, 1-tailed Student t-test, 2 sample equal variance.

B: Percent growth inhibition. Values were normalised for SLF-independent growth (* in A) and represented as 100%-(% treated sample growth / % control sample treated growth).

A**B**

genuinely due to the amount of KIT^S present in the culture (Figure 4.8 (A)). Allowing for the reduced SLF used in this experiment and the non-specific inhibitory effect seen at the highest concentration of the control (Figure 4.8 (B)) the specific inhibitory effects of KIT^S were approximately the same, 50%, as the previous experiment (compare Figure 4.8 (B) with Figure 4.7 (B)). No inhibition was seen with a KIT^S concentration of 2.5µg/ml. Some non-specific inhibition of proliferation was seen in this experiment and others (see below) at the highest control concentration points (Control and CD14-Ig). This may be due to the dilution of the media and nutrients in the well as all concentrated KIT^S and control samples were stored in PBS/0.1% BSA. Background inhibition by controls was not seen at lower concentrations as dilutions were carried out using media containing 10% FBS. In hindsight, indirect inhibition by high concentrations of protein could have been avoided by equilibrating the stock protein solutions with assay medium.

Having established that KIT^S was able to inhibit the proliferation of the low c-Kit expressing cell line TF-1, its ability to prevent growth of murine FDC-P1 cells expressing the G^NNK⁺ isoform of human c-Kit was examined. These cell lines had been generated previously in the laboratory (Dr G. Caruana) and lines expressing c-Kit at a range of levels were available. A high and a low c-Kit expressing clone were chosen, FD^{G^NNK⁺#1} and FD^{G^NNK⁺#2} respectively. The c-Kit levels expressed by these cells were determined by indirect immunofluorescence assay using 1DC3 as the anti-c-Kit mAb. FD^{G^NNK⁺#1} displayed a MFI of 103 and FD^{G^NNK⁺#2} a MFI of 22.6. By comparison M07e had an MFI of 66 and TF-1 had an MFI of 6.9. KIT^S-IGc was unable to prevent the proliferation of FD^{G^NNK⁺#1} in response to a subsaturating concentration of SLF (25ng/ml) (Figure 4.9 (A)). However, some inhibition, approximately 33%, was seen when FD^{G^NNK⁺#2} cells were treated with 40µg/ml of KIT^S (Figure 4.9 (B)). Serial dilution of the KIT^S protein resulted in less inhibition at lower KIT^S concentrations: the ³H-thymidine incorporation experiment was repeated with the FD^{G^NNK⁺#2} clone, titrating KIT^S-IGc from 80µg/ml down to 1.25µg/ml in the presence of SLF at a concentration of 25ng/ml (Figure 4.10). At 80µg/ml, KIT^S-IGc was able to inhibit the proliferation of FD^{G^NNK⁺#2} cells, cultured in the presence of SLF at 25ng/ml, by 80% compared to 24% by the negative control (Figure 4.10). This inhibition decreased slightly to 73% when KIT^S-IGc was diluted to 40µg/ml and at a similar dilution the negative control had

Figure 4.8: Proliferative capacity of TF-1 cells cultured in the presence of KIT^S-IGc.

TF-1 cells were washed and starved of growth factors for 3 hours and then cultured for 3 days in the presence of SLF_b at 3ng/ml with KIT^S or controls at various concentrations. Proliferation was determined by ³H-thymidine incorporation in the last 16 hours of culture (section 2.3). Data presented as the mean ± S.E.M. of quadruplicate wells. Control represents cells incubated with an equivalent amount of supernatant processed from untransfected COS cells. * cells incubated in the absence of SLF_b, ** cells incubated in the presence of SLF_b only.

A: ³H-thymidine incorporation. #: p<0.00005, ##: p<0.0005, ###: p<0.05, &: p<0.05, compared to treatment with SLF_b only (**), 1-tailed Student t-test, 2 sample equal variance. p<0.00005, comparison of # to &, 1-tailed Student t-test, 2 sample equal variance.

B: Percent growth inhibition. Values were normalised for SLF-independent growth (* in A) and represented as 100%-(% treated sample growth / % control sample treated growth).

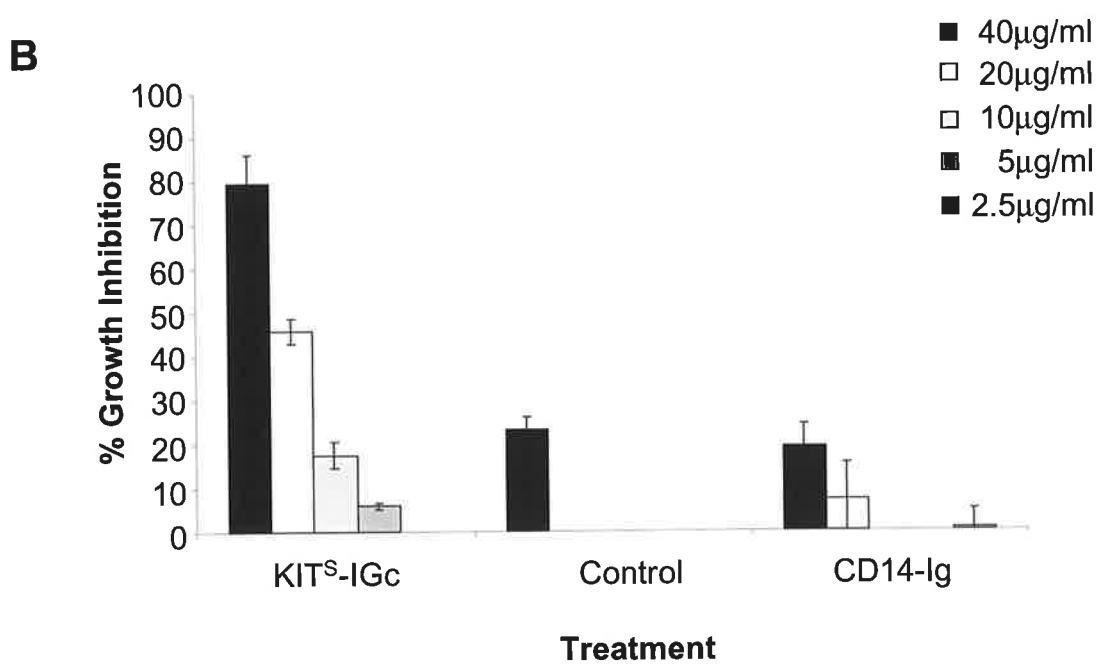
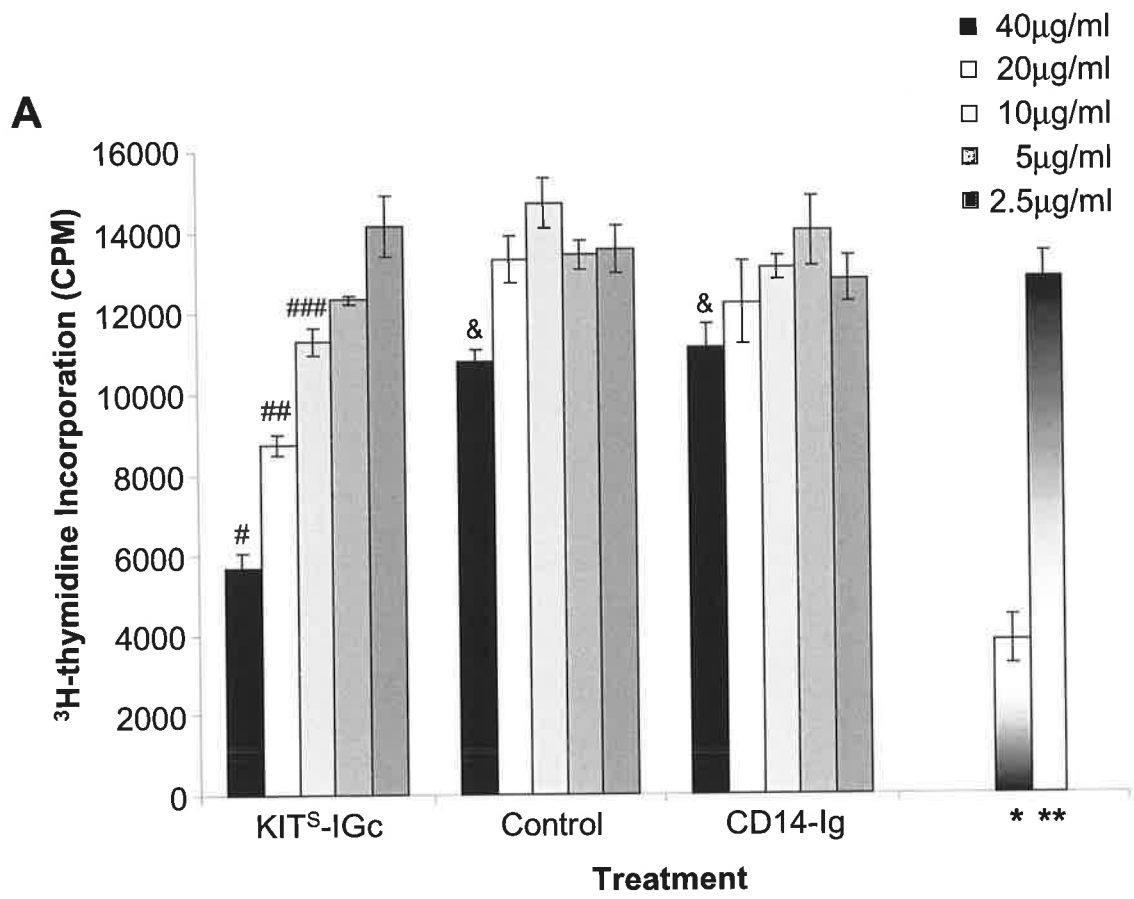


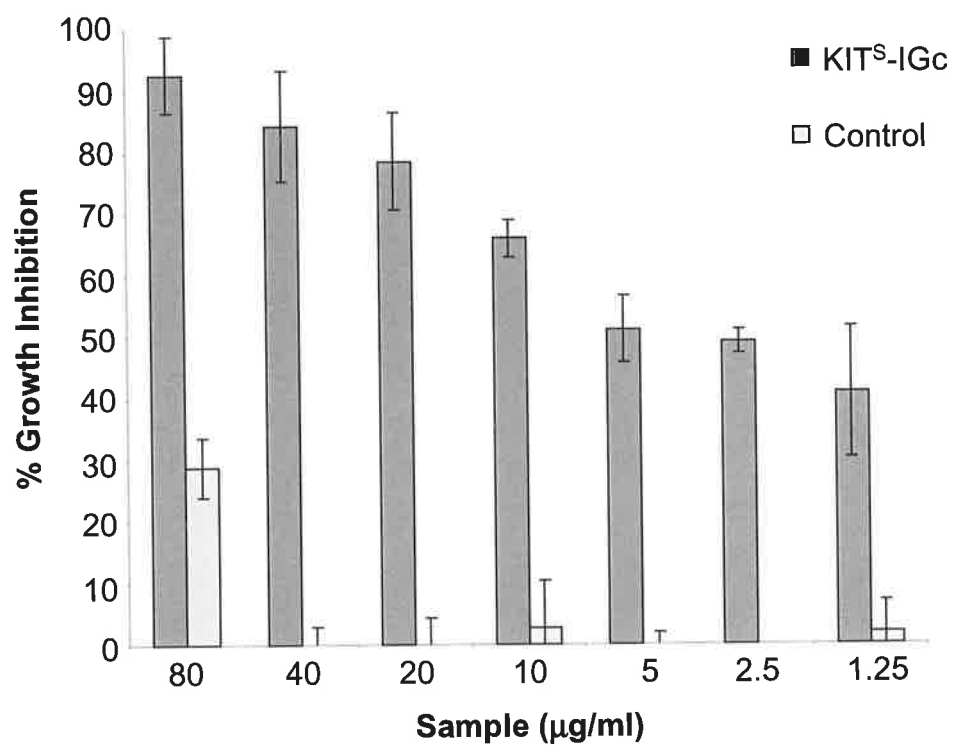
Figure 4.9: Proliferative capacity of FDC-P1 cells expressing the GNNK+ c-Kit isoform (FD^{GNNK+}) cultured in the presence of KIT^S-IGc. FD^{GNNK+} cells were washed and starved

of growth factors for 3 hours and then cultured for 3 days in the presence of SLF_b at 25ng/ml with KIT^S or controls at various concentrations. Proliferation was determined by ³H-thymidine incorporation in the last 16 hours of culture (section 2.3). Data are presented as the mean ± S.E.M. of quadruplicate wells. Control represents cells incubated with an equivalent amount of supernatant processed from untransfected COS cells. * cells incubated in the absence of SLF_b, ** cells incubated in the presence of SLF_b only.

A: FD^{GNNK+}#1; **B:** FD^{GNNK+}#2. #: p<0.001, ##: p<0.01, &: p>0.05, relative to treatment with SLF_b only (**), 1-tailed Student t-test, 2 sample equal variance.

p<0.001, KIT^S-IGc (20μg/ml) compared to control (20μg/ml), 1-tailed Student t-test, 2 sample equal variance.

Figure 4.10: Responsiveness of FD^{GNNK+}#2 cells to KIT^S-IGc titrated from 80 to 1.25µg/ml. FD^{GNNK+}#2 cells were washed and starved of growth factors for 3 hours and then cultured for 3 days in the presence of SLF_b (25ng/ml) with KIT^S-IGc or the control (supernatant purified from untransfected COS cells) at concentrations from 80µg/ml to 1.25µg/ml. Proliferation was determined by ³H-thymidine incorporation in the last 16 hours of culture (section 2.3). Values were corrected for growth in the absence of SLF and represented as 100%-(% treated sample growth / % untreated cellular growth). Data are presented as the mean ± S.E.M. of quadruplicate wells.



no influence on the proliferative rate of the cells. Even at 1.25µg/ml the proliferative capacity of the FD^{GNNK+}#2 cells had been reduced by approximately 35%. Similar results were obtained when the cells were cultured in the presence of SLF at a concentration of 12.5ng/ml (data not shown).

4.4 Comparison of the ability of bSLF_y to bind to or induce the proliferation of factor dependent cell lines

The inability of KIT^S to inhibit M07e or FD^{GNNK+}#1 proliferation whilst it could inhibit TF-1 and FD^{GNNK+}#2 cell proliferation appeared to be due to the level of receptor expression. If the extent of proliferation is determined by the absolute number of receptors occupied by ligand rather than the percentage of receptors occupied, and assuming the affinity of the receptor for ligand is the same on all cells, then at low ligand concentrations a cell expressing large numbers of receptors is more likely to reach the required threshold of receptor occupancy than a low expressing cell. That is, cells expressing high receptor numbers, e.g. M07e, would require a lower concentration of ligand to achieve maximum proliferation, and hence a higher concentration of KIT^S would be required to inhibit their proliferation. To investigate if this hypothesis was correct SLF was titrated on the different c-Kit expressing cells and SLF binding and SLF induced cellular proliferation were determined.

To this end, M07e, TF-1 and FD^{GNNK+}#2 cells were incubated with various concentrations of biotinylated SLF_y (bSLF_y) in two different assays. On the same day, with the same batch of bSLF_y, these cells lines were assayed for their proliferative capacity in response to bSLF_y at final concentrations of 100ng/ml down to 1.56ng/ml, and the amount of bSLF_y bound to these cells at the same concentrations was measured by indirect immunofluorescence assay. Since the binding assay measures the immediate interaction of the ligand with the receptor and the proliferation assay measures the cumulative effect of SLF binding to the surface receptor over three days the methodology for the proliferation assay was altered. Cells were cultured with ligand for 24 hours prior to the addition of ³H-thymidine and the culture continued for a further 24 hours. This allowed the measurement of proliferation from an earlier time point. It was argued, measurement of proliferation for the final 16 hours of 64 hours culture would miss cellular responses to low concentrations of ligand as the source of ligand may be

exhausted by this time. Figure 4.11 depicts the data obtained for M07e (A), TF-1 (B) and FD^{GNNK+}#2 cells (C) proliferation and binding of bSLF_y to the cell surface receptors. The levels of bSLF, approximately 5ng/ml, required to saturate cell surface binding and proliferative responses were similar in the case of M07e and TF-1 cells but, surprisingly, substantially more bSLF, greater than 25ng/ml, was required for maximum proliferation than for maximum binding, approximately 5ng/ml, in the case of FD^{GNNK+}#2 cells. These data do not support the simple hypothesis presented above, and other explanations relating to the nature and duration of the assays are considered more likely to account for the proliferation data.

4.5 Inhibition of c-Kit Phosphorylation

Activation of c-Kit following SLF binding results in receptor dimerisation and transphosphorylation. Phosphorylation of the receptor permits recruitment of many proteins involved signal transduction. Therefore, the proliferation inhibition observed above was assumed to be due to blocking this phosphorylation event and subsequent protein association. Using a soluble c-Kit molecule, KitX, Lev *et al.* (1992), prevented SLF induced phosphorylation of c-Kit resulting in a 90% decrease in PI3'K association with the receptor. A 100-fold excess of KitX over SLF was required to generate this response. The amount of inhibition seen was dependent upon the SLF concentration.

M07e cells were starved of factor for 3 hours and then incubated with SLF (80ng/ml) for 2 minutes in the presence of KIT^S (95µg/ml) or PBS. Work by colleagues in the laboratory had shown maximal induced phosphorylation of c-Kit on M07e cells occurred two minutes after the addition of SLF (A.C. Cambareri and L.K. Ashman, unpublished). Phosphorylation of the receptor was determined by immunoprecipitation of the c-Kit molecules from the NP40 treated cell lysates, SDS-PAGE and Western blotting with anti-phospho-tyrosine antibodies, PY20 and 4G10. Phosphorylation of the receptor did not occur in the absence of factor (Figure 4.12, (A) lane 1) but was easily detected after stimulation with 80ng/ml of SLF (Figure 4.12, (A) lane 2). If the cells were stimulated with SLF in the presence of KIT^S, the amount of phosphorylation seen greatly diminished (Figure 4.12, (A) lane 3 and Figure 4.12 (C)). Incubation with the control, PBS, had no effect on the level of receptor phosphorylation,

Figure 4.11: Comparison of the ability of various concentrations of bSLF_y to bind to FD^{GNNK+}#2, M07e and TF-1 cells and its ability to induce proliferation of these cells at the same concentration. For the proliferation assay, data depicted in the left panels, 1x10⁴ cells/well were incubated in the presence of bSLF_y for 48 hours. Proliferation was determined by ³H-thymidine incorporation in the last 24 hours of culture (section 2.3). Data are presented as the mean ± S.E.M. of quadruplicate wells. * cells incubated in the absence of bSLF_y. Binding of bSLF_y to the cells (2x10⁴/tube), data depicted in the right hand panels, was determined using a PE conjugated-streptavidin complex (SA-PE) and flow cytometric analysis (section 2.2.6). **A:** M07e; **B:** TF-1; **C:** FD^{GNNK+}#2.

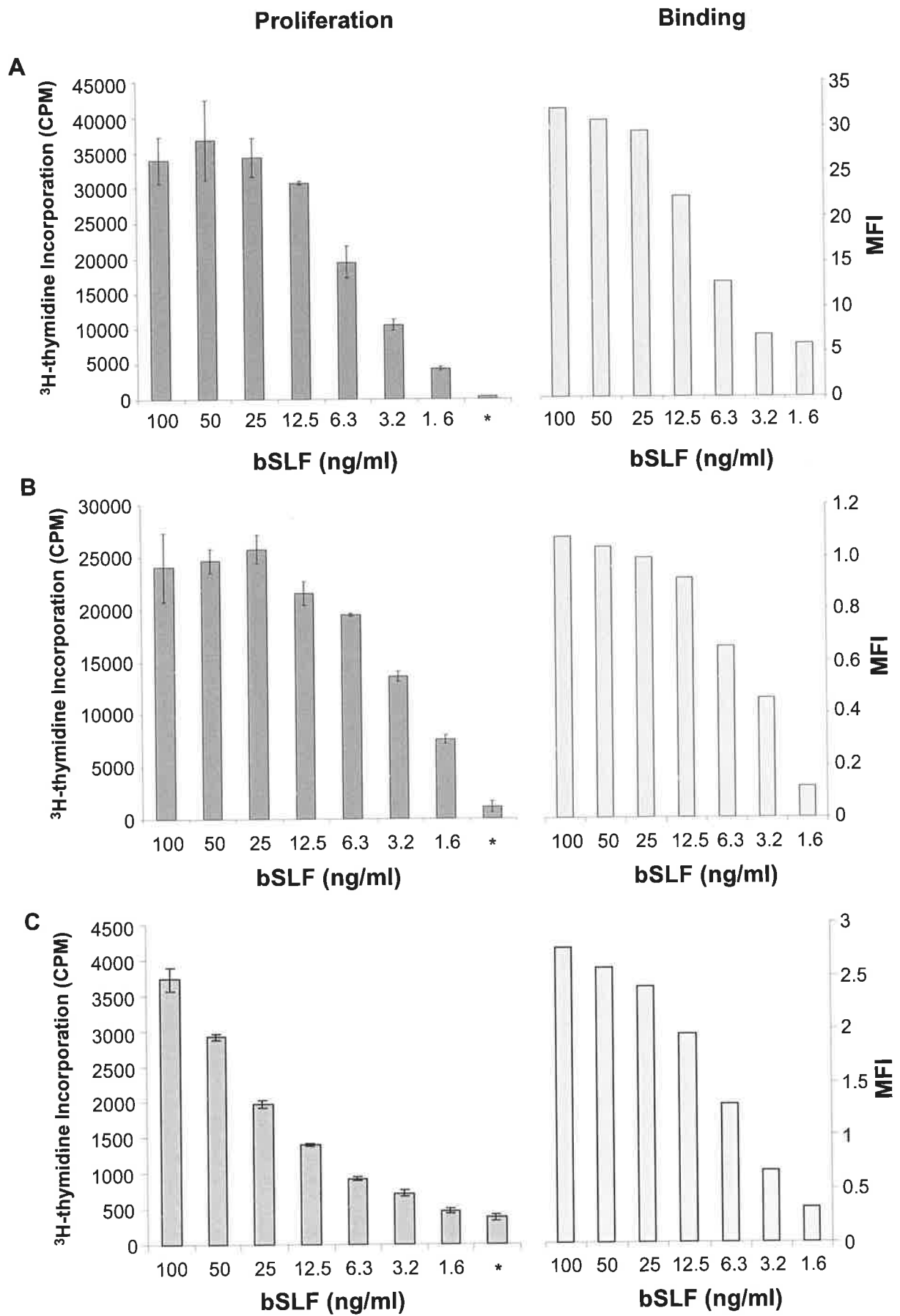
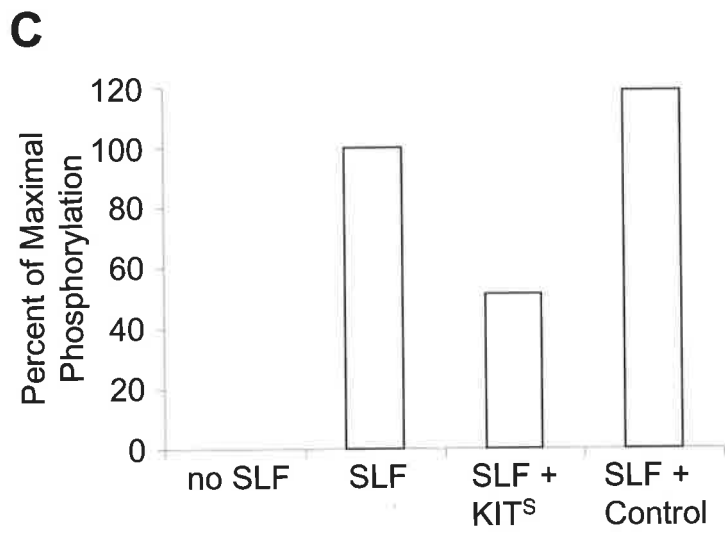
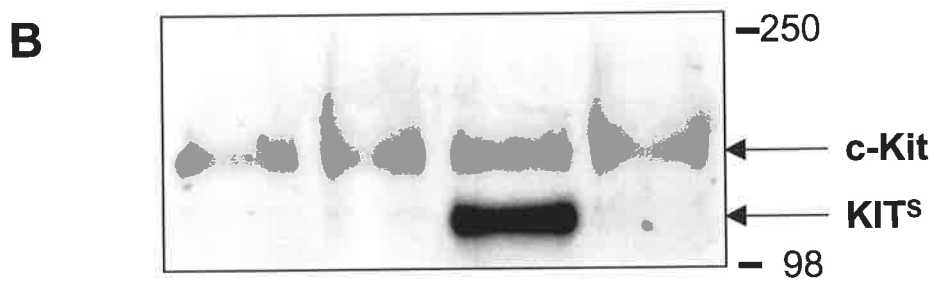
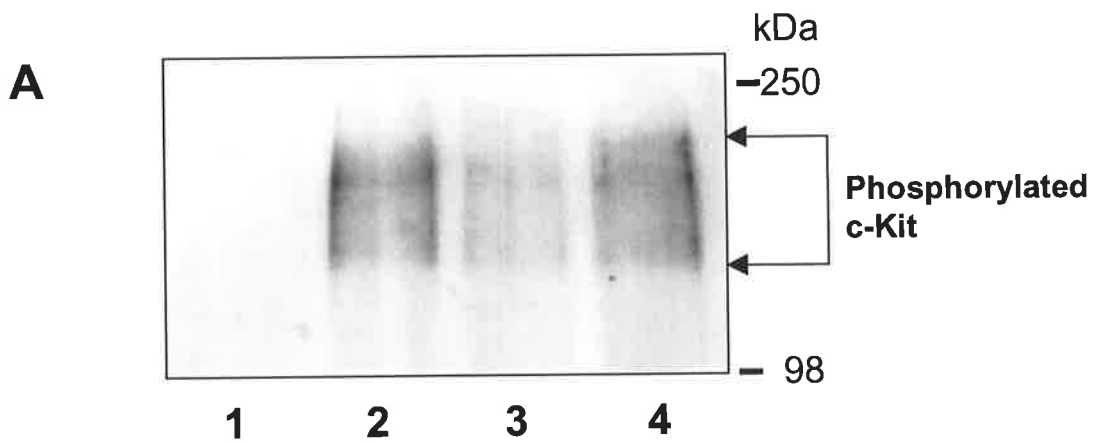


Figure 4.12: SLF dependent phosphorylation of c-Kit in the presence of KIT^S-IGc. M07e cells were incubated for 2 mins in the presence of SLF (80ng/ml) in the presence or absence of KIT^S or control. Lysates of stimulated cells were immunoprecipitated with anti-c-Kit mAb, Kit4, (5µg/ml) and 20µl of Protein A coupled Sepharose, overnight at 4°C. Washed Sepharose was boiled and loaded onto a 8% SDS-polyacrylamide gel. Separated proteins were transferred to PVDF and subjected to Western Blot (section 2.9). **Lane 1:** Cells incubated in the absence of SLF, **Lane 2:** cells incubated in the presence of SLF, **Lane 3:** cells incubated in the presence of SLF and KIT^S (95µg/ml), **Lane 4:** cells incubated in the presence of an equivalent amount of supernatant processed from untransfected COS cells. **A:** Western Blot with anti-phospho-tyrosine mAbs PY20 and 4G10. The larger molecular weight protein reacting with the anti-phospho-tyrosine antibodies is ubiquitinated c-Kit (A.C. Cambareri and L.K. Ashman, unpublished). **B:** Western blot with anti-c-Kit mAb 1C1.HF. **C:** Quantitated fluorescence units (obtained using a Fluorimager, and analysed using ImageQuant software, Molecular Dynamics) of data from **A**, depicted as a percentage of signal from cells stimulated with SLF alone.



lane 4. The larger molecular weight protein reacting with the anti-phospho-tyrosine antibodies is ubiquitinated c-Kit (A.C Cambareri and L.K. Ashman, unpublished). Probing of a duplicate blot with the anti-c-Kit mAb, 1C1.HF, demonstrated that all lanes contained equivalent amounts of c-Kit (Figure 4.12 (B)).

4.6 Effect of KIT^S on M07e Adhesion to SLF

Mast and progenitor cells isolated from *W/W* mice fail to adhere to fibroblasts isolated from normal mice. Conversely, these cells expressing wild-type c-Kit fail to adhere to fibroblasts isolated from *Sl/Sl* mice (Kaneko *et al.*, 1991; Adachi *et al.*, 1992). This indicates stromal cell expressed membrane-bound SLF binding to c-Kit expressed by mast cells and haemopoietic progenitor cells may directly mediate adhesion between these cells. In addition, SLF signalling through c-Kit results in the upregulation of the avidity of the β 1 integrins, VLA-4 and VLA-5, for fibronectin (Dastyh and Metcalfe, 1994; Kinashi and Springer, 1994; Kovach *et al.*, 1995; Lévesque *et al.*, 1995). Thus, adhesion mediated by the c-Kit/SLF interaction may maintain cells in the correct microenvironment and also provide normal and leukaemic cells with survival and proliferative signals by synergising with integrins (Bendall *et al.*, 1998). It was therefore possible that KIT^S could prevent this process.

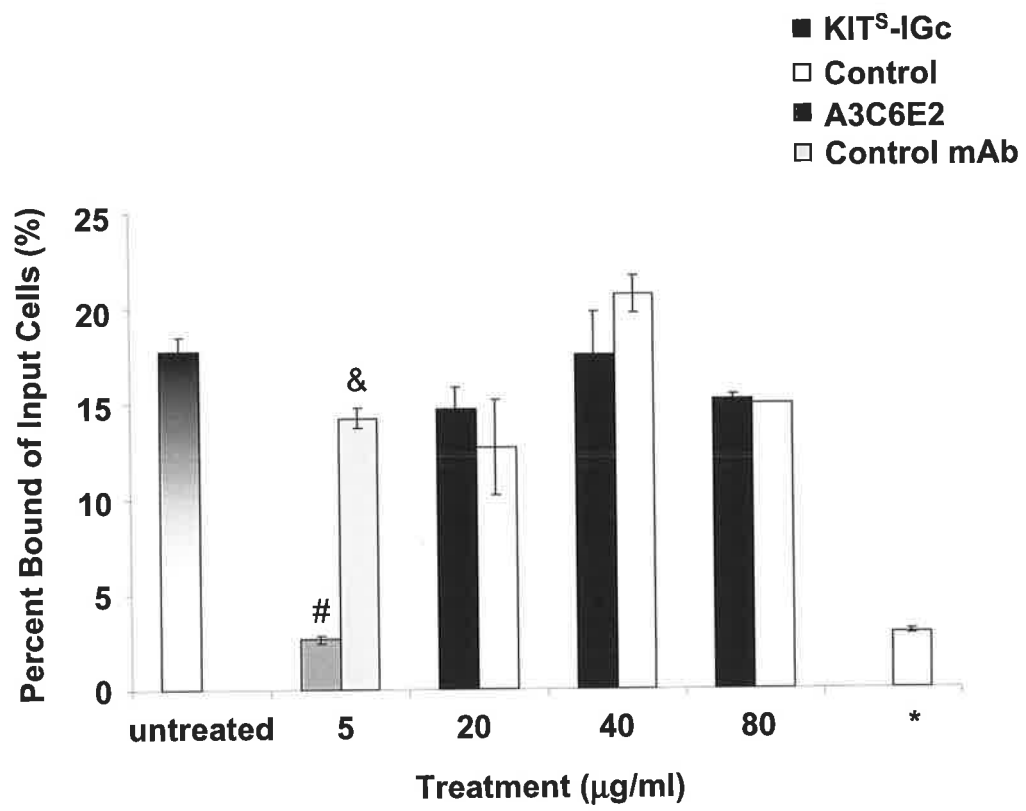
As a first step to see if KIT^S could indeed modulate this process, its effect on the adhesion of c-Kit expressing cells to immobilised SLF was examined. Attempts to observe the adhesion of M07e or TF-1 cells to wells coated with purified SLF were unsuccessful. However, M07e cells were able to bind to CHO cells expressing the membrane associated form of SLF (CHO^{SLF} cells) (Figure 4.13). In this assay, CHO^{SLF} cells were allowed to adhere to the bottom of the wells of a 96 well plate. M07e cells were labelled with Calcein-AM, a fluorescent, membrane permeant dye. The labelled M07e cells were then incubated with the adherent CHO^{SLF} cells in the presence of KIT^S-IGc or negative control. After 2 hours, the non-adhered cells were washed away, the adhered cells were lysed with 1% SDS and the wells were analysed for the presence of fluorescence using a Fluorimager with a 570nm filter. It was found only 15-50% of the M07e cells loaded into the wells adhered to the untreated CHO^{SLF} cells and the data is therefore represented as the percent of cells bound to the CHO^{SLF} cells of the input cells. Background binding of M07e cells to untransfected CHO cells was

Figure 4.13: Influence of KIT^S-IGc upon the adhesion of M07e cells to CHO^{SLF} cells.

Growth factor starved M07e cells labelled with calcein-AM were added to wells coated with a confluent monolayer of CHO^{SLF} cells, which had been pre-incubated with either KIT^S or control (PBS) for 30 mins. As a positive control, M07e cells were incubated with the anti-c-Kit mAb A3C6E2 (kindly provided by Dr H-J. Bühring, Transplantation Immunology and Immunohematology, University of Tübingen, Germany) for 30 min prior to the addition of the M07e cells to the wells. The binding of A3C6E2 to c-Kit prevents SLF from interacting with c-Kit (H-J. Bühring, personal communication). After washing, to remove non-adhered cells, the contents of the wells were lysed with 1% SDS and the plates scanned using a Fluorimager (Molecular Dynamics) using the 570nm filter (section 2.4). Data are depicted as the percentage of input cells bound to the monolayer. * Non-specific binding of M07e cells to untransfected CHO cells. Data represented as the mean \pm S.E.M. of three replicate wells. Data from one experiment shown, experiment repeated three times in total.

#: $p < 0.00005$, &: $p < 0.05$, relative to "untreated", 1-tailed Student t-test, 2 sample equal variance.

$p < 0.0001$, comparison of # to &, 1-tailed Student t-test, 2 sample equal variance.



observed at approximately 3-15% of the total input cells. As a positive control, M07e cells were incubated with the anti-c-Kit mAb A3C6E2 (kindly provided by Dr H-J. Bühring, Transplantation Immunology and Immunohematology, University of Tübingen, Germany) for 30 min prior to the addition of the M07e cells to the wells. The binding of A3C6E2 to c-Kit prevents SLF from interacting with c-Kit (H-J. Bühring, personal communication). Preincubating A3C6E2 with M07e cells reduced their binding to the CHO^{SLF} cells to approximately 3% of the input cells (Figure 4.13). Based on previous results 40µg/ml of KIT^S-IGc was required to block SLF binding to M07e (Figure 4.3); 40µg/ml of KIT^S-IGc was required to inhibit TF-1 proliferation by 50% (Figure 4.7); and at least 20µg/ml of KIT^S-IGc was required to block FD^{GNNK⁺#2} proliferation (Figure 4.10). However, incubating the CHO^{SLF} cells with up to 80µg/ml of KIT^S-IGc for 30 min prior to the addition of the labelled M07e cells, failed to prevent adhesion of the M07e cells to the CHO^{SLF} cells (Figure 4.13).

4.7 Discussion

Determination of KIT^S function was achieved using several different assays. The ability of KIT^S to recognise SLF was established by measuring (by indirect immunofluorescence) KIT^S binding to CHO cells expressing the membrane bound isoform of human SLF. The capacity of KIT^S to prevent SLF from binding to cells expressing the c-Kit molecule was shown by an immunofluorescence assay in which c-Kit expressing cell lines were incubated with bSLF_γ and KIT^S. KIT^S was able to block the binding of SLF to these cells but this assay also demonstrated that the affinity of KIT^S for SLF was weaker than that of membrane bound receptor, as a large excess of KIT^S was required to prevent SLF from binding to the cells. For both KIT^S-His and KIT^S-IGb a 195 fold molar excess of KIT^S was required for 50% inhibition of binding of 3.3nM bSLF_γ to c-Kit positive cells. KIT^S-IGc was more efficient at blocking SLF binding requiring only a 12 fold molar excess for 50% inhibition of bSLF_γ binding to membrane bound c-Kit. The relative activities in the ELISA and indirect immunofluorescence assay are compared in Table 4.1. Due to the low activity of the two KIT^S proteins produced by the Baculovirus expression system only KIT^S-IGc was used in subsequent experiments.

Table 4.1: Comparison of the activity of KIT^S-His, KIT^S-IGb and KIT^S-IGc in the sandwich ELISA and the inhibition of bSLF_γ binding to c-Kit expressing cell lines.

	ELISA (sandwich)		IFA: Inhibition of bSLF binding	
	Limit of Detection	50% Detection	50% Inhibition	Maximal Inhibition
KIT^S-His	310ng/ml (5nM)	2µg/ml (32nM)	40µg/ml (645nM)	275µg/ml (4435nM)
KIT^S-IGb	80ng/ml (0.6nM)	600ng/ml (4.2nM)	90µg/ml (642nM)	>350µg/ml (2500nM)
KIT^S-IGc	40ng/ml (0.16nM)	200ng/ml (0.8nM)	10µg/ml (40nM)	>40µg/ml (160nM)

The ability of KIT^S to prevent SLF from inducing a proliferative response in factor dependent cell lines was measured using ³H-thymidine incorporation assays. KIT^S-IGc, at a concentration of 10µg/ml (29 fold molar excess) or 40µg/ml (160 fold molar excess) was unable to inhibit the proliferation of cell lines M07e or FD^{GNNK+}#1 respectively, which express c-Kit on their surface at high levels. However, the proliferation of cell lines expressing low to moderate surface c-Kit levels was retarded by the presence of KIT^S in the culture supernatant. At a concentration of 40µg/ml (160nM), KIT^S-IGc could inhibit the proliferation of TF-1 in response to 3ng/ml (0.16nM) SLF by 60% and could inhibit the proliferation of FD^{GNNK+}#2 cells in response to 25ng/ml (1.4nM) SLF by 80%. Again, this highlights the low affinity KIT^S has for SLF since these figures represent a 1000 and 114 fold molar excess of KIT^S required to achieve this level of inhibition of cellular proliferation. Interestingly, lower levels (10 fold excess) of KIT^S could inhibit, by approximately 50%, the phosphorylation of the receptor expressed by M07e cells during a 2 minute incubation period. Results obtained here were comparable to those of Lev and colleagues (Lev *et al.*, 1992) using a soluble c-Kit molecule produced by transfected CHO cells (KitX). By Scatchard analysis, KitX had a dissociation constant of 0.7nM and only 1nM was required to inhibit 50% of ¹²⁵I-SLF (0.16nM) binding to a c-Kit expressing cell line, representing a 6.25 fold molar excess of KitX. A 100 fold molar excess of KitX prevented receptor transphosphorylation and resulted in a 90% inhibition of PI3'K activation (Lev *et al.*, 1992). This inhibition was measured between 10 and 15 minutes after the addition of SLF and KitX to the cells. The extent of inhibition was dependent upon the amount of SLF used. While 16nM KitX inhibited 0.16nM SLF, 16nM KitX only inhibited the PI3'K signal by 30% when the cells were stimulated with SLF at a concentration of 3.3nM (Lev *et al.*, 1992). Unfortunately, the authors did not investigate the inhibitory properties of KitX over a longer culture period or look at its ability to prevent cellular proliferation, as was the case in the experiments described here. These data suggest there was only a 2 fold difference in SLF binding avidity between KIT^S-IGc and KitX.

However, the KitX used by Lev and colleagues (1992) was monomeric and the inhibition measured in experiments described in this chapter was achieved with dimeric KIT^S. Thus, by comparison to KitX, dimeric KIT^S-IGc might have been expected to be a better inhibitor since

it could bind twice as much SLF. Attempts were made to examine the difference in inhibitory properties of a dimeric and monomeric KIT^S molecule. Both the monomeric, KIT^S-His, and dimeric, KIT^S-IGb, were produced using the Baculovirus expression system. Unfortunately, the majority of the protein produced from the KIT^S-IGb expression construct was found to be monomeric, see Figure 3.11, possibly due to inefficient splicing of the mRNA encoding the KIT^S and Fc domain of human IgG sequences. In both the ELISA and immunofluorescence assay, the KIT^S-IGb protein had a greater activity than the monomeric form. By Western blot, Figure 3.12, some KIT^S-IGb protein was detectable in a dimeric state and it is possible the increased activity over monomeric form was attributable to this small amount of dimeric protein. If this was the case, then the dimeric KIT^S protein had a much greater ability to bind SLF than the monomeric form. KIT^S-IGb activity was inferior to the dimeric protein produced in transfected COS cells, KIT^S-IGc. Initially, the difference in activity was thought to be due to the different expression system, one using insect cells, the other using a mammalian cell expression system, possibly reflecting differences in glycosylation. However, the total protein stain, Figure 3.11, suggests the difference in activity seen was due to the very small amount of dimeric KIT^S present in the KIT^S-IGb preparation.

Surprisingly, concentrations of KIT^S-IGc that were capable of substantially blocking the binding of SLF to c-Kit expressing cells failed to inhibit the proliferation of factor-dependent M07e cells in response to SLF. These cells express very high levels of c-Kit relative to normal CD34⁺ bone marrow cells of patient's AML cells (Cole *et al.*, 1996). However, similar concentrations of KIT^S-IGc did inhibit proliferation of TF-1 cells that express c-Kit at a more physiological level. To explore further the importance of cellular c-Kit level in determining the ability of KIT^S-IGc to inhibit SLF-driven proliferation, two additional cell lines were examined: these had been derived by transducing murine factor-dependent early myeloid FDC-P1 cells with cDNA encoding human c-Kit (Caruana, 1996) and expressed widely differing levels of the receptor. The c-Kit levels expressed by these cells were determined by indirect immunofluorescence assay using 1DC3 as the anti-c-Kit mAb. FD^{GNNK+}#1 displayed a MFI of 103 and FD^{GNNK+}#2 a MFI of 22.6. By comparison M07e had an MFI of 66 and TF-1 had an MFI of 6.9. Analysis of the effect of KIT^S-IGc on the

proliferation of these cell lines in response to SLF confirmed its inhibition in the case of low but not high c-Kit expressing lines.

The possibility that the cells expressing high cell surface receptor may need a lower level of receptor occupancy to give maximal proliferative response was considered as an explanation for this discrepancy. This would mean that more SLF would need to be sequestered by $\text{KIT}^{\text{S}}\text{-IGc}$ to inhibit the proliferation of high c-Kit expressing cell lines than low expressing cell lines. To test this, the relative levels of bSLF required to saturate binding to and proliferation of three cell lines, M07e, TF-1 and $\text{FD}^{\text{GNNK}^+}\#2$, was examined. As shown in Figure 4.11, the levels of bSLF required to saturate cell surface binding and proliferative responses were similar in the case of M07e and TF-1 cells but, surprisingly, substantially more bSLF was required for maximum proliferation than for maximum binding in the case of $\text{FD}^{\text{GNNK}^+}\#2$ cells. These data do not support the simple hypothesis presented above, and other explanations relating to the nature and duration of the assays are considered more likely to account for the proliferation data. For example, the level of SLF may change during the course of the proliferation assay due to its consumption and degradation and this may vary for different cell lines. It is possible that $\text{KIT}^{\text{S}}\text{-IGc}$ may act as a reservoir for SLF in the culture, preventing its degradation and thus masking any inhibitory effect. Attempts to measure the amount of SLF at the end of the culture failed to provide consistent data. Interestingly, although $\text{KIT}^{\text{S}}\text{-IGc}$ was not effective in inhibiting M07e proliferation, it did substantially block cellular c-Kit tyrosine phosphorylation in a short-term assay (Figure 4.12).

As discussed in section 4.6 and 1.3.2 several studies have shown the importance of the interaction of c-Kit with SLF to provide adhesion between the c-Kit expressing cell and its microenvironment. It was therefore possible KIT^{S} could prevent the adhesion of c-Kit expressing cells, M07e, to CHO cells transfected with the cDNA encoding SLF. Figure 4.13 illustrates that $\text{KIT}^{\text{S}}\text{-IGc}$, at a concentration range of 20 to 80 $\mu\text{g/ml}$, was unable to prevent M07e cells from adhering to SLF expressed by CHO cells. Adhesion of the low c-Kit expressing cell line TF-1 to CHO^{SLF} cells could not be detected in this assay. It was previously noted that $\text{KIT}^{\text{S}}\text{-IGc}$ was not very effective at binding to CHO^{SLF} cells (Figure 4.2) possibly accounting for its inability to block the adhesion of M07e to the CHO^{SLF} cells. By comparison, $\text{KIT}^{\text{S}}\text{-IGc}$, at 10 $\mu\text{g/ml}$, was able to block the binding of soluble bSLF to M07e

cells by more than 50%. It is possible the fixed structure of KIT^S-IGc, i.e. permanent dimerisation of the KIT^S molecule, reduced the interaction of KIT^S with membrane bound, possibly dimerised SLF (Tajima *et al.*, 1998) and that these SLF dimers might be different from those formed in solution.

5. Biology of naturally occurring KIT^S

5.1 Introduction

As discussed in chapter 1, it has been shown that soluble c-Kit exists naturally and can be detected in human serum at an average concentration of approximately 320ng/ml (Wypich *et al.*, 1995). It is also detectable in the culture supernatant of c-Kit expressing human haemopoietic cell lines and cultured endothelial cells (Broudy *et al.*, 1994; Kawakita *et al.*, 1995; Turner *et al.*, 1995). In patients with haematological diseases, the amount of KIT^S present in the serum differs depending upon the disease. Compared to normal levels, patients with lymphoid malignancies showed a decrease in serum KIT^S level. Patients with chronic phase CML showed a wide range of KIT^S levels while patients with acute disease had greatly elevated levels although one patient with lymphoid blast crisis had lower serum KIT^S levels (Kawakita *et al.*, 1995). In AML, the level of KIT^S in the serum correlated with phenotype of the disease. Patients with leukaemic blasts of an immature phenotype had higher levels of KIT^S in their serum than those with more mature phenotypes. The concentration of KIT^S in serum decreased upon chemotherapy and this reduction correlated to a decrease in leukaemic cell number (Kawakita *et al.*, 1995). KIT^S levels in serum were significantly lower in patients with moderate to severe acute graft versus host disease (Hashino *et al.*, 1995) or in patients with delayed engraftment following bone marrow transplantation.

The release of KIT^S into the culture supernatant or serum appears to be due to proteolytic cleavage of the cell surface receptor as a mRNA transcript encoding a soluble form of the receptor has not been identified. Indeed, proteolytic cleavage and release of the extracellular domain of c-Kit after phorbol ester stimulation has been demonstrated for murine (Yee *et al.*, 1993) and human c-Kit (Brizzi *et al.*, 1994). It has been suggested that the alternatively spliced four amino acid insert or deletion of the c-Kit (Reith *et al.*, 1991; Vandebark *et al.*, 1992) may partly encode the proteolytic cleavage site but at present there is no published evidence for this (Wypich *et al.*, 1995). This hypothesis appears based on the observation that PKC has been shown activate specific cleavage of the CSF-1 receptor, a molecule related to c-Kit, at a site just before the transmembrane domain (Downing *et al.*, 1989). Purified, serum derived soluble c-Kit, which is believed to be monomeric, appeared to have a 7 fold lower affinity for SLF (6nM), determined by 50% competitive inhibition of ¹²⁵I-SLF binding to

membrane bound c-Kit (Wypich *et al.*, 1995), than recombinant KIT^S (0.7nM) (Lev *et al.*, 1992) and full length cell-associated human and murine c-Kit (1nM) (Flanagan and Leder, 1990; Huang *et al.*, 1990) although this was not directly compared and experimental differences cannot be excluded. Recently, it has been reported that bacterially expressed, recombinant SLF is predominantly monomeric at *in vivo* concentrations (Hsu *et al.*, 1997) and thus, despite the apparent decreased affinity of serum KIT^S for soluble SLF, the high concentrations of KIT^S in human serum suggests it may effectively bind circulating SLF. The experiments of Hsu *et al.*, (1997) were carried out using bacterially expressed SLF, which therefore would be unglycosylated, and serum or transfected CHO cell derived SLF is glycosylated. However, it has been demonstrated that glycosylation of asparagine residues in the binding core of SLF actually interferes with SLF dimerisation and decreases its affinity for c-Kit (Philo *et al.*, 1996; Zhang *et al.*, 2000).

While purified KIT^S from serum has been shown to interact with recombinant SLF, the association of KIT^S and SLF in human serum has not been demonstrated experimentally. It is important to address this question with respect to the likely function of KIT^S and its potential use as a therapeutic agent. If KIT^S in serum does associate with endogenous SLF then the likelihood of additional KIT^S being of benefit is small. However, monovalent recombinant KIT^S was found to have a lower avidity for SLF than a divalent form (Liu *et al.*, 1993; results in chapter 4) and thus recombinant dimeric KIT^S, see chapter 4, may be more effective than the preexisting monomeric form, removing the free SLF available in serum. In this chapter experiments were carried out to try to determine whether SLF in serum is complexed with KIT^S.

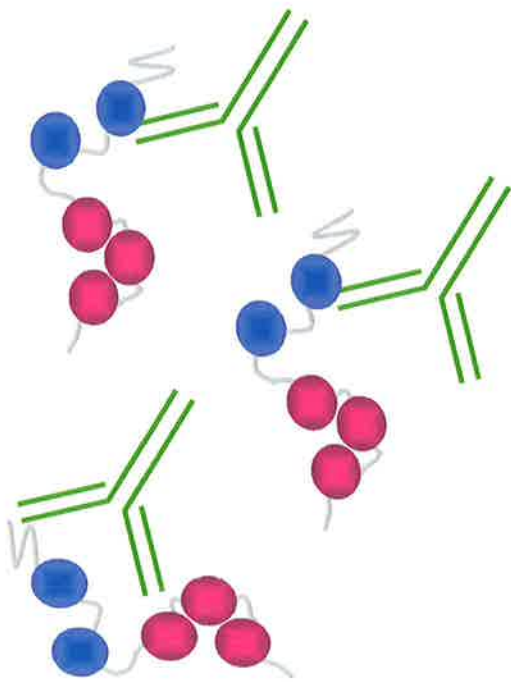
Blood progenitor cell mobilisation from discrete microenvironments within the bone marrow to the peripheral blood involves the modulation of progenitor cell:BM stroma interaction, migration to the marrow sinuses and egress through the basement membrane and the endothelial layer. While the mechanisms involved in this movement of progenitor cells are not fully understood, modulation of the interaction of c-Kit expressed by the progenitor cells and SLF expressed by the stroma appears to be required for the egress into the blood stream to occur (reviewed in To *et al.*, 1997). The importance of this interaction is highlighted by several clinical trials. G-CSF was shown to be far less efficient at mobilising

progenitors from either *Sl* or *W* mice, which have defects in the production of SLF or receptor activity respectively, than wild-type mice, suggesting that the actions of G-CSF in mobilisation may involve endogenous SLF as well (Cynshi *et al.*, 1991). Indeed, it has been shown in mice and baboons that a combination of G-CSF and SLF is more efficient at the mobilisation of long term reconstituting cells than G-CSF alone (Briddell *et al.*, 1993; Andrews *et al.*, 1994). In addition, mobilisation in mice has also been achieved with blocking antibodies to the $\beta 1$ integrin VLA-4 or its cellular ligand, VCAM, and this process requires functional c-Kit expression, indicating integrin/receptor cross talk (Papayannopoulou *et al.*, 1998). Flow cytometric analysis of mobilised CD34⁺ progenitor cells revealed markedly reduced expression levels of c-Kit on these cells compared with the levels expressed by steady state bone marrow and peripheral blood CD34⁺ cells (Simmons *et al.*, 1994; To *et al.*, 1994). This downregulation of c-Kit expression was observed with all six mobilisation protocols studied and the extent of c-Kit downregulation correlated with progenitor cell yield. The mechanism of the observed down regulation is not known. It is possible that it is due to internalisation of the receptor or down regulation of mRNA and hence protein expression. The other possibility is that the extracellular domain is shed into the extracellular environment. Since clinical trials of new chemotherapy and mobilisation regimes have been conducted at the Hanson Centre for Cancer Research, serum samples from patients treated with progenitor cell mobilising agents were available to examine whether the down regulation of c-Kit by mobilised cells might be due to receptor shedding. The second part of this chapter details experiments examining this possibility.

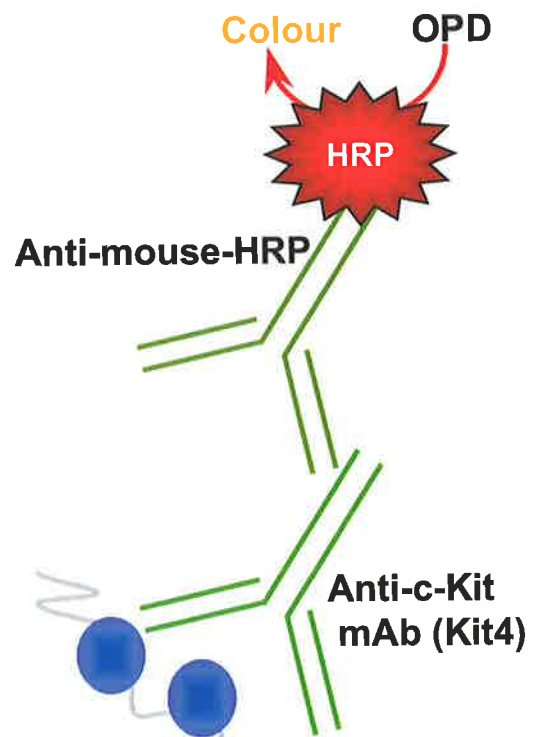
5.2 Competitive ELISA

While the sandwich ELISA allowed the detection of KIT^S in culture supernatants it was decided that this method was neither sensitive enough nor accurately quantitative for the experiments proposed above. A competitive ELISA was therefore established. This system relied on KIT^S in solution binding to an anti-c-Kit mAb preventing the antibody from binding to immobilised KIT^S (Figure 5.1). Known concentrations of purified KIT^S in solution were used to establish a standard curve and unknown KIT^S sample concentrations were determined using this standard curve. Briefly, wells of a 96 well plate were coated with 1 μ g/ml of KIT^S

Figure 5.1: Diagrammatic representation of the competitive ELISA technique. Wells of a 96 well plate were coated with KIT^S, blocked and a solution of KIT^S and anti-c-Kit mAb Kit4 added. The binding of Kit4 to the immobilised KIT^S was detected using an anti-mouse mAb coupled to horseradish peroxidase. Altered substrate colour was detected using Bio-Rad microplate reader. Unknown KIT^S concentrations were determined by comparison to a standard curve generated by KIT^S of known concentration.



KIT^S bound to well



KIT^S bound to well

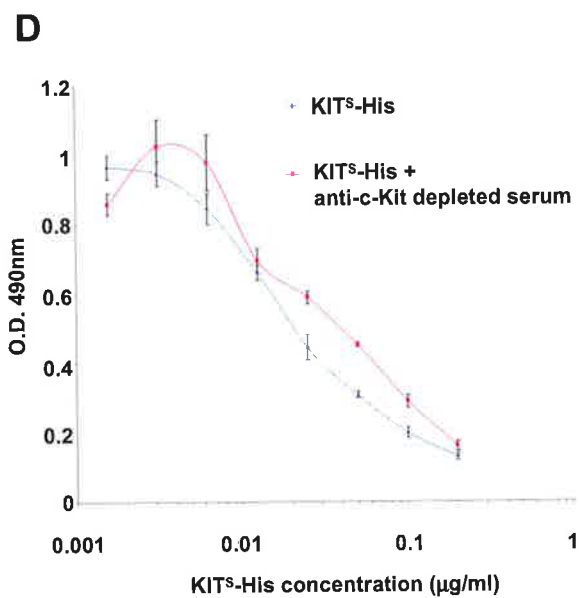
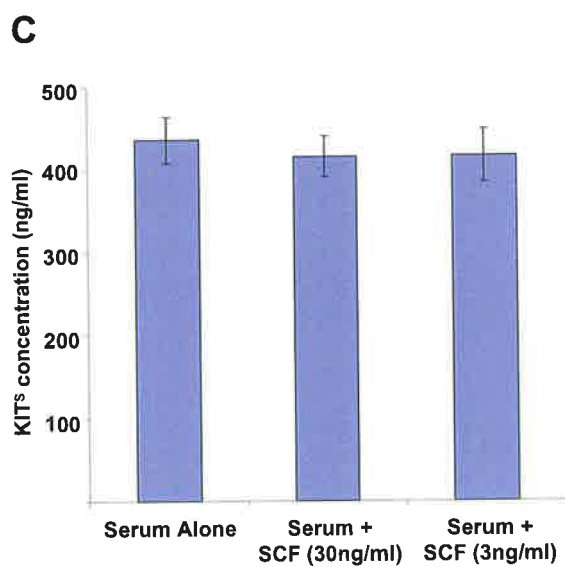
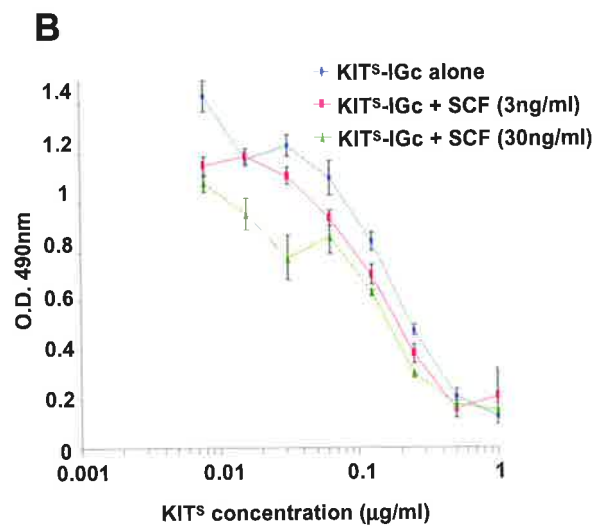
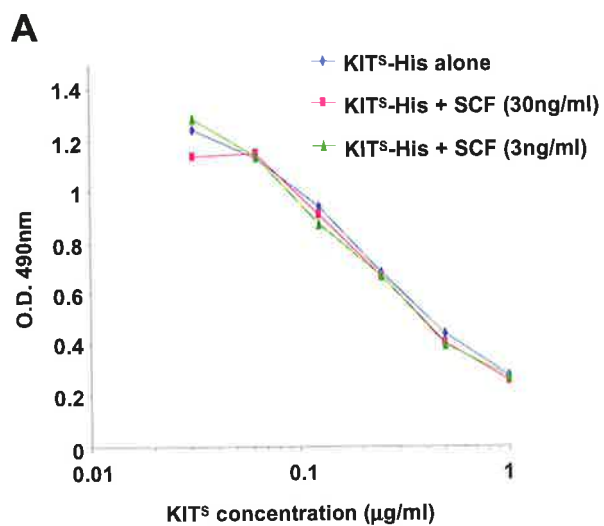


for 2 hours at room temperature. The wells were then washed and blocked overnight with 1% BSA in PBS, pH 8.0. The KIT^S sample to be analysed was incubated with 60ng/ml of an anti-c-Kit mAb, Kit4, for 2 hours prior being added to the coated plate for 60 mins. The concentrations for the coating KIT^S and the anti-c-Kit mAb had been determined by titration and these concentrations resulted in a signal that was 70% of the maximum. Kit4 binding was detected using an anti-mouse IgG coupled to horseradish peroxidase followed by the addition of the substrate o-phenylenediamine dihydrochloride (OPD). The competitive ELISA was slightly more sensitive than the sandwich ELISA able to detect 20-30ng/ml of KIT^S-IGc compared to 40-50ng/ml using the sandwich assay.

Since this ELISA was to be used to measure the concentration of KIT^S in serum samples it was important to show that SLF in serum or other serum components did not interfere with the interaction of the competitive ELISA components. Results obtained with either recombinant monomeric KIT^S (Figure 5.2 A) or serum samples (Figure 5.2 C) were not altered by the presence of bSLF_γ at either 3ng/ml (the level detected in normal serum) or 30ng/ml. However, in chapter 4 it had been shown that KIT^S-His did not have a high affinity for SLF_β. The addition of bSLF_γ to the ELISA using KIT^S-IGc as competitor appeared to result in a slight decrease in the signal detected (B) but this was not related to the concentration of SLF. Since the KIT^S found in serum is monomeric, KIT^S-His was used as the competitive molecule. Similarly, the addition of bSLF_γ at concentrations as high as 4μg/ml did not affect the quantitation of human serum derived or CHO cell derived soluble c-Kit molecule (Wypich *et al.*, 1995). To determine if other serum components interfered with the competitive ELISA, normal serum was immuno-depleted of KIT^S using an anti-c-Kit mAb, Kit4, coupled to Sepharose. A known concentration of recombinant KIT^S, KIT^S-His, was then added and serially diluted in the KIT^S depleted serum. The signal generated by KIT^S-His in the mAb depleted serum was then compared to the signal detected with the same batch of KIT^S-His serially diluted in PBS (Figure 5.2 D). The difference in signal detected was minimal and the slight increase in signal when the depleted serum was used can be accounted for by a trace of Kit4 mAb leaking from the Sepharose beads since the competitive ELISA measures the amount of Kit4 binding to immobilised KIT^S. Using the competitive ELISA, the serum KIT^S concentration was determined from 12 normal donors. The median

Figure 5.2: Influence of SLF on the detection of KIT^S by ELISA. Monomeric KIT^S-His, dimeric KIT^S-IGc or human serum were titrated in a competitive ELISA alone or in the presence of SLF at 3ng/ml or 30ng/ml. **A:** KIT^S-His titration, **B:** KIT^S-IGc titration, **C:** KIT^S serum concentration of one normal human donor, derived using the competitive ELISA assay, alone or in the presence of SLF at 3ng/ml or 30ng/ml, **D:** KIT^S-His was titrated in a competitive ELISA using PBS or human serum depleted of KIT^S as diluent. The serum was depleted of KIT^S by incubation overnight with anti-c-Kit mAb Kit4 in the presence of Protein A Sepharose.

Data represented as the average \pm S.E.M.



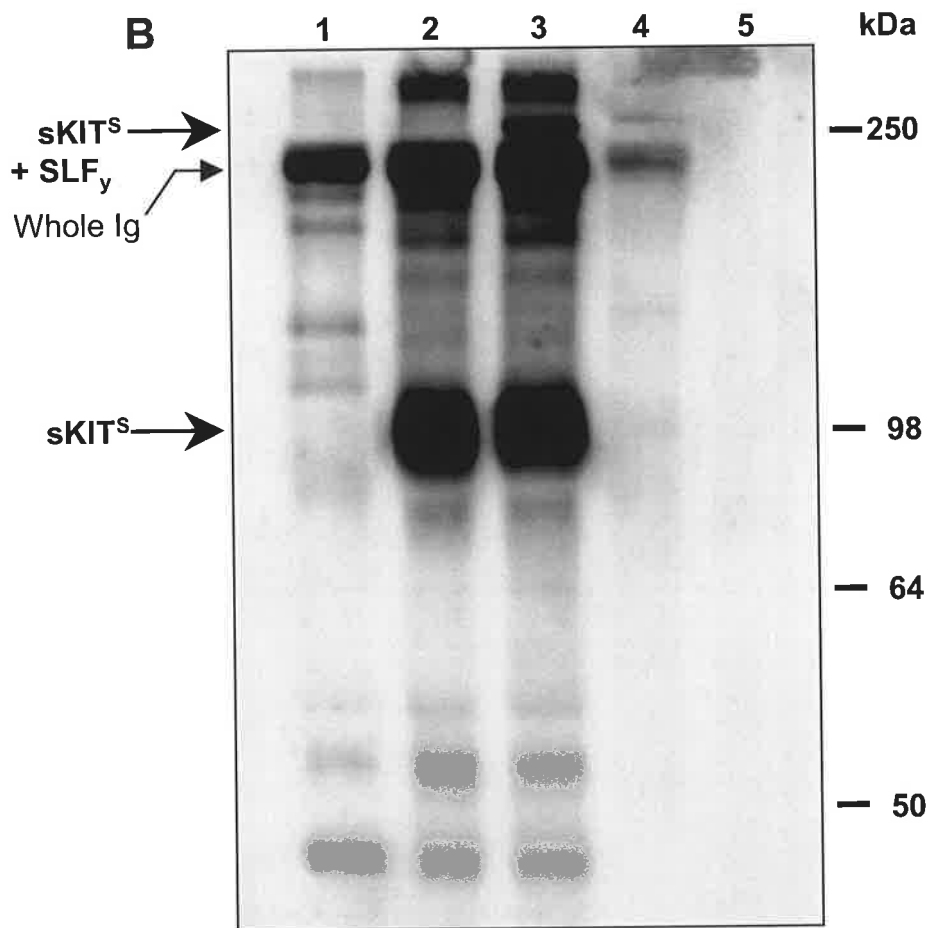
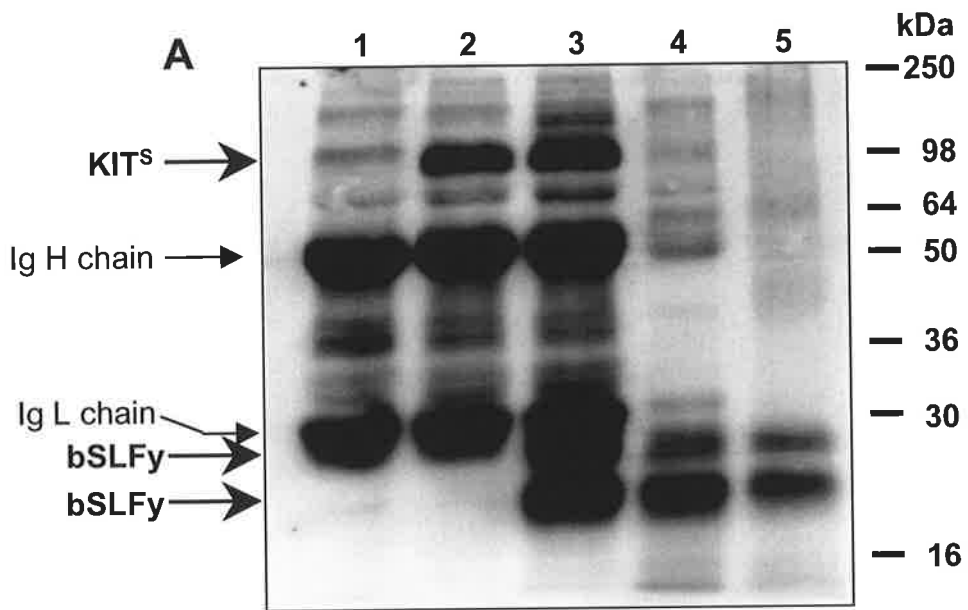
concentration was determined to be 365ng/ml and the concentrations ranged from 164ng/ml to 1110ng/ml. The concentration of KIT^S in these samples, except one, fell within the normal range (163-788ng/ml) published by Wypich *et al.* (1995).

5.3 Interaction of serum KIT^S with SLF

Initial experiments attempted to detect SLF in normal human serum by immunoprecipitation using the anti-SLF mAb 7H6 (Turner *et al.*, 1992; Langley *et al.*, 1993) and then Western blot using 7H6 also. 7H6 has been shown to both immunoprecipitate and Western blot SLF (Zannettino *et al.*, 1997). The average concentration of SLF in human serum was determined to be 3.3ng/ml (Langley *et al.*, 1993). Using 7H6, the limit of detection of recombinant SLF by Western blot was approximately 0.78ng (data not shown). However, it was not possible to detect SLF in 1ml of serum by immunoprecipitation and Western blot using 7H6 (data not shown). Thus it was not possible to determine whether endogenous SLF is associated with KIT^S in serum. To circumvent this problem, biotinylated SLF (the SLF was expressed in yeast), bSLF_y, was added to normal human serum. This serum was incubated with either an anti-c-Kit mAb, or an isotype matched negative control mAb directly coupled to Sepharose. After washing, the immunoprecipitated molecules were boiled in reducing gel loading buffer, separated by electrophoresis and transferred to PVDF. KIT^S was detected with anti-c-Kit mAb, 1C1.HF, and bSLF_y was detected with streptavidin coupled to alkaline phosphatase. Figure 5.3(A) shows the presence of immunoprecipitated serum KIT^S bands in lanes 2 and 3. KIT^S was not immunoprecipitated with the isotype matched negative control mAb, lane 1. Small streptavidin interacting proteins were detected in lane 3. Lane 3 contained the anti-c-Kit immunoprecipitated material from serum to which bSLF_y had been added. These bands were not seen in lane 2 which did not contain bSLF_y. Similarly, bSLF_y and a small amount serum KIT^S were co-immunoprecipitated using streptavidin coupled to agarose beads (Figure 5.3 (A), lane 4). No band in the position of the co-immunoprecipitating KIT^S was seen in lane 5 in which the serum had been replaced with PBS. The serum used in the experiment had been precleared with streptavidin coupled to agarose beads so it is unlikely that the band co-immunoprecipitating with bSLF_y in lane 4 was an irrelevant serum-derived protein. Thus, although complexes of endogenous SLF with KIT^S

Figure 5.3: Immunoprecipitation and Western Blot of serum KIT^S with or without bSLF_y. 1ml of normal human serum was incubated with either an anti-c-Kit mAb, Kit4, or isotype matched negative control mAb, indirectly coupled to Protein-A-Sepharose, lanes 1, 2 and 3, or streptavidin coupled to agarose beads, lanes 4 and 5, with or without the addition of bSLF_y (300ng) as indicated. Immunoprecipitated proteins were separated on a polyacrylamide gel and transferred to PVDF. The blot was probed with an anti-c-Kit mAb, 1C1.HF, and streptavidin coupled to alkaline phosphatase, and the presence of 1C1.HF binding was detected with anti-mouse Ig coupled to alkaline phosphatase. Immunoreactive proteins were detected with ECF substrate and scanned using a Fluorimager 595 (Molecular Dynamics) (section 2.8). **A:** 12% polyacrylamide gel run under reducing conditions. **B:** 8% polyacrylamide gel run under non-reducing conditions. Serum KIT^S (sKIT^S) and bSLF_y are indicated by arrows. **Lane 1:** human serum + negative control mAb + bSLF_y, **Lane 2:** human serum + Kit4, **Lane 3:** human serum + Kit4+ bSLF_y, **Lane 4:** human serum + bSLF_y + streptavidin coupled to agarose beads, **Lane 5:** bSLF_y, in PBS + streptavidin coupled to agarose beads.

Serum was precleared by incubation overnight with streptavidin coupled to agarose beads or isotype matched negative control mAb coupled to Sepharose.



could not be demonstrated these results demonstrate that the KIT^S in serum is indeed able to interact with bSLF_y suggesting it is also capable of interacting with the circulating SLF in human serum.

Although it has been reported that SLF in serum is likely to be in a monomeric form and that KIT^S also circulates as a monomer, the possibility existed that the binding of KIT^S to SLF in serum could result in the production of more stable, higher order structures involving two KIT^S and two SLF molecules. If this structure does exist then the possibility of recombinant dimeric KIT^S being a more effective competitor of SLF binding to membrane c-Kit is less likely. In Figure 5.3 (B) lane 3 of an identical gel to that in Figure 5.3 (A) but that had been run under non-reducing conditions, a band can be clearly seen at an approximate molecular weight of 260kDa. This band was not detected when either bSLF_y or soluble c-Kit from normal serum, were absent (lanes 2 and 5 respectively). This molecular weight corresponds to the weight of 2 soluble c-Kit and 2 SLF molecules complexed together. Interestingly, the majority of the KIT^S immunoprecipitated from the serum was not associated with the higher order structure (lane 3). A band is also visible at an approximate molecular weight of 120kDa in lane 3 of Figure 5.3 (A). This is possibly a heterocomplex of 1 KIT^S molecule and 1 SLF molecule. From these results it appears that the KIT^S in serum is capable of interacting with SLF and that heterocomplexes containing two molecules of each can form.

5.4 Shedding of KIT^S from cell surface

The lack of an alternately spliced mRNA transcript encoding only the extracellular domain of c-Kit and the results of pulse-chase experiments, detecting the accumulation of extracellular protein only after the full length molecule is synthesised, suggest the soluble c-Kit detected in culture supernatants of c-Kit positive cell lines and in human serum is derived by proteolytic cleavage of the full length molecule (Turner *et al.*, 1995; Wypich *et al.*, 1995). The alternate splicing of c-Kit mRNA results in the insertion or deletion of four amino acids, GNNK, in the extracellular, juxtamembrane region of c-Kit. This site appears to lie close to the proteolytic cleavage site that results in the production of KIT^S and it has been suggested that the proteolytic cleavage site may include this sequence (Wypich *et al.*, 1995). In an attempt to determine whether the proteolytic cleavage site includes the GNNK sequence,

the culture supernatants from transfected FDC-P1 cells expressing either the GNNK⁺ (FD^{GNNK⁺}) isoform or the GNNK⁻ (FD^{GNNK⁻}) isoform of human c-Kit were collected, concentrated and analysed for KIT^S presence by competitive ELISA. As a control the culture supernatants from c-Kit expressing cell lines M07e and TF-1 were also collected. Nine FD^{GNNK⁺} clones and six FD^{GNNK⁻} clones were examined for KIT^S production. These clones expressed cell surface c-Kit at various levels as measured by indirect immunofluorescence. Relative mean fluorescence intensity levels ranged from 49-268 for the FD^{GNNK⁺} clones and 64-223 for the FD^{GNNK⁻} clones. Cultures of the cells were established at 1×10^5 cells/ml and allowed to proliferate until cell densities of approximately 1×10^6 cell/ml were achieved. For M07e and TF-1 cell lines this took approximately 96 hours, whereas the FDC-P1 cells achieved this cell density in 48 hours. Culture supernatants, approximately 40ml, were cleared of cells and debris by centrifugation and concentrated to approximately 1ml, i.e. 30-50 fold concentration. The amount of KIT^S in the concentrated supernatants was determined by competitive ELISA, section 5.2, using monomeric recombinant KIT^S-His as the standard. The results obtained are displayed in Figure 5.4. Since the supernatants were obtained from cultures at slightly different cellular densities and were concentrated to slightly different extents, the results were standardised by expressing the results as the amount of KIT^S per 10^6 cells at the time of harvesting. The results should not be interpreted as the amount of KIT^S produced by 10^6 cells as both KIT^S concentration and cellular density increased during the culture period. More KIT^S was produced by M07e cells than TF-1 cells, 4ng/ 10^6 cells and 0.8ng/ 10^6 cells respectively. This result was not surprising since M07e cells display more c-KIT^S on their surface, refer to Figure 4.6. The value obtained for M07e was a little lower than reported by Turner *et al.* (1995), 34.1ng/ml, but from a culture density of 3.2×10^6 /ml, equating to 10.6ng/ 10^6 cells. While the trend appeared to be that FD^{GNNK⁺} cells produce more KIT^S/ 10^6 cells than FD^{GNNK⁻} cells, more clones, which are not available, would need to be studied to achieve statistical significance.

Although a molecule reactive with anti-c-Kit mAb was detected in the supernatant of c-Kit expressing cells it could not be ruled out that this immunoreactive molecule was intact full length c-Kit protein, released into the culture supernatant because of cell lysis. Despite high culture viability some dead cells were detected, and it is likely that during the course of

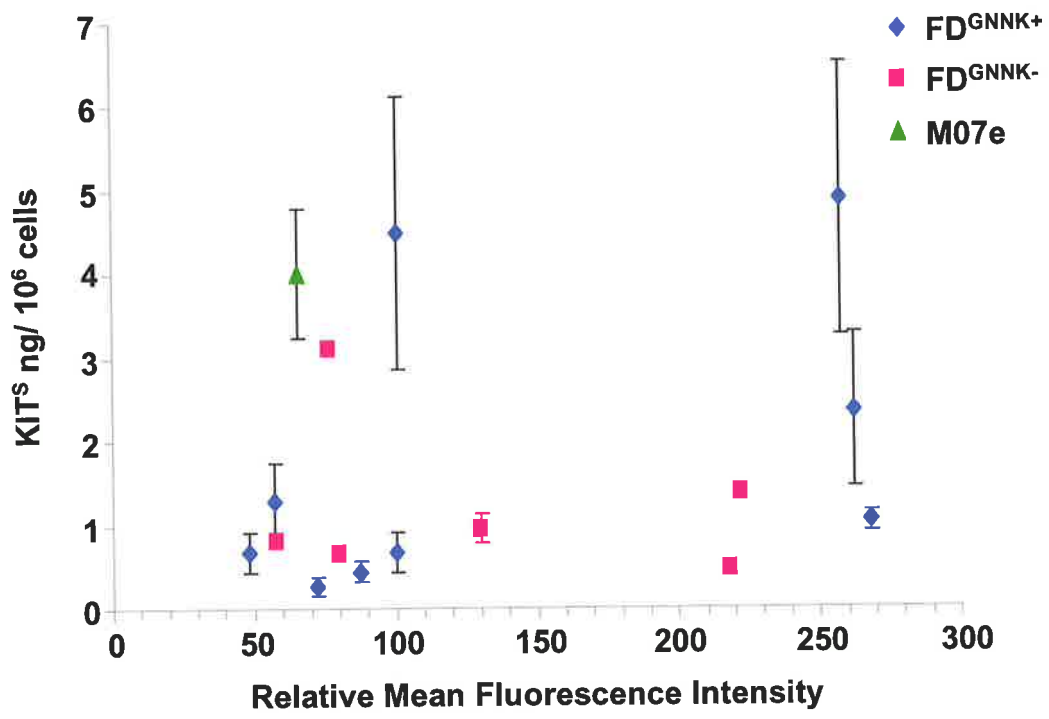
Figure 5.4: Comparison of KIT^S production by c-Kit expressing cell lines. The amount of KIT^S produced was determined by competitive ELISA using concentrated culture supernatants (section 2.2.8). Surface expression level of c-Kit on each of the cell lines was determined by indirect immunofluorescence assay (section 2.2.6). Sample points are depicted as the average of triplicate samples \pm the S.E.M.

FD^{GNNK+}: Spearman Rank correlation coefficient: $R^2=0.22$, $p>0.05$, 1-tailed test

FD^{GNNK-}: Spearman Rank correlation coefficient: $R^2=0.096$, $p>0.05$, 1-tailed test

Therefore no significance between relative mean fluorescence intensity and KIT^S produced/
 10^6 cells

To determine if there was a significant difference in KIT^S production by the two c-Kit isoforms samples were subjected to a Mann-Whitney U test for small samples. $p>0.01$, 2-tailed test, ranking FD^{GNNK+} vrs FD^{GNNK-}.

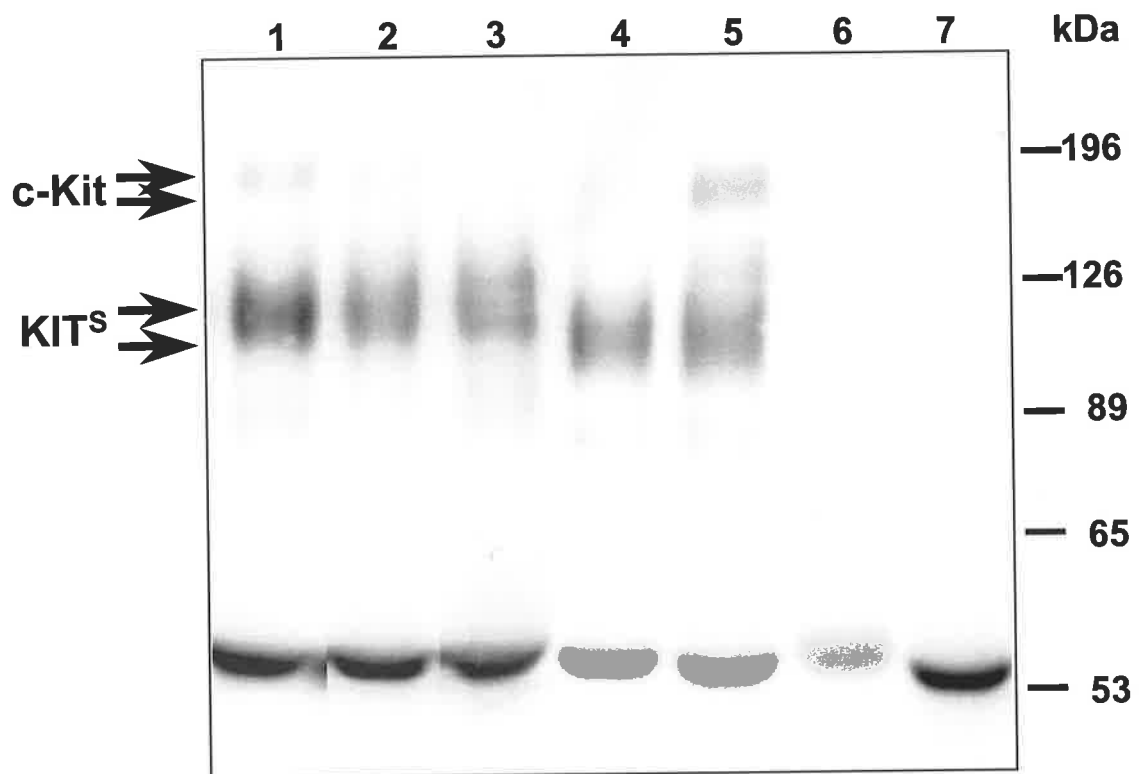


proliferation over 48-96 hours, some cell death occurred. Therefore, 750 μ l aliquots of approximately 40 fold concentrated supernatant from M07e, and FDC-P1 clones FD^{GNNK+}#2 and FD^{GNNK-}#1 and #2 were incubated overnight with Protein A coupled to Sepharose and an anti-c-Kit mAb, Kit4. Immunoprecipitated proteins were resolved by SDS-PAGE under reducing conditions and transferred to PVDF. Figure 5.5 shows the result of probing the blot with an anti-c-Kit mAb. A anti-c-Kit reactive protein of approximately 100-120kDa was detected in lanes 1-5, which had all been immunoprecipitated with Kit4. Lanes 1-3 contained proteins immunoprecipitated from supernatants of the transfected FDC-P1 c-Kit cell lines; and lanes 4 and 5 were derived from M07e culture supernatants. The absence of a band from M07e supernatant immunoprecipitated with isotype matched negative control mAb, 3D3, lane 6, and supernatant from empty vector only control transfected FDC-P1 cells, lane 7, confirmed the specificity of the immunoprecipitation. A larger molecular weight band of approximately 145kDa was detected in lanes 1, 2 and 5. This was believed to be full length c-Kit, originating from lysed cells. To confirm this M07e cells were cultured at a density of 1x10⁶ cells/ml for 24 hours in the absence of factor, which resulted in a decrease in cell viability down to 51%. The immunoprecipitated protein from this culture was loaded onto lane 5. A full length c-Kit protein band is present in lane 5 that was not seen in lane 4, which contains the immunoprecipitated protein from a 92% viable M07e culture, confirming the full length c-Kit protein band detected in lanes 1,2 and 5 originated from lysed cells. Surprisingly, both the KIT^S and full length receptor expressed by the transfected FDC-P1 cells were of a higher molecular weight than those produced by M07e cells. This probably reflects variations in glycosylation by the different cell lines. This Western blot result verifies that the c-Kit detected by ELISA in the culture supernatant of c-Kit expressing cell lines was indeed KIT^S.

5.5 Shedding of KIT^S from stimulated cells

As discussed in section 1.10.1, stimulation of c-Kit expressing bone marrow derived mast cells, M07e and TF-1 cells with phorbol ester resulted in the release of the extracellular domain of c-Kit into the culture supernatant (Yee *et al.*, 1993; Brizzi *et al.*, 1994). All of these cells express both the GNNK+ and GNNK- isoforms of c-Kit. It was possible however, that only one of these isoforms was capable of giving rise to the extracellular domain of c-Kit

Figure 5.5: Immunoprecipitation and Western blot of KIT^S from c-Kit expressing cell lines. 750µl of 40x concentrated culture supernatant was incubated overnight with 5µg of mAb Kit4 and Protein A coupled to Sepharose beads. Immunoprecipitated protein was separated on an 8% reduced polyacrylamide gel and transferred to PVDF. The blot was probed with an anti-c-Kit mAb, 1C1.HF, and the presence of 1C1.HF binding was detected with anti-mouse Ig coupled to alkaline phosphatase. Immunoreactive proteins were detected with ECF substrate and scanned using a Fluorimager 595 (Molecular Dynamics) (section 2.7). **Lane 1:** FD^{GNNK⁺}#2, **Lane 2:** FD^{GNNK⁻}#1, **Lane 3:** FD^{GNNK⁻}#2, **Lane 4:** M07e, **Lane 5:** M07e (51% viability), **Lane 6:** M07e + negative control mAb, 3D3, **Lane 7:** FDC-P1 cells transfected with vector only.



upon stimulation. Therefore, clones of FDC-P1 cells transfected with either isoform of human c-Kit, FD^{GNNK+} and FD^{GNNK-} cells (generated by Dr G. Caruana) were stimulated with SLF_b (100ng/ml), FBS (40%) or phorbol ester (PMA) (200ng/ml and 10ng/ml) and released KIT^S levels in the culture supernatant measured. These clones were determined to have equivalent surface c-Kit expression by indirect immunofluorescence, data not shown. Figure 5.6 depicts the results. Incubation of the cells alone for 30 min at 37°C resulted in some KIT^S being released into the supernatant. Neither SLF_b or FBS resulted in KIT^S production by FD^{GNNK+} cells but some KIT^S was released by FD^{GNNK-} cells with SLF_b stimulation. The most dramatic response was seen when the c-Kit expressing cells were stimulated with phorbol ester. Both FD^{GNNK+} and FD^{GNNK-} cells released KIT^S into the supernatant upon PMA stimulation and the amount released was dependent upon the amount of PMA used to stimulate the cells. Irrespective of the treatment, the FD^{GNNK-} cells produced more KIT^S than the FD^{GNNK+} cells. In similar experiments, Yee *et al.* (1993) showed surface expression of c-Kit on bone marrow derived mast cells was decreased to 50% after incubation with PMA for 30 minutes and this correlated with the detection of the extracellular domain of c-Kit in the culture supernatant. The results, together with those in section 5.4, clearly demonstrate that both isoforms of c-Kit can give rise to KIT^S.

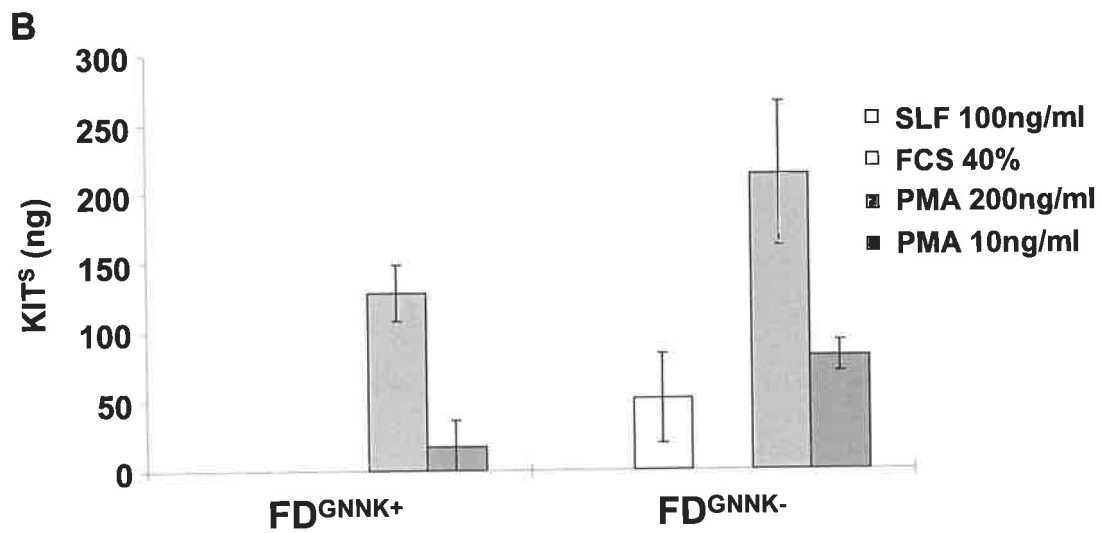
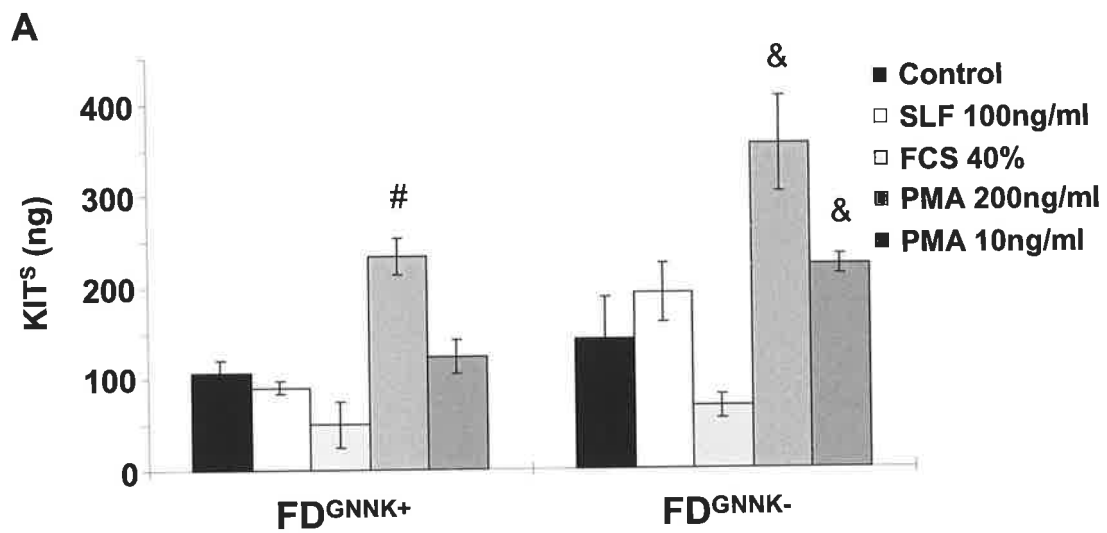
5.6 KIT^S levels in serum of patients treated with cytokines

Modulation of the interaction of c-Kit expressed by haemopoietic progenitor cells and SLF expressed by stromal cells appears to be required for the egress of the progenitors into the blood stream (reviewed in To *et al.*, 1997). G-CSF has been shown to be less efficient at mobilising the progenitor cells from *Sl* or *W* mice compared to wild-type mice and mobilisation using blocking antibodies to VLA-4 required functional c-Kit expression also (Cynshi *et al.*, 1991; Papayannopoulou *et al.*, 1998) indicating a key role for the SLF/c-Kit interaction in mobilisation. Clinical trials conducted at the Hanson Centre for Cancer Research examined the effectiveness of either G-CSF alone or G-CSF and SLF together to mobilise CD34⁺ stem and progenitor cells from the bone marrow into the peripheral blood. The patients recruited to the trial were breast cancer patients undergoing stem cell mobilisation prior to the administration of high dose chemotherapy. During the process of the

Figure 5.6: KIT^S production by stimulated of FD^{GNNK+} and FD^{GNNK-} cells. Cells (10⁷) were stimulated with SLF (100ng/ml), FBS (40%), PMA (200ng/ml) or PMA (10ng/ml) for 30 mins at 37°C. The cells were pelleted and the supernatant analysed for the presence of KIT^S by competitive ELISA.

A: Amount of KIT^S produced by each treatment. #: p<0.01, &: p<0.0005, compared to “control”, 1-tailed Student t-test, 2 sample equal variance.

B: Change in KIT^S production above the control. Data are represented as the average of triplicate samples ± S.E.M.



clinical trials serum samples from the patients were collected. Patients #1-7 were treated with 5µg/kg of G-CSF/day for 6 or 7 days. Patients #8-10 were treated with 10µg/kg of SLF/day for 10 days and 12µg/kg G-CSF for 6 days starting at day 3 of SLF administration. Since one of the possible ways of down regulation of the surface c-Kit levels is shedding of the extracellular, ligand-binding domain, serum samples from these patients were analysed, by competitive ELISA, for KIT^S levels during the course of the mobilisation regime. It should be noted that variations in serum SLF levels do not influence this assay (Figure 5.2C)

In a pilot experiment serum samples taken at days 0, 3 and 6 from the patients treated with G-CSF alone were assayed for KIT^S levels and the results are presented in Figure 5.7. KIT^S levels of patients #1, 3, 5 and 7 increased at day 3 but returned to starting levels at day 6. KIT^S levels dropped at day 3 for patient #2 but returned to starting levels at day 6. A slight increase in KIT^S concentration was seen for both patient #4 and 6 over the time course. However, a consistent alteration of KIT^S levels was not seen over the period of cytokine administration.

Samples taken from four patients at daily intervals over the complete time course of peripheral CD34⁺ cell harvest were available for assay. Since surface expression of c-Kit is downregulated on mobilised CD34⁺ cells, it was postulated that the serum KIT^S levels might increase during the course of mobilisation of the CD34⁺ progenitors from the bone marrow into the peripheral blood. While the number of CD34⁺ cells detected in the peripheral blood of cytokine treated patients increased dramatically during the course of treatment, no significant change in the KIT^S level was seen over the same period (Figure 5.8). All of the serum KIT^S levels measured in these assays were within the range defined by other groups (Wypich *et al.*, 1995).

5.7 Discussion

In this chapter some of the physical characteristics of naturally occurring KIT^S were examined. Attempts were made to detect the interaction of KIT^S and endogenous SLF in serum, but techniques available were not sensitive enough to detect the low level of SLF (3ng/ml) present in serum. However, addition of labelled bSLF_y to human serum enabled the demonstration that KIT^S in serum is capable of interacting with SLF. Using an anti-c-Kit

Figure 5.7: Serum KIT^S levels from patients treated with G-CSF for 6 days. Breast cancer patients were treated with G-CSF at 5µg/kg for 6 days. Serum samples taken on days 0, 3 and 6 of treatment were analysed for KIT^S levels by competitive ELISA using monomeric KIT^S-His as a standard.

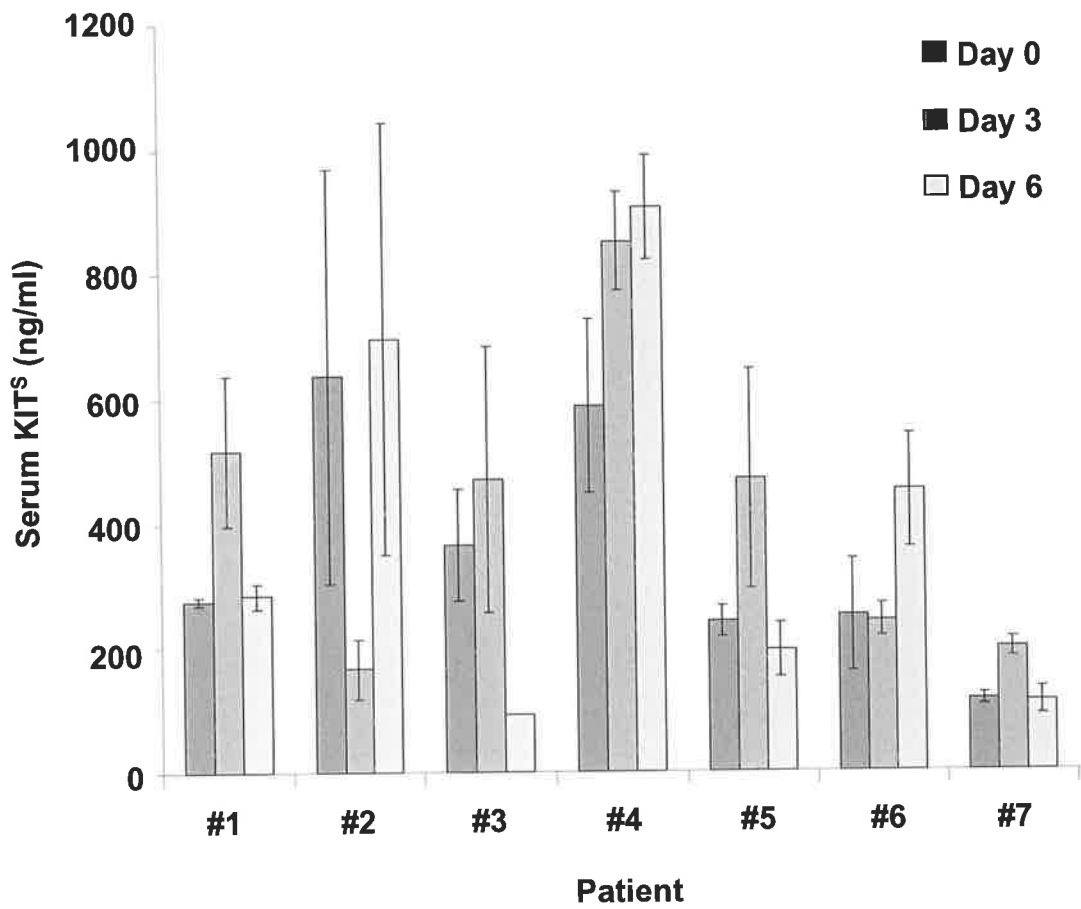
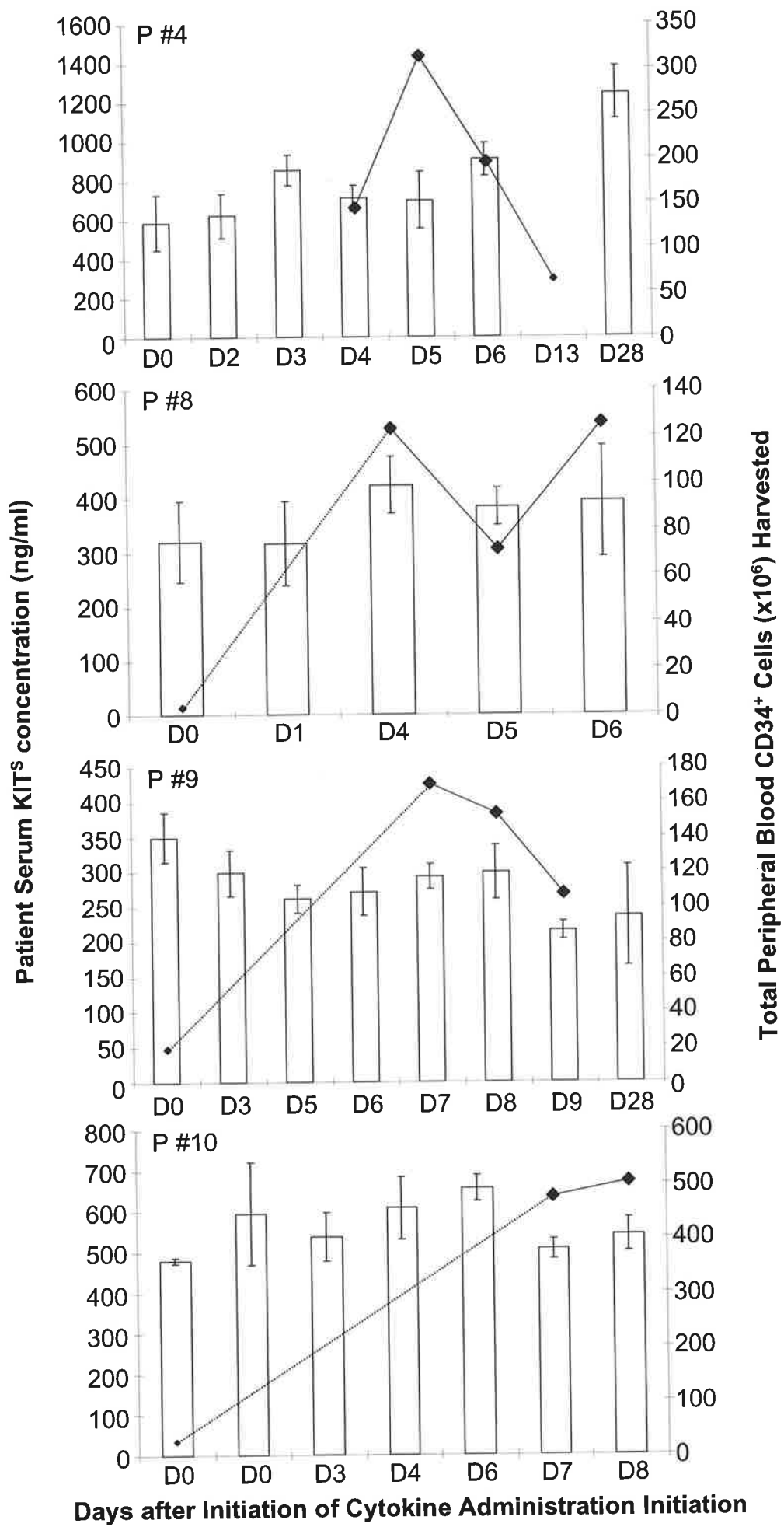


Figure 5.8: Comparison of peripheral blood CD34⁺ cells and serum KIT^S levels from patients treated with G-CSF for 6 days of G-CSF and SLF for 10 days. Breast cancer patients were treated with either G-CSF at 5µg/kg for 6 days, P#4, or G-CSF at 5µg/kg for 6 days and SLF at 10µg/kg for 10 days P#8, 9, 10. Serum samples were analysed for KIT^S levels by competitive ELISA using KIT^S-His as a standard, Bar graph, left hand side Y axis. Peripheral blood CD34⁺ levels were determined and supplied by the Transplantation laboratory of the Division of Haematology, IMVS, Line graph, right hand side Y axis.



mAb, labelled bSLF_γ was immunoprecipitated along with KIT^S. Even after the samples had been boiled a band of the approximate molecular weight expected for 2 KIT^S molecules and 2 SLF molecules was seen in the anti-c-Kit immunoprecipitated lanes, suggesting a multimeric complex involving KIT^S and SLF can form. The stability of this complex suggests KIT^S and SLF may be disulphide bond linked however analysis of the crystal structure of SLF with c-Kit does not indicate disulphide bonds are involved in the interaction (Jiang *et al.*, 2000; Zhang *et al.*, 2000). The use of gel filtration, separating native molecular structures based on molecular weight, may resolve how much of the KIT^S and SLF in human serum are complexed together and the stoichiometry of such complexes.

The function of the different c-Kit isoforms is not fully understood. Most cells seem to express both the GNNK+ and GNNK- isoforms although the shorter isoform is predominant (Crosier *et al.*, 1993). Experiments using transfected cells expressing each isoform individually have shown they have different signalling and activation properties (Caruana *et al.*, 1999). Due to the positioning of these amino acids several groups have suggested they make up part of the proteolytic cleavage site and that protease activity at this site results in the release of KIT^S into the milieu (Yee *et al.*, 1993; Turner *et al.*, 1995; Heaney and Golde, 1996). The culture supernatant from FDC-P1 cells expressing either the GNNK+ or GNNK- isoform was collected and tested for the presence of KIT^S. KIT^S was detected in the culture supernatant of both transfected cell lines. Treatment of these cells for 30 minutes with SLF failed to reproducibly induce the release of KIT^S but treatment with phorbol ester did induce the release of KIT^S. The detection of KIT^S in the supernatant of GNNK- transfected cells implies that GNNK is not an essential part of the cleavage site.

Treatment of patients with certain cytokines, including G-CSF and SLF, results in the movement of CD34⁺ progenitor cells from the bone marrow, where they reside, into the peripheral blood. While this process is not fully understood, it is known that altered integrin function is required and it is associated with a down regulation of c-Kit expression (Lévesque *et al.*, 1994; Papayannopoulou *et al.*, 1998). This down regulation of c-Kit may occur at the level of gene expression or through internalisation and degradation of the receptor but may also involve the proteolytic cleavage of the extracellular domain. Serum samples from breast cancer patients treated with G-CSF or G-CSF and SLF were analysed for KIT^S levels. While

changes in the KIT^S levels were seen in some patients over the time course of treatment, a consistent change was not seen and there was no correlation with the large increase of CD34⁺ cells detected in the peripheral blood of these patients between days 4 and 9 of treatment. These studies provide no evidence of a role of extracellular domain cleavage in downregulation of c-Kit expression accompanying mobilisation of haemopoietic stem and progenitor cells to the peripheral blood.

6. GENERAL DISCUSSION

Cytokine receptor tyrosine kinase overexpression has been linked to transformation of cells and induction of cancers in many cases, (Slamon *et al.*, 1987, Ashman *et al.*, 1988; Slamon *et al.*, 1989; Maa *et al.*, 1995 and references within). Indeed, forced overexpression of unaltered receptor tyrosine kinases by immortalised cell lines, often results in transformation (Hudziak *et al.*, 1987; Di Fiore *et al.*, 1987a,b; Velu *et al.*, 1987; Roussel *et al.*, 1987; Rohrschneider *et al.*, 1989; Alexander *et al.*, 1991; Caruana *et al.*, 1998, 1999). The transforming potential of c-Kit was based on an earlier study in our laboratory, which suggested that high levels of c-Kit expression on the cell surface of AML cells might play a role in leukaemogenesis (Ashman *et al.*, 1988). Using more sensitive techniques, recent data from our group and that of other groups have demonstrated that c-Kit mRNA and protein levels expressed by AML blast cells were generally lower than in normal CD34⁺ fractionated bone marrow cells. Of the blasts analysed, the approximate 20% that did express higher levels of c-Kit were generally of the undifferentiated phenotype (FAB M1 and M2) (Cole *et al.*, 1996). The increased expression levels detected may simply reflect an earlier stage of differentiation arrest of these cells. However, results from our group, (Caruana *et al.*, 1998, 1999) have shown expression of c-Kit at levels only moderately higher than physiological, compare 2x10⁴ copies/cell for CD34⁺ human progenitor cells (Cole *et al.*, 1996) to 5x10⁴ copies/cell for transfected NIH3T3 cells (Caruana *et al.*, 1998), resulted in transformation of NIH3T3 cells as measured by anchorage independent growth. In addition, ectopic expression of murine c-Kit by the factor dependent cell line 32D, rendered these cells leukaemogenic when injected into syngeneic mice, and it was shown cells expressing low levels of receptor were capable of transformation (Hu *et al.*, 1995). Therefore, expression of c-Kit at levels higher than physiological may not be required to cause transformation. The accumulation of cells constitutively expressing c-Kit, and for some reason unable to undergo maturation with concomitant downregulation of c-Kit expression, may contribute to leukaemogenesis. Deregulated c-Kit expression may provide these cells with a competitive advantage, over their normal counterparts in the bone marrow microenvironment, by increased SLF induced signalling. The association of c-Kit expression levels and poor remission rates may not be direct but may be explained by the correlation of c-Kit levels and multi-drug efflux activity

(Sincock and Ashman, 1997). Thus, methods that can prevent leukaemic growth outside of the conventional chemotherapy regimes may provide better means of managing the disease. In recent years, many groups have concentrated on the use of soluble receptors as a method of inhibiting the cytokine induced proliferative responses of transformed cells. It has been envisaged that these molecules would be better than other methods, for example, monoclonal antibodies, as they should have a high affinity for the cytokine, allow better distribution due to their smaller size and should not evoke immune responses. It was for these reasons that this study examined the possibility of using a soluble c-Kit molecule to inhibit SLF dependent cellular growth.

The biggest challenge in examining the functional activity of KIT^S was to find an expression system capable of producing enough KIT^S to carry out the desired experiments. Chapter 3 highlights the lengths pursued to produce useable quantities of KIT^S. Other groups had used a variety of expression systems to produce soluble c-Kit. Initially, soluble murine c-Kit was expressed as a fusion protein with alkaline phosphatase under the control of a Molony virus long terminal repeat. NIH3T3 cells co-transfected with this vector and pSV7neo, were reported to produce 24µg of KIT^S per ml of culture (Flanagan and Leder, 1990). The first human soluble c-Kit molecule was expressed in *dhfr* deficient CHO cells stably transfected with the pSV-*dhfr* vector. Stably transfected cells were subjected to increasing concentrations of methotrexate, up to 1µM, resulting in a transfected cell line producing up to 4µg/ml in 24 hours (Lev *et al.*, 1992). Several groups expressed KIT^S in CHO cells from a vector pDSR, in roller bottles for up to 7 days (Turner *et al.*, 1995; Philo *et al.*, 1996). Finally, Liu *et al.* (1993) and Lemmon *et al.* (1997) used the Baculovirus expression systems to produce KIT^S. Using the vector pVL1992 from Invitrogen, Liu *et al.* (1993) reported production of up to 25µg/ml of KIT^S under the control of the polyhedrin promoter in 48 hours. As they became available, similar vector expression systems to those listed above were tried. These included a bacterial expression system, using the pFlag expression vector, several eukaryotic expression systems, including pcDNA-1/Neo, pRSV009/A⁺, pEE14 and pIG expression vectors and the Baculovirus expression system. The pcDNA-1/Neo vector was based on the highly successful CDM8 vector used by Seed and Aruffo (1987) for expression cloning. pRSV009/A⁺ and pEE14 are vectors derived from

published vectors, containing genes coding for particular drug resistance. These three systems utilised stable transfection and expression in target cells, which was considered preferable for large-scale KIT^S production. Transfection of CHO cells with KIT^S sequence in pcDNA-1/Neo resulted in a cell line stably resistant to G418, and close to 100% of the cells expressing KIT^S, as determined by mAb binding. However, after 4 weeks of continuous culture, while still resistant to G418, these cells no longer expressed KIT^S. Similarly, using vectors carrying drug resistance genes allowing expressing cells to grow in increasing concentrations of drug, pRSV009/A⁺-methotrexate and pEE14- methionine sulfoximine, resulted in CHO cell lines resistant to the drug but they were negative for KIT^S expression. It is unknown why KIT^S expression was down regulated as the expression systems used were very similar to those published. The most obvious explanation is that forced KIT^S expression was toxic to the cells. However, as mentioned above, other groups were able to generate cell lines capable of expressing KIT^S at concentrations up to 25µg/ml. Finally, two transient expression systems, Baculovirus, using the polyhedrin promoter to control KIT^S expression and the COS cell/pIG expression system, were settled upon. Although inconvenient, requiring multiple transfections, they did enable the production useable quantities of KIT^S.

KIT^S was able to bind to SLF expressed on the surface of CHO cells. However, KIT^S-IGc did this poorly although it was more effective at blocking the binding of soluble SLF to cells. This may be due to an unfavourable orientation of KIT^S, in the dimer, for interacting with dimeric SLF in the membrane (Tajima *et al.*, 1998). KIT^S-His and KIT^S-IGb were able to prevent SLF binding to c-Kit expressed by cells by 50% but only when in excess by approximately 200 fold. KIT^S-IGc was shown to be more active, since only a 12 fold excess was required to inhibit SLF binding to c-Kit expressing cells by 50%. By comparison, a 6.25 fold excess of KitX (Lev *et al.*, 1992) was needed to block SLF binding and only 4 fold excess was required for Baculovirus expressed, dimeric KIT^S to block SLF binding (Liu *et al.*, 1993). In cell signalling and proliferation assays, a 1000 fold excess of KIT^S-IGc was required to block the proliferation of TF-1 cells by 60% and 140 fold excess was required to block FD^{GNNK+}#2 proliferation by 80% (Figures 4.8, 4.10). However, dimeric KIT^S was reportedly required only in excess by 20 fold to block murine myeloid leukaemia NFS60 cells from proliferating for 48 hours in response to SLF (Liu *et al.*, 1993). A 42-fold excess of KIT^S was

required to block the SLF induced phosphorylation of c-Kit expressed by M07e cells by 55% (Figure 4.12). This compares favourably with the results of Lev *et al.* (1992), where a 100 fold excess of KitX was required to block PI3'K activation by 90%. Both the results obtained here and those of Liu and colleagues (1993) indicate that a dimeric KIT^S molecule was more effective at blocking the interaction of c-Kit and soluble SLF. However, there appeared to be a large difference between the activity of KIT^S described here and that of the protein produced by Liu *et al.* (1993). Unfortunately, Liu *et al.* (1993) did not include any controls in their assay. Without the controls it is impossible to say that the inhibition was due to the presence of KIT^S.

All of the published figures on KIT^S activity may be influenced by the purity of both the KIT^S and the SLF used. Nevertheless, the activity of KIT^S described here appeared to be lower than that discussed by others. The purity of all of the KIT^S samples used in the experiments described in this text was determined by SDS-PAGE and total protein stain. Adjustments were made to the calculated concentration based on the purity. At worst the samples were 50% pure, resulting in only a two-fold difference in the values detailed in the text. The entire cDNA encoding KIT^S was sequenced and base mismatches were not found. Additionally, the expression systems used, transient and stable expression in COS and CHO cells, or Baculovirus were similar to those used by other groups. Thus, the difference in activity between our KIT^S molecules and those produced by other groups was not due to differences in cloning or expression system. It is possible some inactivation occurred during the purification process but the relatively high yield of immunologically active KIT^S (see Table 4.1) suggests this was not the case. The main difference between the different KIT^S molecules used by the different groups is the position of the C-terminal amino acid with respect to the published sequence. All of the KIT^S proteins start at the same methionine. The KIT^S used in the studies described here terminates at amino acid 515 (glutamine) encoded by bases 1563-1566 (based on published sequence Yarden *et al.*, 1987). The soluble c-Kit molecule used in the experiments described by Lev *et al.* (1992c) terminated at amino acid 508 (phenylalanine) encoded by bases 1543-1545. The termination point of the KIT^S used by Broudy *et al.* (1992) and Wypich *et al.* (1995) is not stated but the same group describe the production of KIT^S in another paper, Turner *et al.* (1995) and it is assumed that the same

molecule was used. This KIT^S terminated at amino acid 520 (threonine) or base 1581 in the published sequence. This corresponds to the last amino acid of the extracellular domain before the transmembrane domain is reported to start. Likewise the murine KIT^S used by Liu and colleagues (1993) also terminates at the last amino acid N-terminal of the transmembrane domain at position 1583 or amino acid 518 (threonine) as per published sequence (Qiu *et al.*, 1988). The published murine sequence does not contain the four amino acid insert, GNNK (Qiu *et al.*, 1988). Therefore, the difference in activity between the KIT^S used in the experiments described here and those published by others may be due to the termination point of the protein. However, since the ligand binding site is localised to Ig domains 1 to 3, this seems unlikely at least in the case of monomeric KIT^S. It is possible the addition or removal of amino acids at the C-terminus of KIT^S may result in changes in the relative orientation of the SLF binding sites in dimeric KIT^S, KIT^S-IGc, possibly affecting the affinity for SLF.

The other difference between the two dimeric KIT^S molecules used in this study, KIT^S-IGb and KIT^S-IGc, and that of Liu *et al.* (1993) is the molecule used by the latter comprised of a direct fusion of KIT^S sequence to the Ig sequence. This alleviates the requirement for the mRNA to be spliced together to produce the fusion protein. Failure of the insect cell machinery to correctly splice the mRNA together may account for the lack of full length, Protein A binding KIT^S-IGb protein, although insect derived mRNA contains introns.

The results obtained from the proliferation assays with TF-1 and FD^{GNNK⁺#2} cells, which express levels of c-Kit comparable with those on normal haemopoietic progenitor cells and most AML cells (chapter 4), were encouraging. However, the large excess of KIT^S required to inhibit cellular proliferation suggest *in vivo* studies, or clinical trials with KIT^S are unlikely to be successful. Furthermore, although it blocked binding of SLF, KIT^S was ineffective at inhibiting proliferation of cells such M07e and FD^{GNNK⁺#1} that express high levels of c-Kit. *In vitro*, it appears KIT^S maybe acting as a reservoir, binding SLF and maintaining equilibrium between SLF binding to cell surface c-Kit and SLF in solution. As the cells proliferate in response to SLF they may deplete the culture supernatant of free SLF, resulting in its release from the KIT^S/SLF complex. Since KIT^S has a lower affinity for SLF than the membrane bound c-Kit, the equilibrium favours the proliferating cell, allowing continued proliferation. The proliferation assay takes 3-4 days and it is possible that interpretation is

complicated by changes in the level of SLF, especially at lower concentrations. Attempts to measure the amount of SLF remaining in the culture supernatant at the end of the assay were unsuccessful but the majority of the KIT^S was still detectable (data not shown). Even though an effect of KIT^S on the proliferation of M07e could not be demonstrated with the assay employed, it did substantially block c-Kit signalling as determined by receptor phosphorylation.

In chapter 5 the biology of naturally occurring KIT^S was examined. KIT^S detected in serum was shown to be capable of binding to SLF although attempts to determine whether endogenous SLF in serum is complexed with KIT^S were not successful. Several groups have suggested the KIT^S in serum is produced from the cleavage of membrane bound c-Kit by a protease, possibly at a site that includes the amino acids sequence GNNK (Wypich *et al.*, 1995), a site influenced by alternate splicing. However, little difference was seen in the amount of KIT^S detected in the culture supernatants of cells expressing only the GNNK+ or GNNK- isoform of c-Kit either spontaneously or following PMA stimulation suggesting that the protease site does not depend on this sequence. Mobilisation of haemopoietic stem cells from the bone marrow into the peripheral blood involves the downregulation of c-Kit expression (reviewed in To *et al.*, 1997). It was a possibility that this downregulation was partially achieved by shedding of the extracellular domain of c-Kit. Serum samples from breast cancer patients undergoing stem cell mobilisation with G-CSF and/or SLF were assayed for KIT^S levels. A consistent alteration to serum KIT^S levels was not seen, nor did the serum KIT^S levels correlate with CD34⁺ cell mobilisation. Interestingly, a correlation between the numbers of CD34⁺ cells detected in the peripheral blood of 16 patients treated with G-CSF and serum KIT^S levels was reported recently by Ishiga and colleagues (2000). In this case, serum KIT^S levels peaked at day 19 of cytokine treatment and peripheral blood CD34⁺ levels peaked at day 17, a time course considerably different from that observed with the patients in this study.

For KIT^S to be of clinical use, alterations to its structure would need to be assessed. While others have reported that the affinity of KIT^S for SLF is similar to membrane bound receptor, mutation of the residues involved in generating the SLF binding site to create a molecule with higher affinity for SLF may assist administered KIT^S to more effectively

compete for SLF binding. Deciding which amino acids to target is now easier since the crystal structure of SLF has been resolved to 2.2-2.3-A (Jiang *et al.*, 2000; Zhang *et al.*, 2000). The crystal structure of c-Kit has not yet been published. Attachment of KIT^S to a structure that induced cellular engulfment could be used to remove SLF from circulation. Mutation of KIT^S such that a covalent bond is generated when it interacts with SLF would prevent SLF disassociation, altering KIT^S function from acting as a reservoir to removing SLF from circulation.

The use of soluble receptors in the treatment of disease must overcome many hurdles before being used successfully in the clinic. Each soluble receptor needs to be studied extensively by experimental methods prior to making any judgements of its efficacy. This is very important especially since their biological role is still unclear. The most probable outcome will be that different soluble receptors have different roles (discussed in detail in chapter 1, 1.10). If their function is to act as carriers or sinks of growth factors, then they are likely to have little therapeutic benefit, since the introduction of these soluble receptors into a patient may merely prolong the half-life of the factor that is partially responsible for causing the disease. Even worse, as has been demonstrated with the soluble IL-6R, they may interact with the cytokine and cell-associated receptor and initiate the normal cellular signalling cascade. Also, the existence of large amounts of naturally occurring soluble receptors is of concern. Will the recombinant soluble receptor be able to compete with the existing soluble receptor? Is the majority of the soluble growth factor detected in serum already complexed to a carrier and will the addition of another make little difference? In addition, some soluble receptor levels are elevated in disease states, e.g. sIL-2R and sTNFR, and failure to respond to the treatment correlates with a failure to decrease the levels of soluble receptor. Furthermore, the signalling cascades of the different receptor complexes are so complicated that one needs to take into account the effect of the other molecules involved in the complex, e.g. IL-1ra and sIL-1R or sIL-6R and sgp130. The *in vivo* stability of the recombinant soluble receptor in serum, its ability to home to the area required and the route of administration all need to be considered. The success of some soluble receptors *in vitro* and *in vivo* must be accepted with caution. In most experimental systems the soluble receptor was administered at the same time

as the tumour inducing cells and its effect is greatly diminished if it is not. These models do not mimic the real-life situation.

Several soluble receptors have been tested for therapeutic potential in phase I and II clinical trials. While they are well tolerated, with side effects similar to those seen with most chemotherapeutic regimes, and success was achieved in animal models, little evidence of activity in these trials, at the concentrations tested, were observed. These studies were carried out some years ago and very little has been published as to the efficacy of soluble receptors as treatment since. Rather than being used as a therapeutic tool, soluble receptor levels, measured in the serum of patients with particular diseases, are being used to predict outcome or severity of disease.

Currently, the use of monoclonal antibodies engineered to prevent cross-species immunogenicity ("humanised") appears to be a better therapy to treat cancers. Clinical trials have been established using antibodies that target tumour cell antigens such as B-cell idiotypes, CD20 on malignant B cells, CD33 on leukaemic blasts and HER2/neu on breast cancer (reviewed in Weiner, 1999a,b). One major advantage of monoclonal antibodies is that they have a serum half-life greater than 24 hours, whereas, soluble receptors have a half-life of three to six hours. A lot of progress has been achieved with the treatment of breast cancers that over express the receptor HER2/neu. One antibody to HER2/neu, 4D5, has been fully humanised, and is termed "trastuzumab" (Herceptin; Genentech, San Francisco, USA). Clinical trials, phase I, II and III, show trastuzumab is well tolerated and at effective doses objective tumour regression was seen in 10-20% of patients (Pegram *et al.*, 1998, 1999; Baselga *et al.*, 1999; Goldenberg, 1999; Osaba and Burchmore, 1999; Shak, 1999). The tumour inhibition was greatest in those patients with highest HER2/neu expressing tumours. In combination with a standard chemotherapy drug, cisplatin, trastuzumab synergistically enhanced the cytotoxic effects of the drug (Pegram *et al.*, 1998). Experiments in mice show that trastuzumab has synergistic effects with cyclophosphamide, doxorubicin, paclitaxel, methotrexate, etoposide and vinblastine, suggesting these are all combinations that should be tested in human clinical trials (Pegram *et al.*, 1999). So far the biggest drawback of trastuzumab treatment is the effect on cardiac tissue. Twentyeight percent of patients receiving an anthracycline (doxorubicin) and trastuzumab developed heart failure,

cardiomyopathy. HER2/neu receptors seem to be cardio-protective because they mediate activation of important cardiac survival pathways (Ewer *et al.*, 1999; Feldman *et al.*, 2000).

An alternate approach to blocking the function of growth factor receptors involved in cancer with antibodies or soluble receptors is the use of cell-permeant, small molecule, kinase inhibitors. Exciting results have been reported with STI 571, a 2-phenylaminopyrimidine derivative that acts as a competitive inhibitor of ATP binding to kinase domains. STI 571 has been shown to inhibit c-abl, bcr-abl and PDGFR (Buchdunger *et al.*, 1996; Druker *et al.*, 1996) and is being evaluated in clinical trials for the treatment of CML (Lauder *et al.*, 2000; Mauro *et al.*, 2000; Ottmann *et al.*, 2000; Sawyers *et al.*, 2000; Talpaz *et al.*, 2000; Druker *et al.*, 2001a; Druker *et al.*, 2001b). Incubation of M07e cells with STI 571, in the presence of SLF, resulted in inhibition of c-Kit autophosphorylation in a dose dependent manner (Heinrich *et al.*, 2000a). c-Kit induced activation of MAP kinase and Akt phosphorylation was also inhibited without affecting the protein expression levels of c-Kit, MAP kinase or Akt (Heinrich *et al.*, 2000a). Inhibition of c-Kit decreased SLF induced proliferation of M07e and induced the cells to undergo apoptosis (Heinrich *et al.*, 2000a). The inhibitory effects of STI 571 are specific for bcr-abl, PDGFR and c-Kit. STI 571 did not inhibit activation of CSF-1R, Flt-3, Flt-1 or Tek kinases (Buchdunger *et al.*, 2000) nor did it inhibit GM-CSF induced proliferation of M07e cells (Heinrich *et al.*, 2000a). STI 571 was unable to inhibit the kinase activity of the c-Kit expressing the D816V mutation found in HMC-1 and many human mastocytomas or the equivalent mutation (V814) in the murine cell line P815 (Akin *et al.*, 2000; Heinrich *et al.*, 2000b; Zermati *et al.*, 2000). This suggests that STI 571 will be ineffective in the treatment of mastocytomas. The effect of STI 571 on c-Kit expressing small cell lung cancers (SCLC) has also been studied. STI 571 inhibited SLF induced proliferation and motility of SCLC cells with associated inhibition of MAP kinase activation and Akt phosphorylation and induction of apoptosis (Krystal *et al.*, 2000; Wang *et al.*, 2000). Therefore STI 571 may be suitable for the treatment of diseases involving the abnormal activation of c-Kit or PDGFR (Heinrich *et al.*, 2000a).

The search for new therapies to treat cancer continues. Their discovery and implementation, alone or in combination with known or yet to be tried compounds, requires

continued research into the understanding of the fundamental biology of cancers and the surrounding normal tissue.

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8. APPENDICIES

8.1 APPENDIX 1: COMPANIES

Company	City	State	Country
Ajax Chemicals	Sydney	N.S.W.	Australia
Amersham, now Amersham Pharmacia Biotech		Buckinghamshire	England
Beckman Coulter	Palo Alto	California	USA
Becton Dickinson	Mountainview	California	USA
Bio-Rad	Hercules	California	USA
Boehringer Mannheim (now Roche Biochemicals)		Mannheim	Germany
Caltag Laboratories	San Francisco	California	USA
Clontech Laboratories	Palo Alto	California	USA
Corning Costar	Cambridge	Massachusetts	USA
Dako	Carpinteria	California	USA
Difco, now part of Becton Dickinson	Sparks	Maryland	USA
Dow Chemicals	Midland	Michigan	USA
Falcon, part of Becton Dickinson	Franklin Lakes	New Jersey	USA
FMC	Rockland	Maine	USA
Forma Scientific	Marietta	Ohio	USA
Gelman Sciences	Ann Arbor	Michigan	Australia
Geneworks (formerly Bresatec)	Adelaide	South Australia	Australia
Gibco BRL	Rockville	Maryland	USA
Greiner	Frickenhausen		Germany
Hunter Antisera	Sydney	N.S.W.	Australia
ICN Biomedicals	Aurora	Ohio	USA
Invitrogen	Palo Alto	California	USA
Kodak Eastman	Rochester	New York	USA
Merck	Melbourne	Victoria	Australia
Millipore Corporation	Bedford	Massachusetts	USA
Molecular Dynamics, now part of Amersham Pharmacia Biotech			
Molecular Probes	Eugene	Oregon	USA
Nalge Nunc International	Naperville	Illinois	USA
PA Biologicals	Sydney	N.S.W.	Australia
Perkin Elmer Applied Biosystems	Boston	Massachusetts	USA
Pharmacia Biotech, now merged with Amersham		Uppsala	Sweden
Pierce	Rockford	Illinois	USA
Progen Industries	Brisbane	Queensland	Australia
Promega	Madison	Wisconsin	USA
Schleicher and Schuell	Dassel	Vertrieb	Germany
Shandon Scientific	Runcorn	Cheshire	England
Sigma	St Louis	Missouri	USA
Silenus, part of AMRAD	Melbourne	Victoria	Australia
Skatron Instruments	Dolasletta	Lier	Norway
Southern Biotechnology Associates	Birmingham	Alabama	USA
Stratagene	Austin	Texas	USA
Techno-Plas	Adelaide	South Australia	Australia
Transduction Laboratories, part of Becton Dickinson			
Upstate Biotechnology	Lake Placid	New Jersey	USA
Zymed Laboratories	San Francisco	California	USA

8.2 APPENDIX 2: REAGENTS

8.2.1 General Reagents

Phosphate Buffered Saline (PBS) consisted of 0.14M NaCl, 3mM KCl, 8mM Na₂HPO₄.12H₂O and 1mM KH₂PO₄, pH 7.4. The solution was sterilised by autoclaving.

PBS/azide: 0.14M NaCl, 3mM KCl, 8mM Na₂HPO₄.12H₂O, 1mM KH₂PO₄, 0.1% w/v NaN₃, pH7.4.

PBS/BSA/azide: 0.14M NaCl, 3mM KCl, 8mM Na₂HPO₄.12H₂O, 1mM KH₂PO₄, 1% w/v bovine serum albumin (Fraction V) (Sigma, USA, Cat. No. A-7906) and 0.1% w/v azide. The pH of the solution was 7.4 and was filtered through 0.45µm filter (Millipore, USA, Cat. No. HAWG 047 A0).

PBS-T: PBS + 0.1% Tween 20

0.1M Na⁺-phosphate buffer: mixture of 0.1M Na₂PO₄ and 0.1M NaHPO₄, pH 8.2 supplemented with 0.1% w/v sodium azide (NaN₃).

0.1M Citrate buffer (Na⁺): mixture of 0.1M tri-sodium citrate and 0.1M citric acid. Solutions made ranged from pH 3.5-6.5.

8.2.2 Reagents for Tissue Culture

DMEM: To make Dulbecco's Modified Eagle Medium (DMEM), one sachet of DMEM powder (Gibco BRL, Cat. No. 12800-017) along with 3.7g were dissolved in 900mls sterile Milli-Q purified water. Sterile stock solutions of N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) (Boehringer Mannheim, Cat. No. 737151) pH 7.2, penicillin (Sigma, Cat. No. P.3032) and streptomycin sulphate (Sigma, Cat. No. S 9137) were added to give final concentrations of 15mM, 100 i.u./ml and 100µg/ml respectively. The pH was adjusted to 7 using 4 mls of 1M HCl and the volume made up to 1 litre with Milli-Q-purified water. This medium was filter sterilised using a Sterivex GS 0.22µm filter unit with filling

bell (Millipore, Cat. No. SVGB1010). The pH after filtering was 7.0-7.4. The medium was stored in the dark at 4°C.

Ham's F12 medium: consisted of one dissolved sachet of Ham's F12 powder (Gibco BRL, Cat No. 21700-075) along with 1.176g of NaHCO₃ in approximately 900ml of Milli-Q H₂O. Sterile stock solutions of penicillin and streptomycin sulphate were added to the final concentrations as above for DMEM and the pH adjusted to 7.0 with 1M HCl. The volume was adjusted to 1L and the medium filtered and stored as above.

IMDM: Iscove's Modified Dulbecco's Medium (IMDM) was prepared by adding one sachet of IMDM powder (Gibco, Cat. No. 12200-036) and 2g of NaHCO₃ in 900ml of Milli-Q H₂O. Sterile stock solutions of penicillin and streptomycin sulphate were added to the final concentrations as above for DMEM and the pH adjusted to 7.4 with 1M HCl. The volume was adjusted to 1L and the medium filtered and stored as above.

RPMI 1640 medium: was prepared by dissolving one sachet of RPMI 1640 powder (Gibco, Cat. No. 31800-02) along with 2g NaHCO₃ in 900 ml of Milli-Q-purified H₂O. Sterile stock solutions of HEPES, streptomycin sulphate and penicillin were added to give the final concentrations as above for DMEM and the pH adjusted to 7.4 with 2.5 ml 1M HCl. The volume was made up to 1 litre and the medium filtered and stored as above.

Before use all of the above media were supplemented with glutamine (BDH, Cat. No. 37107) to a final concentration of 2mM, and 5-10% v/v of heat-inactivated foetal bovine serum (FBS) as indicated (Gibco BRL, Cat. No. 200-6140PJ, Batch No. 660; PA Biologicals, Aust, Cat. No. 3000, Batch No. 80693 and 300795). The FBS was heat inactivated by incubation at 56°C for 30 min. After 7 days of storage medium was re-supplemented with glutamine (2mM final).

Insect cell medium: Graces insect medium (Gibco BRL) supplemented with 5-10% FBS, 2mM glutamine, 100i.u./ml penicillin (Sigma, Cat. No. P.3032) and 100µg/ml streptomycin sulphate (Sigma, Cat. No. S 9137).

Insect cell medium: Sf-900 II SFM medium (Gibco BRL, Cat. No. 10902-088). Complete optimised serum-free medium for insect cell culture that required no additional nutrients. Purchased as ready to use liquid.

Hank's balanced salt solution (HBSS): consisted of 0.14M, 5mM, 0.3mM Na₂HPO₄.12H₂O, 0.4mM KH₂PO₄, 4.2mM NaHCO₃, 5.5mM glucose, 1% Phenol Red (M & B, England, Cat. No. P152/18/61) (in 0.1M NaOH) in Milli-Q-purified H₂O, pH 7.4 The medium was sterilised by autoclaving.

Trypsinisation solution: consisted of 0.054% w/v trypsin (Difco, Cat. No. 0152-13-1) and 0.54mM ethylenediaminetetra-acetic acid (EDTA) in HBBS. The solution was filtered through a low protein binding 0.22µm filter (Millipore, Cat. No. SLGV025LS) immediately after dissolving the trypsin powder in the EDTA/HBBS, aliquoted and stored at -20°C.

Methyl cellulose: Methyl cellulose powder (8.1g A4M premium grade 4000 centipose, Dow Chemicals), was added to a 500ml glass bottle and autoclaved at 15psig (121°C) for 15 min. To this, 270ml sterile 1x IMDM was added, mixed and left stirring for 48 hours with occasional shaking to assist mixing. When the medium was homogeneous, 180ml sterile FBS and 60ml BSA were added and the mixture was allowed to stir for a further 3 hours. The methyl cellulose mixture was then stored at -20°C in 25ml aliquots.

8.2.3 Reagents for APAAP

Standard fixative: Mix 47.5ml acetone with 47.5ml methanol and 5ml formaldehyde.

Tris Buffered saline (TBS): 50mM Tris-HCl, pH 7.6, 120mM NaCl

Substrate: Prepared immediately before use. Dissolved 20mg naphthol ASMX phosphate free acid (Sigma, Cat. No. L9756) in 2ml dimethylformamide (Merck, Cat. No. 10322) and made up to 10ml with 0.1M Tris-HCl, pH 8.2. To this added 100µl 1M levamisole (Sigma, Cat.

No. L9756, dissolved in water) and 100mg Fast Red TR salt (Sigma, Cat. No. F1500) and filtered through 3M Whatman filter paper.

Gill's haematoxylin: Reagents to be added in the following order: 730ml distilled H₂O, 250ml ethylene glycol, 2.0g Haematoxylin (anhydrous/monohydrate), 0.2g sodium periodate (NaIO₄), 17.6g aluminium sulphate (Al₂ (SO₄)₃.18H₂O), 20ml glacial acetic acid. Allow solution to stir for 1 hour at room temperature. Stock matures with age. Allow to mature for 4 weeks prior to use. Staining times have to be adjusted according to the age of the stock.

Scott's gentle alkaline solution: Dissolve 3.5g NaHCO₃ and 20g MgSO₄.7H₂O in 1L H₂O.

glycerol-glycine: 1.4g glycine/100ml H₂O, pH to 8.6 with NaOH. To 30ml glycine buffer add 70ml glycerol.

8.2.4 Reagents for SDS-PAGE

Resolving Gel

MW separating range	50-200kDa	20-150kDa	10-60kDa
% gel	7.5	10	12
1.5M Tris-HCl, pH 8.8	25%v/v	25%v/v	25%v/v
30% Acrylamide-bis	25%v/v	33%v/v	40%v/v
SDS	0.001%w/v	0.001%w/v	0.001%w/v
Ammonium persulphate (in H ₂ O)	0.0004%w/v	0.0004%w/v	0.0004%w/v
TEMED	0.0008%v/v	0.0008%v/v	0.0008%v/v
made up in H ₂ O.			

4% Stacking Gel

0.5M Tris-HCl, pH 6.8	25%v/v
30% Acrylamide-bis	13%v/v
SDS	0.001%
Ammonium persulphate (in H ₂ O)	0.0005%
TEMED	0.0008%
made up in H ₂ O.	

Sample Buffer (non reducing): 62.5mM Tris-HCl pH 6.8, 10% glycerol, 2%w/v SDS, 0.00125% Bromophenol blue; for reducing sample buffer 5% β-Mercaptoethanol was included.

Electrophoresis Buffer: 0.025M Tris, 0.192M Glycine, 0.1% SDS, should be pH 8.3 without further adjustment.

8.2.5 Bacterial Culture

Luria Broth: 1% Bactotryptone, 0.5% Bacto Yeast extract, 1% NaCl.

Dissolve 10g of Bacto-tryptone, 10g NaCl and 5g Bacto-yeast in Milli-Q water, pH to 7-7.2 with NaOH, and make up to a final volume of 1L. Autoclave.

Luria Broth Agar Plates: Melt 7.5g Bacto-agar/500mL Luria Broth by autoclaving. Allow agar to cool to ~55°C prior to the addition of antibiotic (at appropriate concentration) and then pour into petri dishes (Techno-Plas Cat. No. S-9014-520). Allow to set, then dry and store at 4°C.

Super Broth: 3.2% w/v Bactotryptone, 2.0% Yeast extract, 0.5% NaCl

S.O.C. medium: Final concentrations of 2% Bacto-tryptone, 0.5% Bacto-yeast extract, 10mM NaCl and 2.5mM KCl were dissolved in 97mls H₂O and autoclaved. When the medium was cool, a final concentration of MgCl₂/MgSO₄ (10mM each final) and 20 mM glucose were added. The medium was filter sterilised through a 0.22µm filter unit. The final pH should be 7.0±0.1.

Ψa agar plates: 5g/L Bacto-yeast extract (Difco, Cat. No. 012-01-7), 20g/L Bacto-tryptone (Difco, Cat. No. 0127-01-7) 5g/L MgSO₄, pH 7.6 with KOH and 14g/L Bacto-agar (Difco, Cat. No. 0140-01).

Ψb medium: as for Ψa agar plates in the absence of Bacto-agar.

Tfb I Buffer: 30mM Potassium acetate, 100 mM KCl, 10mM CaCl₂ (2H₂O), 50 mM MnCl₂ (4H₂O), 15% (v/v) glycerol, pH 5.8 with acetic acid. Filter sterilise.

Tfb II Buffer: 10mM MOPS (Sigma, Cat. No. M-9381) (or PIPES (Sigma, Cat. No. P-9291), 7mM CaCl₂, 10mM KCl, 15%(v/v) glycerol; pH 6.5 with 1 M KOH. Filter sterilise.

Note KCl in Tfb I and Tfb II solutions is a replacement for RbCl in the original method.

8.2.6 DNA Purification

NaCl-TE saturated isopropanol: Add an equal volume of isopropanol to 5M NaCl made in 10 mM Tris-HCl, 1mM EDTA, pH 7.4 (Top layer = isopropanol).

Sterile dialysis tubing: Boil 1x in 2% NaHCO₃, 1mM EDTA for 10 min. Wash in sterile H₂O, 1mM EDTA, pH 8.0. Store in 1mM EDTA, pH 8.0, 4°C.

Equilibration of Phenol

Molecular biology grade phenol (Merck, Cat. No. 101884Y) and 0.1% 5-hydroxy-quinoline were melted at 65°C. An equal volume of 0.5M Tris-HCl (pH 8.0) was added and the mix stirred on a magnetic stirrer 15 min at room temperature in the dark. The stirrer was then turned off and the phases allowed to separate. The aqueous (upper) phase was aspirated using a vacuum line and an equal volume of 0.1M Tris-HCl (pH 8.0) added. This was again stirred for 15 min and phases then allowed to separate. The extractions of the phenol continued alternating with 0.5 M and 0.1M Tris-HCl (pH 8.0) until the pH of the phenol was >7.8 as measured by pH indicator paper. The equilibrated phenol was then stored under 0.1 volume of 0.1M Tris-HCl (pH 8.0) in the dark at 4°C.

8.2.7 Manual Sequencing, Resolving gel Preparation

46% urea solution: Dissolve 460g of urea (Merck, Cat. No. 10290) in Milli-Q water to a final volume of 1L. Add 20-30g of Analytical Grade Mixed Resin AG 501-X8 (Bio-Rad, Cat. No. 142-6424) and stir for at least 30 min. Store above the resin at room temperature.

10x TBE: Dissolve 121g of Tris base, 7g of EDTA and 53.4g boric acid in Mill-Q water to a final volume of 1L. The pH should be 8.3.

20% acrylamide solution: 467g urea was dissolved in 500ml of a 40% acrylamide solution (19:1, acrylamide : N, N'-methylene-bisacrylamide (Bio-Rad, Cat. No. 161-0144) and made up to final volume of 1L in Milli-Q H₂O. 20-30g of Analytical Grade Mixed Bed Resin AG 501-X8 was added and stirred for at least 30 min. This was filtered through Whatman and stored at room temperature in the dark.

Sequencing Gel (70ml): 35ml of 46% Urea, 28ml 20% acrylamide, 7ml 10xTBE, 350µl ammonium persulphate, 70µl TEMED. Mixed and poured between taped glass plates using a 50ml syringe and a 19 gauge needle.