

**Ca²⁺ and Phosphoinositides Regulations
in α -actinin-4 F-actin Binding**

by

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Summary

α -actinin-4 is a non-muscle isoform of α -actinin that belongs to the spectrin superfamily. It comprises three functional regions: an N-terminal actin-binding region that consists of two calponin homology (CH) domains, a central region that consists of four copies of the spectrin-like repeat domain and a C-terminal calmodulin-like domain that is predicted to bind Ca^{2+} . α -actinin-4 is organised as an antiparallel homodimer formed by the interaction of four spectrin-like repeats between the two monomers, giving a rod-like shape, with actin binding regions at both ends.

α -actinin-4 is an abundant actin-bundling protein, which provides a direct link between actin filaments and integrins, and is believed to play an important role in stabilising cell shape and adhesion and regulating cell migration. It also acts as a tumor suppressor and influences the metastatic potential and invasiveness in human cancers. A cluster of three actin binding motifs have been identified in the CH domains (2X CH) from other members of the spectrin superfamily, utrophin and dystrophin. Two of them reside in the CH1 domain and the third resides in the first α -helix of the CH2 domain. In addition, a PIP2 binding site has been mapped on a region adjacent to actin-binding site-3. These observations imply the F-actin binding activity would be regulated by phosphoinositides. Five mutations of α -actinin-4, K122N, an alternative splice variant, K255E, T259I and S263P, have been reported to be involved in three human diseases, non-small lung cancer (NSCLC), small cell lung cancer (SCLC) and focal segmental glomerulosclerosis (FSGS). The mutation site within these mutants is located on the actin binding region. Therefore, the actin binding region is presumed to be associated with the progression of human disease.

The aims of this thesis focused on the regulation of the F-actin binding activity of α -actinin-4 by phosphoinositides (PIP2 and PIP3), the calmodulin-like domain and Ca^{2+} , determination of the three-dimensional structure of the CH2 domain in solution and identification of the phosphoinositide binding site on the CH2 domain. In order to investigate the F-actin binding activity quantitatively, a novel *in vitro* F-actin binding assay (solid phase) was established to replace the semi-quantitative actin bundling assay.

Using this novel solid phase F-actin binding assay, Ca^{2+} was shown to enhance the F-actin binding activity of α -actinin-4 in a concentration dependent manner. The presence of 10 mM Ca^{2+} results in a two-fold increase in the F-actin binding activity. Both PIP2 and PIP3 inhibited the F-actin-binding activity of α -actinin-4 in a concentration dependent manner with an approximate IC_{50} of 75 and 45 μM , respectively.

In order to characterise how phosphoinositides regulated the F-actin binding activity of α -actinin-4, the solution structure of α -actinin-4 CH2 domain was determined and the phosphoinositide binding residues within the CH2 domain were identified using NMR spectroscopy. The solution structure of α -actinin-4 CH2 domain contained six α -helices and was similar to that of other spectrin superfamily members. The strategy used in identification of the phosphoinositide binding site was an NMR-based 2D ^1H - ^{15}N HSQC ligand titration assay to replace the traditional semi-quantitative protein-lipid overlay assay. Using the NMR-based ligand titration assay, the recognition site for the inositol head group resides in residues Trp 172, Tyr 265 and His 266 and the binding region of acyl chains resides in the first α -helix structure which is one of the putative F-actin binding sites. In order to examine the interaction of phosphoinositides with this site, Y265A and H266E mutants of α -actinin-4 CH2 domain were generated using site-directed mutagenesis and verified the interaction with phosphoinositides and the inositol head group using an NMR-based ligand titration assay. These results confirmed the phosphoinositide binding site on the CH2 domain and residues, Tyr 265 and His 266, are critical for interacting with phosphoinositides.

Wildtype and mutants (Y265A and H266E) of α -actinin-4 were expressed in mammalian cells as EGFP-fusion proteins. Wildtype α -actinin-4 was shown to be co-localised with focal adhesions and actin stress fibres. However, Y265A and H266E mutants of α -actinin-4 were co-localised with actin stress fibres but poorly co-localised with focal adhesions. Moreover, both Y265A and H266E mutants of α -actinin-4 were co-localised with actin in the cytoplasm rather than localised along the cell membrane after EGF stimulation for 30 minutes. These results suggested that PIP2 assists the co-localisation of α -actinin-4 with focal adhesions.

Taken together, the results described in this thesis concluded that Ca^{2+} enhanced the F-actin binding activity of α -actinin-4 *in vitro*. However, phosphoinositides (PIP2 or PIP3) inhibited the F-actin binding activity *in vitro*. Moreover, the results described in this thesis provided a phosphoinositide binding site on α -actinin-4 CH2 domain. Binding to PIP2 is important to the localisation of α -actinin-4 in focal adhesions.

Declarations

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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List of Abbreviations

2D	two-dimensional
3D	three-dimensional
4D	four-dimensional
ABD	Actin binding domain
Amp	ampicillin
ARIA	Ambiguous Restraints of Iterative Assignment
Arp 2/3	actin-related protein 2/3
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C-	carboxyl-
CamLD	calmodulin-like domain
CCPNMR	Collaborative Computing Project for the NMR
cDNA	complementary DNA
CH	Calponin Homology
CTLs	cytotoxic T lymphocytes
COSY	Correlation Spectroscopy
CSI	chemical shift index
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOL	degree of labeling
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	enhanced chemiluminescence
ECM	extra-cellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	Epidermal growth factor

EGFP	enhanced green fluorescent protein
ENTH	epsin N-terminal homology
ER	endoplasmic reticulum
Erk 1/2	extracellular signal-related kinase 1/2
ES	embryonic stem
EtBr	ethidium bromide
F-actin	filamentous actin
FAK	focal adhesion kinase
FBS	fetal bovine serum
FERM	4.1-ezrin/radixin/moesin
FITC	fluorescein isothiocyanate
FP	fluorescence polarisation
FRET	forster resonance energy transfer
FSGS	focal segmental glomerulosclerosis
FYVE	the first letters of four proteins, Fab1p, YOTB, Vac1p and EEA1
G-actin	globular actin
GLB	gel loading buffer
GST	glutathione-S-transferase
HSQC	Heteronuclear Single Quantum Coherence
ILK	integrin-linked kinase
IP3	inositol 1,4,5-trisphosphate
IP4	inositol 1,3,4,5-tetraphosphate
IPTG	isopropyl- β -D-thiogalactopyranoside
Kan	kanamycin
kb	kilobase pair
Kd	equilibrium dissociation constant
kDa	kilo Dalton
LB	Luria broth
LCC	large cell lung cancer
LPA	lysophosphatidic acid
mA	milli-amperes

MEKK 1	mitogen-activated protein/extracellular signal-related kinase kinase 1
N-	amino-
NMR	nuclear magnetic resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
NSCLC	non-small cell lung cancer
OD _{600nm}	optical density at 600 nm wavelength
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PDGF	Platelet-derived growth factor
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP 5K	phosphatidylinositol-4-phosphate 5-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonylfluoride
PPD	<i>p</i> -Phenyldiamine
ppm	parts per million
PTB	phosphotyrosine binding
PX	Phox homology
RNA	ribonucleic acid
SCLC	small cell lung cancer
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Src homology
SV40	simian virus 40
TAE	Tris-acetate EDTA
TBS	Tris buffered saline
TE	Tris-EDTA

TILs	tumor infiltrating lymphocytes
TOCSY	Total Correlation Spectroscopy
Tris	Tris(hydroxymethyl)aminoethane
TRITC	Tetramethylrhodamine isothiocyanate
TTBS	Tris buffered saline/0.1% Triton X-100
Tween 20	polyethylene-sorbitan monolaurate
UV	ultra violet
V	volts
v	volume
w	weight
X-GAL	5-bromo-4-chloro-3-indoyl-galactopyranoside