# $Ca^{2+}$ and Phosphoinositides Regulations in α-actinin-4 F-actin Binding

by

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## Contents

**CHAPTER 2 Materials and Methods**

## 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 $^{1}H$-$^{15}N$ 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 |
| *E. coli* | *Escherichia coli* |
| 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/moiesin |
| 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 | *p*-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 |