



Pleckstrin Homology and Tec Homology Domains Link Tec Kinase Signalling to the Cytoskeleton

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Declaration

To the best of my knowledge, this thesis does not contain any material previously submitted for a Degree or Diploma in any University or any material previously written or published by any other person except where due reference is given in the text.

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Summary

The Tec family of non-receptor protein tyrosine kinases is involved in the progression of intracellular signalling pathways that involve changes in calcium influx and cellular gene expression, cell growth and differentiation, and cytoskeletal remodelling. The five members of the Tec-family of kinases (Btk, Itk, Bmx, Txk and Tec) are expressed primarily in haematopoietic cells and are responsible for the transmission of signals from a variety of cell surface receptors. This includes antigen receptors such as the B cell, T cell and Fc- γ receptors, cytokine receptors, receptor tyrosine kinases, G-protein-coupled receptors and integrin adhesion receptors. Tec-family kinases are biologically important molecules, since, lack of functional Btk causes the severe human immunodeficiency disease X-linked agammaglobulinemia.

The constituent modular domains of Tec-family tyrosine kinases are responsible for the allosteric regulation of the kinase domain catalytic activity and subcellular targeting. Both intermolecular (with proteins and 3'-phosphatidylinositol lipids) and intramolecular interactions play an important role in this process. Like Src-family kinases, Tec-family kinases contain carboxyl terminal Src homology-3 (SH3), Src homology-2 (SH2) and tyrosine kinase domains. However, Tec-family kinases also contain amino terminal pleckstrin homology (PH) and Tec homology (TH) domains that make them unique amongst tyrosine kinase families.

There is increasing evidence that links the signalling of Tec-family kinases to actin cytoskeleton remodelling. The cytoskeleton provides a dynamic structural framework within cells. As a key part of the contractile apparatus, it plays fundamental roles in the maintenance of cell morphology, cell-cell and cell-matrix adhesion, cell division and phagocytosis. The PH domain plays an integral role in this cytoskeletal connection through interaction with proteins such as filamentous actin (F-actin), focal adhesion kinase and integrin-associated signalling complexes. PH domains, in general, function to target the activated protein to the correct cellular site, such as the cytoskeleton or the cell membrane.

The primary objective of this work was to identify protein ligands of the PH and TH domain pair (PHTH domain) of Tec kinase and to better understand the role of the PHTH domain in Tec signalling pathways. A yeast two-hybrid assay was performed in which the Tec PHTH domain was used as a bait to screen a library of potential protein ligands expressed in human liver tissue; this tissue was chosen since Tec was first identified as a tyrosine kinase expressed in hepatocellular carcinoma. Actinin-4 was identified as a novel ligand of Tec. The

bulk of the work presented in this thesis involves characterisation of the Tec:Actinin-4 interaction using biochemical, molecular biology and cell biology techniques.

Actinin-4 is a newly identified non-muscle α -actinin isoform that belongs to the spectrin-family of proteins. α -actinins are described as actin-bundling proteins that determine the mechanical properties of the actin filament network. They form anti-parallel homodimers with an actin-binding domain at each end, dynamically cross-linking actin filaments by simultaneously binding to adjacent F-actin strands through their distinct actin binding sites. Regulation of F-actin binding by α -actinins plays an important role in cell morphology change. Actinin-4 has been implicated in the metastatic potential of human cancers and is, therefore, an attractive target for anti-cancer therapies.

α -actinins are composed of modular domains; these include dual N-terminal calponin homology (CH) domains, a central rod region that has four copies of the spectrin repeat domain and dual C-terminal calmodulin-like EF-hand domains that are predicted to bind Ca^{2+} and confer sensitivity to intracellular Ca^{2+} concentrations. The F-actin binding region is adjacent to a phosphatidylinositol 4,5-bisphosphate binding site and is contained within the CH domains. In the homodimer, these are juxtaposed against the EF-hand domains of the other molecule. The spectrin repeat domains contain the intermolecular dimerisation interface and contact protein ligands. The Tec binding region of Actinin-4 includes the third spectrin repeat domain and flanking sequences that confer dimerisation.

Co-immunoprecipitation experiments utilizing mammalian cell expressed Tec and Actinin-4 proteins confirmed the interaction in a cellular context. Site-directed mutagenesis was used to create variants for probing the involvement of specific residues predicted to affect the binding of the two proteins. Targeted residues included critical actin binding determinants of the Tec PH domain: K18A/K19A/K20A; as well as regulatory residues predicted to endorse activity-dependent conformations of the Tec molecules and, thus, availability of activation-dependent ligand binding sites: Y187E, K397E and R29C. In Actinin-4, a substitution mutation of a candidate PH-domain ligand known as a HIKE-like motif was created: H602A/I603A/K604A. None of the analysed mutations appeared to abrogate binding.

The subcellular localisations of endogenous Tec and Actinin-4 proteins were investigated using indirect immunofluorescence microscopy. Extensive colocalisation in an ultrafine filamentous network as well as in juxtanuclear regions of the cell were detected. The subcellular localisation of enhanced green fluorescent protein (EGFP)-tagged Tec protein variants, including wild-type Tec as well as the substitution mutants described above, were

investigated using direct fluorescence microscopy of transiently transfected COS-1 cells. Interestingly, they have distinct as well as overlapping subcellular localisation patterns compared with endogenous Tec. These overexpressed fusion proteins were also used in co-immunoprecipitation experiments.

Both Triton-X-100 soluble and insoluble pools of the various EGFP-Tec proteins were identified. This was also the case for endogenous Tec and Actinin-4 proteins. Potentially, the insoluble fraction represents cytoskeletal-translocated protein and suggests that insoluble cytoskeletal proteins are targets of tyrosine kinase regulation. While phosphorylation is a major regulator of Tec-family kinase activation and function, future experiments will be required to address whether or not this is also the case for α -actinins. Given that Tec is implicated in actin rearrangement during phagocytosis, it would be attractive to consolidate the physiological relevance of the Tec:Actinin-4 interaction using phagocytosis experiments.

Since change in cell morphology is a key aspect of oncogenesis, it is important to understand and characterise the cytoplasmic signals that regulate cytoskeletal architecture. Modulation of these signals can be used to combat aberrant or inappropriate signalling that arises in disease states. In an effort to elucidate the Tec binding site of Actinin-4 and, thus, provide a potential target site for chemical interference of the Tec:Actinin-4 interaction, recombinant protein encompassing the third spectrin repeat of Actinin-4 was prepared for structural studies. Preliminary nuclear magnetic resonance spectroscopy studies were performed and indicate that this protein is amenable to structure determination. It is envisaged that the Actinin-4 residues of the interaction interface will be identified by comparison of spectra obtained from PTH domain bound and unbound samples of the Actinin-4 third spectrin repeat. This suggests that a template for design of modulators of Actinin-4 function, through the interference of Tec binding, can be obtained and is a major step toward dissecting the signalling pathway involving Tec and Actinin-4 proteins.

Taken together, the results presented here provide valuable information concerning a potential direct link between Tec tyrosine kinase and regulation of cytoskeletal architecture. Tec could be involved in cell restructuring through interaction with and phosphorylation of cytoskeletal components in processes such as cell adhesion, migration and phagocytosis. Since tyrosine kinase signalling pathways are activated immediately downstream of cellular receptors, this helps to explain the concomitant swiftness of cytoskeletal reorganisation. Lastly, this research has identified new targets for cancer therapies and provided a reference point to manipulate signalling pathways involved in tumour metastasis.

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Contents

Declaration	ii
Summary	iii
Acknowledgments	vi
Contents	vii
List of Figures	xiv
List of Abbreviations	xvi
CHAPTER 1: Introduction And Literature Review	1
1.1 Introduction	2
1.2 Non-Receptor Protein Tyrosine Kinases	2
1.3 Tec-Family Protein Tyrosine Kinases	4
1.3.1 Members and Expression	4
1.3.2 Regulation of Enzyme Activity	5
1.4 Modular Domains	7
1.4.1 Pleckstrin Homology and Tec Homology Domains	7
1.4.2 Src Homology Domains: SH3, SH2 and Kinase	9
1.5 Function of Tec-family Kinases	11
1.5.1 Receptor Initiated Signalling Pathways of Tec Kinases	11
1.5.2 Signalling Pathways That Involve Tec	12
1.6 Ligands of Tec-Family Kinases	13
1.6.1 PHTH Domain Ligands	13
1.6.2 SH3 Domain Ligands	15
1.6.3 SH2 Domain Ligands	15
1.6.4 Kinase Domain Ligands	16
1.7 Downstream Effectors of Tec-family Kinases	17
1.8 Model of Tec-family Kinase Activation	18
1.9 Null Phenotypes of Tec-family Kinases	19
1.10 Yeast Two-Hybrid	21
1.11 Aims	22

CHAPTER 2:	Materials And Methods	23
2.1	Materials.....	24
2.1.1	Sheet Materials.....	24
2.1.2	Other Materials.....	24
2.1.3	Chemicals and Reagents.....	24
2.1.4	Solutions.....	26
2.1.5	Bacterial Strains.....	28
2.1.6	Yeast Strain.....	28
2.1.7	Mammalian Cell Lines.....	29
2.1.8	Bacterial Growth Media.....	29
2.1.9	Yeast Growth Media.....	30
2.1.10	Synthetic-Dropout Amino Acid Mix.....	30
2.1.11	Mammalian Cell Growth Media.....	31
2.1.12	Kits.....	31
2.1.13	Enzymes.....	31
2.1.14	DNA Molecular Weight Standards.....	31
2.1.15	Protein Molecular Weight Standards.....	32
2.1.16	Cloning Vectors and Bacterial Protein Expression Vectors.....	32
2.1.17	Yeast Two-Hybrid Vectors (CLONTECH Laboratories, 1996).....	32
2.1.18	Mammalian Cell Expression Vectors.....	33
2.1.19	Cloned Tec DNA Sequences.....	33
2.1.20	Cloned Actinin-4 DNA Sequence.....	33
2.1.21	Human Liver complementary DNA (cDNA) library.....	34
2.1.22	Oligonucleotide Primers Used in Polymerase Chain Reactions.....	34
2.1.23	Primary Antibodies.....	34
2.1.24	Secondary Antibodies.....	36
2.2	Electronic Resources.....	36
2.2.1	Internet Databases.....	36
2.2.2	Internet Software.....	36
2.2.3	DNA Sequence Analysis.....	37
2.3	Methods.....	38
2.3.1	Molecular Biology Techniques.....	38
2.3.1.1	Primer Design.....	38

2.3.1.2	Polymerase Chain Reaction (PCR)	38
2.3.1.3	Site-Directed Mutagenesis	38
2.3.1.4	Agarose Gel Electrophoresis	39
2.3.1.5	Restriction Endonuclease Digestion of Plasmid DNA.....	39
2.3.1.6	Removal of 5' Phosphate from Linear DNA Fragments	39
2.3.1.7	Endfill Reaction.....	40
2.3.1.8	Purification of Linear DNA Fragments.....	40
2.3.1.9	Ligation Reactions.....	40
2.3.1.10	Preparation of Calcium Chloride Competent Cells	40
2.3.1.11	Preparation of Electro-Competent Cells.....	41
2.3.1.12	Heat Shock Transformation of Competent Cells	41
2.3.1.13	Electroporation Transformation of Competent Cells	41
2.3.1.14	Making Glycerol Stocks.....	41
2.3.1.15	Colony Cracking.....	42
2.3.1.16	Determining cDNA Library Titre.....	42
2.3.1.17	Megadeath Preparation of Plasmid DNA	42
2.3.1.18	Midiprep Preparation of Plasmid DNA.....	43
2.3.1.19	Megaprep Preparation of Plasmid DNA	43
2.3.1.20	Small Scale Kit Preparation of Plasmid DNA.....	43
2.3.1.21	Large Scale Kit Preparation of Plasmid DNA.....	44
2.3.1.22	Cesium Chloride Purification of Plasmid DNA	44
2.3.1.23	Sequencing of Plasmid DNA	44
2.3.2	Protein Chemistry Techniques	45
2.3.2.1	Induction and Preparation of GST or Trx Fusion Proteins	45
2.3.2.2	GST Fusion Protein Affinity Chromatography	45
2.3.2.3	Trx Fusion Protein Affinity Chromatography.....	46
2.3.2.4	Bradford Assay.....	46
2.3.2.5	SDS-PAGE.....	46
2.3.2.6	Concentration and Buffer Exchange of Recombinant Proteins.....	47
2.3.2.7	Thrombin Cleavage of Fusion Proteins.....	47
2.3.2.8	Size Exclusion Chromatography	47
2.3.2.9	GST Pulldown	48
2.3.2.10	Western Blot.....	48

2.3.2.11 NMR Sample Preparation	49
2.3.2.12 NMR Spectroscopy	49
2.3.3 Yeast Two-Hybrid Assay Techniques.....	49
2.3.3.1 Quick Yeast Transformation (QYT)	49
2.3.3.2 Yeast Transformation by Electroporation	50
2.3.3.3 Lithium Acetate Method of Yeast Transformation	50
2.3.3.4 Yeast Protein Extraction.....	51
2.3.3.5 Yeast Two-Hybrid Library Screening	51
2.3.3.6 XGAL Overlay Assay	51
2.3.3.7 Recovering Plasmids From Yeast	51
2.3.3.8 Yeast Colony PCR.....	52
2.3.4 Tissue Culture Techniques	52
2.3.4.1 Culture of Mammalian Cells	52
2.3.4.2 Harvesting Antibodies From Hybridoma Cell Cultures.....	53
2.3.4.3 Transfection of Mammalian Cells.....	53
2.3.4.4 Lysis of Mammalian Cells	53
2.3.4.5 Immunoprecipitation of Proteins Expressed in Mammalian Cells.....	54
2.3.4.6 Preparation of Phagocytic Target For Phagocytosis Assay.....	54
2.3.4.7 Preparation of Phagocytic Cells For Phagocytosis Assay.....	54
2.3.4.8 Phagocytosis by Adherent PMA-U937 Cells.....	54
2.3.4.9 Phagocytosis by Non-Adherent PMA-U937 Cells.....	55
2.3.4.10 Fixing and Immunostaining Cells	55
2.3.4.11 Fluorescence Microscopy.....	56
2.3.5 Digital Imaging.....	56
2.3.6 Photography.....	56

CHAPTER 3: Yeast Two-Hybrid Assay With Tec PHTH Domain	57
3.1 Introduction: Tec-family PHTH Domain	58
3.2 Aims.....	59
3.3 Approach	59
3.3.1 Overview Of Approach	59
3.3.2 Yeast Two-Hybrid System	59
3.3.3 Components of the Yeast Two-Hybrid System.....	60
3.3.3.1 pLexA Vector and Control Vectors Derived From pLexA.....	60

3.3.3.2	pB42AD Vector and Derivatives	60
3.3.3.3	The cDNA Library	61
3.3.4	Preparation For Yeast Two-Hybrid Screen	61
3.3.5	Yeast Two-Hybrid Screen	61
3.3.6	Analysis and Verification of Positive Two-Hybrid Interactions.....	62
3.3.7	Site-Directed Mutagenesis	62
3.4	Results	63
3.4.1	LexA-PHTH Bait and Controls Were Expressed in Yeast.....	63
3.4.2	The Yeast Two-Hybrid System Was Functional.....	63
3.4.3	The Human Liver cDNA Library Was Amplified in Bacteria.....	64
3.4.4	The cDNA Library Has Characteristics of Human Liver Expression.....	64
3.4.5	A Library of Yeast Transformants Was Created.....	65
3.4.6	Isolation of Tec PHTH Domain Potential Protein Ligands.....	66
3.4.7	Yeast Colony PCR Obtained cDNA Library Inserts of Positive Clones	67
3.4.8	Actinin-4 Binds Tec PHTH Domain	68
3.4.9	Site-Directed Mutagenesis of the HIKE-like Motif in Actinin-4 Does not Disrupt Binding of Tec PHTH Domain	69
3.4.10	Minimal PHTH-binding Region of Actinin-4 Contains Spectrin Repeat Three..	70
3.4.11	The PHTH Domain Functional Unit Is Required for Actinin-4-binding	71
3.5	Discussion	72
3.5.1	Identification of Actinin-4 as a Ligand of Tec PHTH Domain.....	72
3.5.2	Actinin-4 is a Cytoskeleton Structural Protein.....	73
3.5.3	Actinin-4 Is A Newly Identified Isoform of α -Actinin	74
3.5.4	Possible Functions Of Actinin-4	75
3.5.5	Ligands of Non-Muscle α -Actinins.....	76
3.5.6	Cytoskeletal Reorganisation Downstream of Tec Signalling.....	77
CHAPTER 4:	Tec And Actinin-4 In Mammalian Cells	79
4.1	Introduction	80
4.2	Aims	82
4.3	Approaches.....	82
4.4	Results	84
4.4.1	Endogenous Tec And Actinin-4 Colocalise In Mammalian Cells	84

4.4.2	Endogenous and Epitope-Labelled Tec and Actinin-4 Have Varying Degrees of Solubility	85
4.4.3	Endogenous Tec and Actinin-4 Both Exist in Soluble and Insoluble Fractions of Mammalian Cell Lysates.....	86
4.4.4	Solubility of Epitope-Tagged Tec and Actinin-4 in COS-1 Cells.....	86
4.4.5	Differing Solubilities Of Various Tec Proteins Expressed As C-Terminal Fusions Of EGFP	87
4.4.6	Mouse and Human Tec PHTH Domain and Mouse Tec4 and Tec3 Were Expressed in COS-1 Cells as N-Terminal Fusions of EGFP	88
4.4.7	Site-Directed Mutagenesis was Used to Alter Specific Residues of Tec Regulatory Domains.....	89
4.4.8	Substitution Mutants Have Different Solubility to Wildtype Tec.....	89
4.4.9	The Majority of Myc Tagged Truncated Actinin-4 Partitions in The Soluble Fraction.....	90
4.4.10	Expression of the EGFP-Tec Proteins was Verified by Fluorescence Microscopy	91
4.4.11	Endogenous Tec And Actinin-4 Interact In Mammalian Cells.....	92
4.4.12	Epitope-Labelled Tec and Actinin-4 Interact In Mammalian Cells	93
4.4.13	EGFP-Tec Variants Have Similar Myc-Actinin-4-binding Characteristics.....	93
4.4.14	Purified Actinin-4 Repeat-3 Binds to EGFP-Tec.....	94
4.4.15	Involvement of Tec and Actinin-4 in Phagocytosis by Differentiated U937 Cells	94
4.5	Discussion	96
4.5.1	The Interaction of Tec and Actinin-4 in Mammalian Cells	97
4.5.2	The Effect of Specific Mutations on Tec Subcellular Localisation and Solubility	98
4.5.3	The Effect of Actin-binding Residues on Tec and Actinin-4 Binding.....	99
4.5.4	Activation of Tec Kinases and Cytoskeletal Association	101
4.5.5	Biological Consequences of Tec and Actinin-4 Interaction.....	102
4.5.6	A Potential Role for Tec and Actinin-4 in Phagocytosis	103
CHAPTER 5:	Actinin-4 Repeat-3 Protein Structure	104
5.1	Introduction	105
5.1.1	Background	105

5.1.2	Structural Determination by NMR Spectroscopy	107
5.2	Aims	108
5.3	Approaches	108
5.4	Results	109
5.4.1	Recombinant PHTH Domain Protein Expression and Purification	109
5.4.2	Recombinant Trx-R817 Protein Expression and Purification	110
5.4.3	Cloning of GST-Rpt3 For Expression and Purification	111
5.4.4	Optimisation of GST-Rpt3 Expression	111
5.4.5	Optimisation of GST-Rpt3 Fusion Protein Purification	112
5.4.6	Optimisation of GST-Rpt3 Thrombin Digestion	113
5.4.7	Purification of Rpt3 from GST	113
5.4.8	Dimerisation Analysis of Actinin-4 Protein Fragments	114
5.4.9	Rpt3 Protein Sample Preparation For NMR Spectroscopy Analysis	114
5.4.10	1D NMR Spectroscopy Experiments	115
5.4.11	2D NMR Spectroscopy Experiments	115
5.4.12	Predicted Structure of PHTH and Rpt3 Proteins	115
5.5	Discussion	116
5.5.1	Production of Recombinant PHTH domain and Rpt3 Domain Proteins	116
5.5.2	Structural Analysis of Actinin-4 Spectrin Repeat-3 Domain	118
CHAPTER 6:	Final Discussion and Future Directions	120
6.1	Final Discussion	121
6.1.1	Mechanism of Activation of Tec-family Kinases	121
6.1.2	Isoforms of Tec	122
6.1.3	Signalling of Tec Kinases to the cytoskeleton	123
6.1.4	The Role of the PHTH Domain in Tec Signalling	124
6.1.5	Actinin-4 is A Novel Binding Protein of Tec PHTH Domain	124
6.1.6	The Implications of Tec and Actinin-4 Binding	128
6.2	Future Directions	129
6.2.1	Are α -actinins Substrates of Tec-family Kinases?	129
6.2.2	Do Tec Kinases and α -actinins Co-redistribute in Stimulated Cells?	129
6.2.3	What are the Determinants of Tec and Actinin-4 Binding?	130
6.2.4	What is the Effect of Preventing Tec Signalling to Actinin-4?	130
6.3	Concluding Remarks	131

List of Figures

Chapter 1

Figure 1.1	Non-receptor Protein Tyrosine Kinases.....	3
Figure 1.2	Sequence Alignment of Tec Family Kinases.....	5
Figure 1.3	Activation of Tec Family Kinases	5
Figure 1.4	PHTH Domain Structure	7
Figure 1.5	SH3 Domain Structure.....	9
Figure 1.6	SH2 Domain Structure.....	10
Figure 1.7	Signalling Pathways Involving Tec Family Kinases	11

Chapter 3

Figure 3.1	Putative Binding Sites of Btk PHTH domain	58
Figure 3.2	Yeast Two-Hybrid Assay	59
Figure 3.3	Nucleotide and Amino Acid Sequence of Tec PHTH Domain	63
Figure 3.4	LexA Fusion Protein Expression in Yeast.....	63
Figure 3.5	Amino Acid Sequence Alignment of Human and Mouse Tec	64
Figure 3.6	Mouse Tec PHTH Domain Model With Substitutions Highlighted.....	64
Figure 3.7	Characterisation of the cDNA Library	65
Figure 3.8	Yeast Two-Hybrid Screen	66
Figure 3.9	Summary of the Yeast Two-Hybrid Screen.....	66
Figure 3.10	Analysis of Yeast Two-Hybrid Potential Positive Clones by YC PCR.....	67
Figure 3.11	Nucleotide and Amino Acid Sequence of Clone R817	68
Figure 3.12	Actinin-4 Protein Domain Structure and PHTH domain Binding Region	69
Figure 3.13	Mutation of the HIKE-like Motif in Clone R817	69
Figure 3.14	Mapping the Interacting Residues of R817 and Tec	70

Chapter 4

Figure 4.1	Colocalisation of Tec and Actinin-4 in Adherent Cells.....	85
Figure 4.2	Western Blot of Endogenous Tec and Actinin-4 Separated into Triton-X100 Soluble and Insoluble Fractions	86
Figure 4.3	Cloning of EGFP-Tec	87
Figure 4.4	Western Blot of COS-1 Cells Transfected With EGFP-Tec Plasmids	87

Figure 4.5	Cloning of Tec-EGFP	88
Figure 4.6	Electropherograms of Tec Nucleotide Sequence Before and After Site Directed Mutagenesis	89
Figure 4.7	Analysis of Solubility of EGFP-Tec Variants	89
Figure 4.8	Cloning of Epitope Tagged Actinin-4 Lacking the Actin Binding Domain, Myc-A4	90
Figure 4.9	Western Blot of COS-1 Cells Transiently Transfected With pXMT2-Myc-A4	90
Figure 4.10	Subcellular Localisation of EGFP and EGFP-PHTH Wild Type and Actin Binding Mutant	91
Figure 4.11	Subcellular Localisation of EGFP-Tec4 and Actin Binding Mutant	91
Figure 4.12	Subcellular Localisation of EGFP-Tec3 Wild Type, Actin Binding Mutant and Y187E Mutant	91
Figure 4.13	Co-immunoprecipitation of Endogenous Tec with Actinin-4	92
Figure 4.14	Co-immunoprecipitation of Epitope Tagged Tec and Actinin-4 From Transiently Co-transfected COS-1 Cells	93
Figure 4.15	Co-immunoprecipitation of Epitope Tagged Tec Variants and Actinin-4 From Transiently Co-transfected COS-1 Cells	93
Figure 4.16	Co-immunoprecipitation of Exogenous GST-Rpt3 With EGFP-Tec From Transiently Transfected COS-1 Cells	94

Chapter 5

Figure 5.1	GST-PHTH Domain Fusion Protein Expression and Purification	109
Figure 5.2	Trx-R817 Fusion Protein Expression and Purification	110
Figure 5.3	Cloning of GST-Rpt3 Expression Plasmid	111
Figure 5.4	Optimisation of GST-Rpt3 Fusion Protein Expression and Purification	112
Figure 5.5	Purification of GST-Rpt3 Fusion Protein	112
Figure 5.6	Optimisation of Thrombin Digestion of GST-Rpt3 Fusion Protein	113
Figure 5.7	Purification of Rpt3 Protein From GST Protein	113
Figure 5.8	Actinin-4 Repeat-3 is Monomeric	114
Figure 5.9	1D ¹ H NMR Spectra of Actinin-4 Rpt3	114
Figure 5.10	2D ¹ H NMR Spectra of Actinin-4 Rpt3	115

List of Abbreviations

Amp	ampicillin
APS	ammonium persulphate
ATP	adenosine triphosphate
BCR	B cell receptor
bp	base pair
BSA	bovine serum albumin
C-	carboxyl-
cDNA	complementary DNA
cFn	cellular fibronectin
CFU	colony forming units
CIP	calf intestinal phosphatase
COSY	correlation spectroscopy
DMEM	Dulbecco's modified eagles medium
DMF	dimethyl fluoride
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DQF-COSY	double-quantum-filtered correlation spectroscopy
DTE	dithioerythritol
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
EPO	Erythropoietin
EtBr	ethidium bromide
F-actin	filamentous actin
FAK	focal adhesion kinase
FBS	fetal bovine serum
Fc- γ R	Fc-gamma receptor
FITC	fluorescein isothiocyanate

G-CSF	granulocyte colony-stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony- stimulating factor
GST	glutathione-S-transferase
HRP	horseradish peroxidase
HSQC	Heteronuclear Single Quantum Coherence
IL-3	Interleukin-3
IP	immunoprecipitation
IP ₃	inositol 1,4,5-trisphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
ITAM	immunoreceptor tyrosine-based activation motif
Kan	kanamycin
kb	kilobase pair
kDa	kilo Dalton
LB	Luria broth
N-	amino-
NOE	peak in NOESY spectrum resulting from dipolar coupling
NOESY	nuclear Overhauser enhancement spectroscopy
OD _{600nm}	optical density at 600 nm wavelength
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PEG	polyethylene glycol
PFA	paraformaldehyde
PH	pleckstrin homology
PHTH domain	PH domain and Btk motif of the TH domain
PI 3,4,5-P3	phosphatidylinositol 3,4,5-trisphosphate
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PLC	phospholipase C
PLL	poly-L-lysine
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonylfluoride

ppm	parts per million
PRR	proline-rich region
PSI	pounds per square inch
PTK	protein tyrosine kinase
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Src homology
TAE	Tris-acetate EDTA
TBS	Tris buffered saline
TCR	T cell receptor
TE	Tris-EDTA
TH	Tec homology
TOCSY	total correlation spectroscopy
Tris	Tris(hydroxymethyl)aminoethane
TRITC	Tetramethylrhodamine isothiocyanate
TTBS	Tris buffered saline/0.1% Triton-X-100
UV	ultra violet
WCE	whole cell extract
XGAL (BCIG)	5-bromo-4-chloro-3-indoyl-galactopyranoside
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

CHAPTER

1

Introduction And Literature Review

1.1 Introduction

The work described in this thesis is focussed on identifying and characterising protein ligands of the pleckstrin homology and Tec homology domain of Tec kinase using biochemical, molecular and cell biology approaches. This introductory chapter summarises signal transduction pathways linked to Tec-family kinases, the modular protein domains that contribute to the regulation of Tec-family kinase activity and ligand interactions of those modular domains. There is particular emphasis on pleckstrin homology domains and their binding partners. Finally, the yeast two-hybrid screen, which was the crucial starting point of this work is summarised.

1.2 Non-Receptor Protein Tyrosine Kinases

Non-receptor protein tyrosine kinases (PTKs) are a subset of intracellular proteins that are involved in signal transduction. These evolutionarily refined molecules underpin one of the most elemental forms of communication, a process that is fundamentally important in the life of biological systems. They function in the accurate propagation and amplification of signals to effect changes in cellular architecture, metabolism and gene expression. Their activity is tightly regulated and, importantly, deregulated or aberrant activation of individual PTKs is known to cause illnesses such as immune deficiencies and cancer (Pawson and Scott, 1997). Diseases can result from mutations in the gene, leading to mutant or null protein expression. Therefore, understanding signalling pathways and how the components interact will aid in the development of therapeutic drugs to modulate inappropriate signal transduction.

At the molecular level, classical signalling to non-receptor PTKs originates from ligand-specific activation of cell surface receptors on target cells (Taniguchi, 1995). Extracellular ligand binding induces receptor conformational change and subsequent receptor activation. The ensuing series of recruitment and activation of certain intracellular molecules, generally kinases and adapter proteins, activates particular biochemical pathways depending on the cell type and the initial stimulus (Taniguchi, 1995).

Proteins are recruited according to the binding preference of their constituent domains, which in non-receptor PTKs include one or more Src homology (SH) domains and a unique region (Pawson and Gish, 1992, Bolen, 1993). SH domains are defined by regions of sequence homology to Src, one of the earliest recognised tyrosine kinases (Koch *et al.*, 1991). In general, domains are independently folding and functional units.

The enzymatic activity of the catalytic or tyrosine kinase (SH1) domain of non-receptor PTKs is influenced by protein or phospholipid interactions mediated with the amino- (N-) terminal region, often containing SH3 and/or SH2 domains (Koch *et al.*, 1991, Bolen, 1993, Pawson, 1995). In Src-family kinases, a myristoylated glycine residue at position 2 immediately precedes the unique region. This functions to tether the molecule at the cell membrane in close proximity to cell surface receptors.

Activation of Src-family kinases is one of the earliest steps of intracellular signalling and requires a number of distinct steps. This includes targeting of the PTK to a specific cellular site, such as the cell membrane, and reversible chemical modification, such as phosphorylation of tyrosine residues (Cooper and Howell, 1993, Pawson, 1997). Ultimately, through the activation of signalling pathways, the production of second messengers combined with, as yet, incompletely defined mechanisms leads to rearrangement of the cytoskeleton, cell growth, cell differentiation, changes in gene transcription or apoptosis. Overlapping, yet distinct subsets of non-receptor PTKs are thought to be activated by different receptors to transmit their signal (reviewed in Ihle, 1995). It is thought that divergence of pathways occurs at the level of relative protein binding and the use of priming sites that bind one set of ligands but not another. Binding of proteins to the priming sites is expected to facilitate further specific protein binding events that lead to the propagation of select signalling pathways.

Currently, eight families of non-receptor PTKs are recognised. Figure 1.1A illustrates a simplified comparison of the known non-receptor PTKs and their recognised common domains (adapted from Taniguchi, 1995) while Figure 1.1B illustrates the Tec kinase family members. The similarity of the common domains is thought to reflect the general functions of the PTK family members. Unique domains are believed to assist in mediating the interactions and activity of individual PTKs. It is likely that all non-receptor PTKs are involved in cellular signal transduction, with their main difference being restriction to specific cell types or lineage-specific expression (Bolen, 1993). For instance, Src-family kinases, including Src, Fyn, Yes and Lyn, are expressed in a broad range of tissues with particularly high levels in haematopoietic and neural cells (Lowell and Soriano, 1996). While they are all expressed during early embryonic development, not all are coexpressed in any given cell type (Lowell and Soriano, 1996).

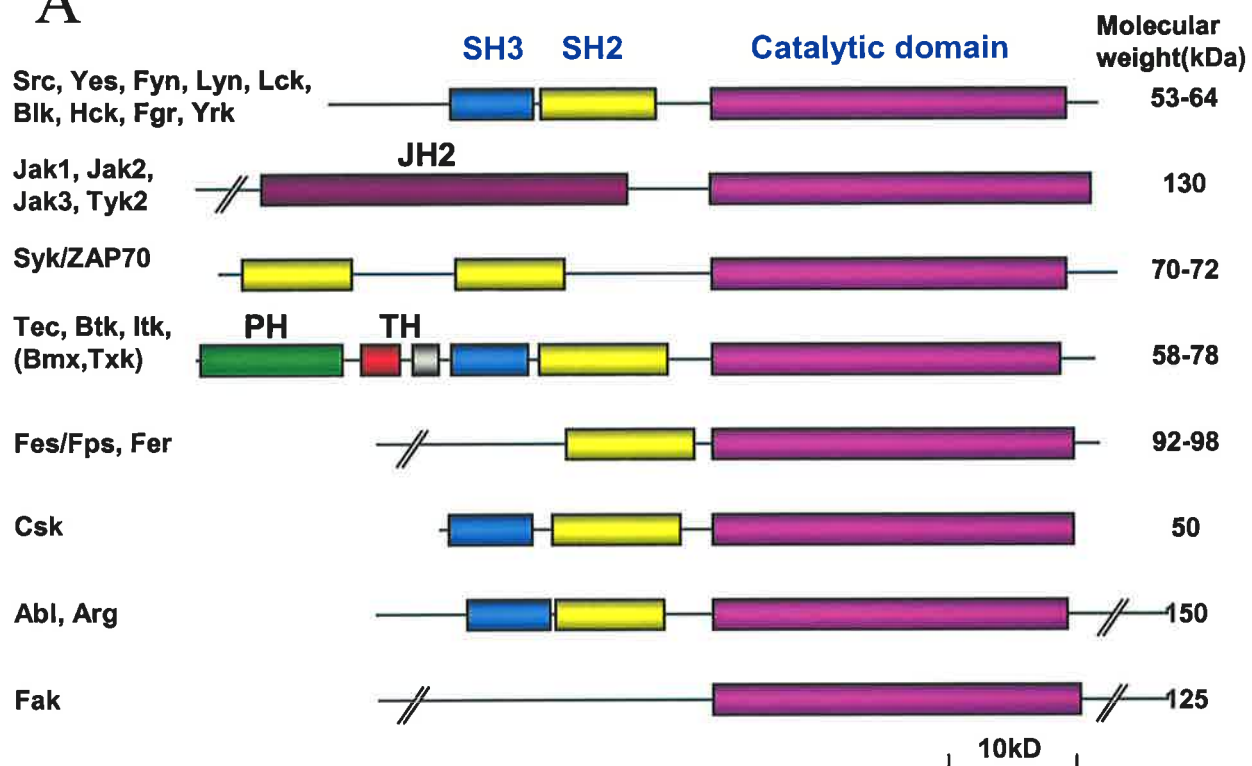
Figure 1.1 Non-receptor Protein Tyrosine Kinases

A The major common domains and the relative molecular weights of the eight different families of non-receptor protein tyrosine kinases are shown (adapted from Taniguchi, 1995).

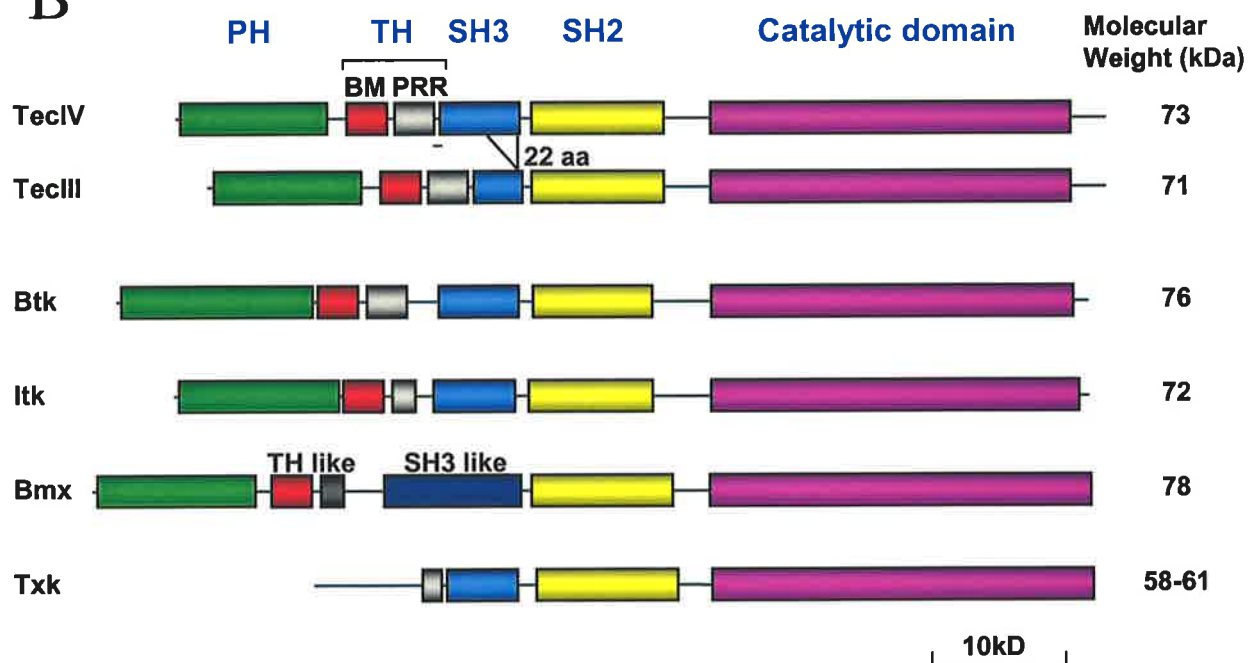
B Linear representation of the domain structure of Tec family of non-receptor protein tyrosine kinases.

SH: src homology
JH: janus homology
PH: pleckstrin homology
TH: Tec homology
BM: Btk motif
PRR: proline rich region

A



B



1.3 Tec-Family Protein Tyrosine Kinases

1.3.1 Members and Expression

Tec tyrosine kinase belongs to the second largest family of non-receptor PTKs, second in size to the structurally related Src-family kinases. Tec-family kinases include Tec (*Tyrosine kinase expressed in hepatocellular carcinoma*) (Mano *et al.*, 1990, Mano *et al.*, 1993), Btk (*Bruton's tyrosine kinase*) (Vetrie *et al.*, 1993, Tsukada *et al.*, 1993), Itk (*Interleukin-2 inducible T cell kinase*) (Siliciano *et al.*, 1992, Heyeck and Berg, 1993, Yamada *et al.*, 1993), Bmx (*Bone marrow expressed kinase*) (Tamagnone *et al.*, 1994) and Txk (*T cell expressed tyrosine kinase*) (Haire *et al.*, 1994, Hu *et al.*, 1995), as well as other non-mammalian homologues (reviewed in Smith *et al.*, 2001). They transmit intracellular signals from a variety of activated cellular receptors and their activation is downstream of that of Src-family-kinases, Syk-family-kinases and phosphatidylinositol-3-kinases (PI3Ks) (August *et al.*, 1997). The stimulatory signals lead to cytoskeletal remodelling upon receptor-mediated interactions, which culminates in cellular gene expression changes. Expression of Btk, Itk and Txk is restricted to haematopoietic cells while Tec and Bmx also exist in non-haematopoietic tissues (Table 1.1, adapted from Smith *et al.*, 2001 and references therein).

Table 1.1 Expression Pattern of Tec-family PTKs (adapted from Smith *et al.*, 2001)

PTK	Expression Pattern
Tec	Tec3 and Tec4 are the major splice forms. Both are found in the embryo (brain, lung, spleen) and in adult intestine and spleen. Tec4 predominates in the placenta while Tec3 predominates in adult liver, kidney and embryonic limb. Tec expression without splice type designation is seen in thymus, bone marrow, endothelial and haematopoietic cells and melanocytes.
Itk	Thymus, spleen, lymph node, T lymphocytes, NK and mast cells.
Btk	Bone marrow, spleen, all haematopoietic cells except T lymphocytes and plasma cells.
Bmx	Endothelium of large arteries, fetal endocardium, adult endocardium of the left ventricle, bone marrow, lung, testis, granulocytes, myeloid cell lines and prostate cell lines.
Txk	Thymus, spleen, lymph node, T lymphocytes, NK cells, mast cell lines and myeloid cell line.

Splice variants that affect the composition of the N-terminal modular domains have been described for Tec and Txk. There are two predominantly expressed Tec isoforms, Tec3 and Tec4 (Mano *et al.*, 1993, Merkel *et al.*, 1999, Atmosukarto, 2001). Tec3 has a 22 amino

acid truncation in the C-terminus of the SH3 domain compared with Tec4 and it is, thus, expected that the two isoforms have different activation characteristics.

Two splice variants of Txk have been described affecting the N-terminal unique region; one which encodes a 5 or 6 cysteine-string motif that can be palmitoylated and another which lacks this motif and has the potential for nuclear localisation (reviewed in Lewis *et al.*, 2001). Although there are significant differences in the N-terminal unique regions of Txk compared with other Tec-family kinases, Txk is included in the Tec-family of non-receptor PTKs due to homology in the SH3-catalytic domain region (Haire *et al.*, 1994). Furthermore, the genes for Tec and Txk are contiguous; they are separated by 2.6 kb on mouse chromosome 5 (Merkel *et al.*, 1999) and 1.5 kb on human chromosome 4p12 (Ohta *et al.*, 1996) and it is therefore expected that one arose by gene duplication of the other.

1.3.2 Regulation of Enzyme Activity

The activation of the carboxyl- (C-) terminal tyrosine kinase domain is allosterically regulated by the N-terminal modular domains and their ligands (Nguyen and Lim, 1997, Brazin *et al.*, 2000). Tec and Src-family kinases have similar domain structure and similar methods of enzymatic regulation. They both contain SH3, SH2 and tyrosine kinase domains. However, Tec-family kinases lack the regulatory C-terminal tyrosine residue and the N-terminal myristoylation present in Src kinases (Tsukada *et al.*, 1993). The Tec-family kinases Tec, Btk and Itk contain pleckstrin homology (PH) and Tec homology (TH) domains in their N-terminal unique region (Figure 1.1B) (Shaw, 1993, Smith *et al.*, 1994) and this distinguishes them from all other non-receptor PTK families. The TH domain is made up of a Zn²⁺-binding (Hyvonen and Saraste, 1997) Btk motif and one or two copies of a proline-rich region (PRR; see Figure 1.2A) (Smith *et al.*, 1994, Vihinen *et al.*, 1994b) that serves as the intramolecular ligand for the adjacent SH3 domain (Andreotti *et al.*, 1997, Pursglove *et al.*, 2002). Bmx and Txk are atypical Tec kinase family members since Bmx lacks a PRR and has a modified SH3 domain while Txk lacks a PH domain and Btk motif (Haire *et al.*, 1994, Tamagnone *et al.*, 1994). Sequence alignment shows that a high level of sequence conservation exists for Tec family kinase proteins, both amongst the different family members and with the SH3-kinase domain region of Src (see Figure 1.2 A-D).

To summarise the activation steps, tyrosine kinase activity is regulated by an ordered series of interactions mediated with the different protein domains of the molecule (see Figure 1.3A). During activation, phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5-P₃) produced by

Figure 1.2 Sequence Alignment of Tec Family Kinases

- A Alignment of mouse Tec family kinase sequences obtained from GenBank generated using the MULTALIN program (Corpet, 1988). Also included is the sequence of mouse Src. The mouse Tec kinase sequences were compared to the Src sequence to determine SH3 (blue), SH2 (orange) and kinase (pink) domain boundaries. The typical poly-proline sequences characteristic of SH3 recognition sites are underlined and where more than one site was identified, additional overlapping sites are marked in italics. The Tec family kinase specific PH domain (red), Btk motif and proline rich region (grey) are indicated. Residues that have a high consensus value (> 90%) are shown in red while residues that have a low consensus value (50%-90%) are shown in blue. All other residues are shown in black. GenBank accession numbers: Tec: P24604, Itk: Q03526, Btk: P35991, Bmx: NP_033889, Txk: P42682 and Src: P05480.
- B Tabulation of amino acid sequence identity between the full-length protein sequences described above.
- C Tabulation of amino acid identity between the C-terminal SH3-kinase domain protein sequences described above.
- D Tabulation of amino acid identity between the N-terminal PTH domain sequences described above.

SH: Src homology

PH: pleckstrin homology

A

		PH domain ->																				120
Tec	MNFNTILEEI	LIKRSQQKKK	TSPLNYKERL	FVLTKSVLSY	YEG--R-AEK	KYRKGVIDIS	KIKCIVEIV--	-----	---KNDDGVIP	CQNKFPFQ--	----VVHDAN	TLYIFAPSPQ										
Itk	MNMFILLEEQ	LIKKSQQKRR	TSPSNFKVRF	FVLTKASLAY	FED--RHGKK	RTLKGSIELS	RIKCIVEIV--	-----	---KSDIS-IP	CHYKYPFQTL	VYLQVVDHNY	LLYVFPADCE										
Btk	MAAVILESI	FLKRSQQKKK	TSPLNFKKRL	FLLTVHKLSY	YEYDFERGRR	GSKKGSIDVE	KITCVETVIP	EKNPPPERQI	PRRGEESSEM	EQISIIERFP	YPFQVVDYBG	PLYVFSPTTE										
Bmx	MESKSIIEEL	LLKKSQQKKK	MSPNNYKERL	FVLTKTSLSY	YEY--DKMKR	GSRKGSIEIK	KIRCVEKV--	-----	---NLEEQTP	VERQYPFQ--	----IVYKDG	LLYVYASNEE										
Txk												M ILSSYSFQS										
Src												MGSNK										
		Btk motif ->										Proline Rich Region ->										240
Tec	SRDRVWKLLK	EEIKNNNNIM	IKYHPKFWAD	GSYQCCRQTE	KLAPGCE---	-----KYNL	FESSIRKTLT	PAPEIKK--R	RPPPPIP---	PEEENTEIV	VAMYDFQATE	AHDLRLERGQ										
Itk	SRQRWVLTLLK	BETRNNSLV	SKYHPNFWMD	GRWRCCSQLE	KPAVGCA---	-----PYDP	SKNASKKPLP	PTPEDNR--R	S-----	-FQEPETLV	IALYDYQTN	PQELALRCDE										
Btk	LKRWIHQLLK	NVIRYNSDLV	QKYHPCFWID	GQYLCCSQTA	KNAMGCQILE	NRNGSLKPGS	SHRKTCKPLP	PTPEEDQILK	KPLPPEPTAA	PISTTELKVV	VALYDYMPMN	ANDLQLRKGE										
Bmx	SRQWLKALQ	KEIRGNPHLL	IKYHSGFFVD	GKFLCCQSC	KAAPGCTLWE	---AYADLHI	AISDEKHRA	TFPERLLKIP	RAVPVLK---	-MDASSSGAI	LPQYDSYSK	SCGSQPTSNI										
Txk	VLCCCCRCRS	VQKRQVRTQI	SLSREEELSE	KHSQRQRPWF	AKLMGKT---	---QSNRGG	VQPSKRKPLP	FLPQE-----	---	PPDERIQV	KALYDFLPR	PGNLALKRAE										
Src	SKPKDASQRR	RSLEPSENVH	GAGGAFPASQ	TPSPKASADG	HRGPSAAFVP	PAAEPKLFEG	FNSSDTVTSP	ORAGAL---	---	AGGVTTTF	VALYDYESRT	ETDLSFKKGE										
		SH2 domain ->																				360
Tec	EYIILEKN--	---DLHWWR	ARDKY-GSEG	YIPSNYV---	-----	-----TGK	KSNNDQYEW	YCRNTNRSA	EQLLRT-EDK	EGGFVVRDSS	QP-GLYTVSL	YTKFGGEGSS										
Itk	EYYLDDSS--	---EIHWWR	VQDKN-GHEG	YAPSSYL---	-----	-----VEK	SPNNLETYEW	YNSISRDKA	EKLLLD-TGK	EGAFVVRDSR	TP-GTYTVSV	FTKAIISENP										
Btk	EYFILEES--	---NLPWWR	ARDKN-GQEG	YIPSNYI---	-----	-----TEA	E-DSIEMYEW	YSKHMTRSQA	EQLLKQ-EGK	EGGFVVRDSS	KA-GKYTVSV	FAKSTGEPQG										
Bmx	RYIPREDC--	---P-DWWQ	VRKLK-SEED	IACSNOLERN	IASHSTSKMS	WGFPSSSSSE	EEENLHAYDW	FAGNISRSQS	EQLLRQ-KGK	EGAFVVRNNS	QM-GMYTVSL	FSKAVNDKKG										
Txk	EYLILERC--	---DPHWWK	ARDRF-GNEG	LIPSNYV---	-----	-----TEN	RLANLEIYEW	YHKNITRNQT	ERLLRQ-EAK	EGAFVVRDSR	HL-GSYTISV	FTARRHTQS										
Src	RLQIVNTRK	VDVREGDWWL	AHSLSTGQTG	YIPSNYV---	-----	-----A	PSDSIQAEW	YFGKITRES	ERLLNAENP	RGTFLVRESE	TTKGYCLSV	-SDFDNAKGL										
		Kinase domain ->																				480
Tec	GFRHYHIKET	ATSPKKYILA	EKHAFGSIPE	IEYHKHNAA	GLVTRLRYPV	STKGKNAPT	AGFSYDKWEI	NPSELTFMRE	LGSGLFVVVR	LGKWRAQYKV	AIKAIREGAM	CEEDFIEBAK										
Itk	CIKHYHIKET	NDSPKRYIVA	EKYVFDISPL	LIQYHQYNGG	GLVTRLRYPV	CSWRQKAPVT	AGLRYGKWI	QPSELTFVQE	IGSGQFGLVH	LGYNLNDKV	AIKTIQECAM	SEEDFIEBAE										
Btk	VIRHYVVCST	PQS--QYILA	EKHLFSTIPE	LINVHQHNSA	GLISRLKYPV	SKQKNAPST	AGLGYGSWEI	DPKDLTFLKE	LGTGQFVVVK	YGKWRGQYDV	AIKMIREGAM	SEDEFIEBAK										
Bmx	TIKHYHVHTN	AEN--KLYLA	ENYCFDSIPK	LIHYHQHNSA	GMITRLRHPV	STKANKVPVS	VALGSGIWEL	KREEITLLKE	LGNGQFVVVK	LGQWKQYDV	AVKMIKEGAM	SEDEFIEBAK										
Txk	SIKHYQIKKN	DSG--QWYIT	ERHLFPSVPE	LIQYHQYNA	GLISRLRYPV	GLLGSLPAT	SGFSYEKWEI	DPSELAFVKE	IGSGQFVVVK	LGWEVRAHIPV	AIKAINEGSM	SEEDFIEBAK										
Src	NVKHYKIRKL	DSG--GFYIT	SRTQFNSLQ	LVAYYSKHAD	GLCHRL---T	TVCPTSKPQT	QGLAKDAWEI	PRESLRLEVK	LQGCFCGEVW	MGTWNGTTRV	AIKTLKPGTM	SPEAFLEBAQ										
																						600
Tec	VMMKLTHPKL	VQLYGVCTQ	KPIYIVTEFM	ERGCLLNFLR	QRQGH-FSRD	MLLSMCQDVC	BGMLEYLERN	FIHRDLAARN	CLVNEAGVVK	VSDFGMARYV	LDDQYTSSSG	AKFPVKWCPP										
Itk	VMMKLSHPKL	VQLYGVCTEQ	APICLVFEFM	EHGCLSDYLR	SQRGL-FAAE	TLLGMCLDVC	BGMAYLEKAC	VIHRDLAARN	CLVGENQVIK	VSDFGMTRFV	LDDQYTSSTG	TKFPVKWASP										
Btk	VMMNLSHEKL	VQLYGVCTKQ	RPIFIITEYM	ANGCLLNLYR	EMRHR-FQTQ	QLEMCCKDVC	EAMEYLESKQ	FLHRDLAARN	CLVNDQGVVK	VSDFGLSRYV	LDDQYTSVSG	SKFPVRSWSP										
Bmx	TMMKLSPKPL	VKFYGVCSKK	YPIYIVTEYI	TNGCLLNLYK	SHGKG-LESC	QLEMCYDVC	BGMAFLESHQ	FIHRDLAARN	CLVSDLSVK	VSDFGMTRYV	LDDQYVSSVG	TKFPVKWASP										
Txk	VMMKLSSHRL	VQLYGVCIQ	KPLIYIVTEFM	ENGCLLDYLR	ERKQG-LQKA	LILLSMCQDVC	BGMAYLERSC	YIHRDLAARN	CLVSSACVVK	ISDFGMARYV	LDDQYVSSSG	AKFPVKWCPP										
Src	VMKKLRHEKL	VQLYAVVSEE	-PIYIVTEYM	NKGSLLDFLK	GETGKYLRPL	QLVDMASQIA	SGMAYVERMN	YVHRDLAARN	ILVGENLVCK	VADFGLARLI	EDNEYTARQ	AKFPVKWASP										
																						702
Tec	EVFNYSRFSS	KSDVWSFGVL	MWEIFTEGRM	PFEKNTNYEV	VTMVTGRHRL	HRPKLASKYL	YEVMLRCWQE	RPEGRPSFED	LLRTIDELVE	CEETFGR												
Itk	EVFSFSRYSS	KSDVWSFGVL	MWEVFSEKGI	PYENRNSNEV	VEDISTGFR	YKPLASCHV	YQIMNHCWKE	KPEDRPPFSQ	LLSQLAEIAE	AGL												
Btk	EVLMSKFS	KSDIWAFGVL	MWEIYSLGKM	PYERFTNSET	AEHIAQGLRL	YRPHLASERV	YTIMYSCWHE	KADERPSFKI	LLSNILDVMD	EES												
Bmx	EVFHYFKYSS	KSDVWAFGIL	MWEVFSLGKQ	PYDLYDNSEV	VVKVSQGHRL	YRQLASDTI	YQIMYSCWHE	LPEKRPTFQ	LLSAIEPLRE	QDKP												
Txk	EVFHFNKYSS	KSDVWSFGVL	MWEVFTEGKM	PFEKNSNLQV	VEAISQGFRL	YRPHLAPMTI	YRVMYSCWHE	SPKGRPTFAE	LLQVLEIAE	TW												
Src	EAALYGRFTI	KSDVWSFGIL	LTELTKGRV	PYPGMVNRV	LDQVERGYRM	PCPECPESL	HDLMCQWRK	EPERPTFEY	LQAFLEDYFT	STEPQYQPGE	NL											

B

	Bmx	Btk	Itk	Tec	Txk
Btk	49%				
Itk	47%	52%			
Tec	47%	55%	57%		
Txk	44%	48%	51%	53%	
Src	32%	33%	34%	35%	33%

C

	Bmx	Btk	Itk	Tec	Txk
Btk	52%				
Itk	50%	54%			
Tec	48%	59%	58%		
Txk	48%	56%	59%	61%	
Src	33%	34%	34%	35%	33%

D

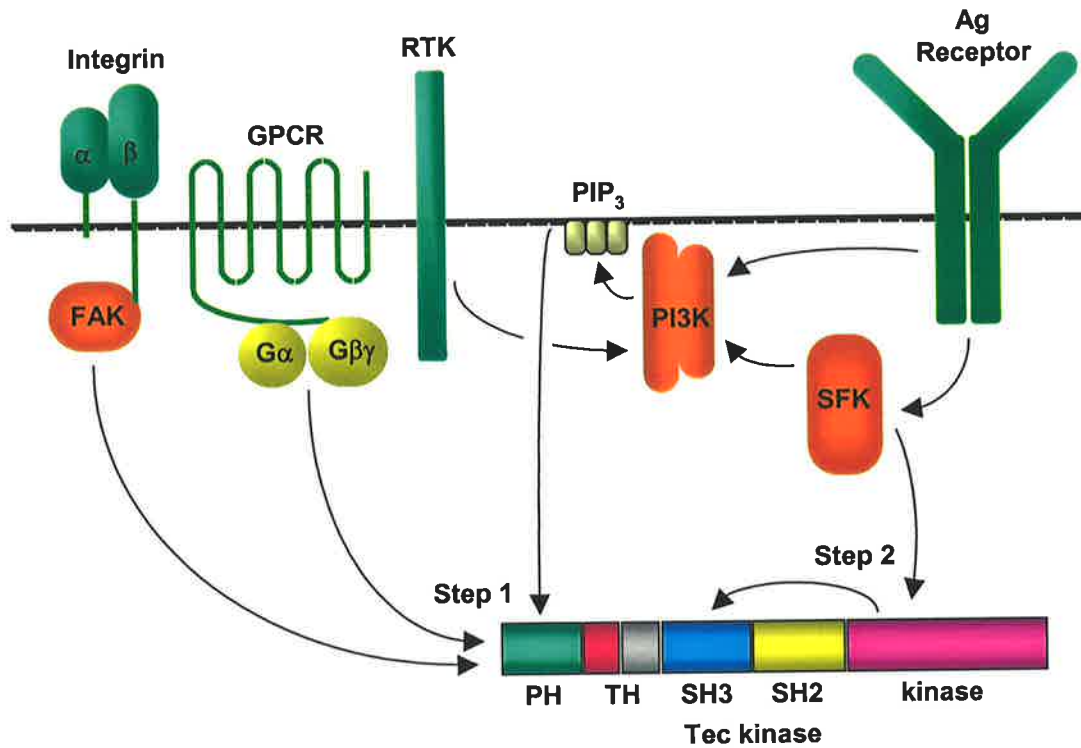
	Bmx	Btk	Itk	Tec	Txk
Btk	40%				
Itk	38%	45%			
Tec	38%	43%	50%		
Txk	7%	17%	22%	14%	
Src	9%	18%	18%	18%	18%

Figure 1.3 Activation of Tec Family Kinases

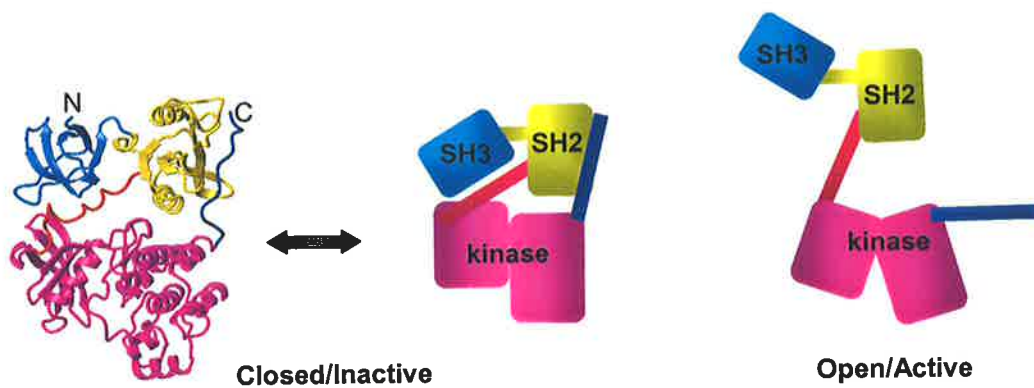
- A Two-step model of Tec kinase activation (adapted from Figure 1 in Lewis *et al.*, 2001). Step 1: The products of PI3K engage and recruit the Tec kinase PH domain to the membrane. Alternatively, protein-protein interactions such as with the FERM domain of FAK or heterotrimeric G-protein subunits, may assist in this step of activation. Step 2: once at the membrane, Tec kinases are phosphorylated on a tyrosine in their activation loop by a Src-family kinase (SFK). Subsequently, the SH3 domain is autophosphorylated.
- B Crystal structure and schematics of the closed and open conformations of Src family kinases. At left is a ribbons diagram showing intramolecular interactions in the tertiary structure of the Src protein tyrosine kinase. The SH3 domain is shown in blue, the SH2 domain is shown in yellow and the tyrosine kinase domain is shown in pink. The linker region between the SH2 and kinase domains, which acts as an intramolecular ligand of the SH3 domain, is shown in red. The carboxyl-terminal residues that contain a phosphotyrosine residue and participate in an intramolecular interaction with the SH2 domain are shown in navy. These intramolecular interactions lock the molecule in a conformation that simultaneously disrupts the kinase active site and sequesters the binding surfaces of the SH2 and SH3 domains and, thus, holds the Src protein in a “closed” inactive state (Xu *et al.*, 1997). The positions of the amino-terminus (N) and carboxyl-terminus (C) of the molecule are indicated. The figure was generated using MOLMOL software (Koradi *et al.*, 1996) by Dr Kasper Kowalski. PDB code: 1fmk (Xu *et al.*, 1997). In the middle is a schematic of the “closed” conformation of Src. At right is a schematic of the “open” conformation.
- C Schematics of two potential “closed” conformations of Tec kinases are shown at the left. In the left picture, an intramolecular interaction is shown between the SH3 domain (blue) and SH2-kinase linker (grey), analogous to the interaction observed in the Src crystal structure. In the middle picture, an intramolecular interaction is shown between the SH3 domain and the PRR (grey) of the TH domain. At right is a schematic of the potential “open” conformation in which the exposed protein domains are able to participate in intramolecular interactions with other proteins and cellular factors.

SH: Src homology
PH: pleckstrin homology
TH: Tec homology
PRR: proline rich region

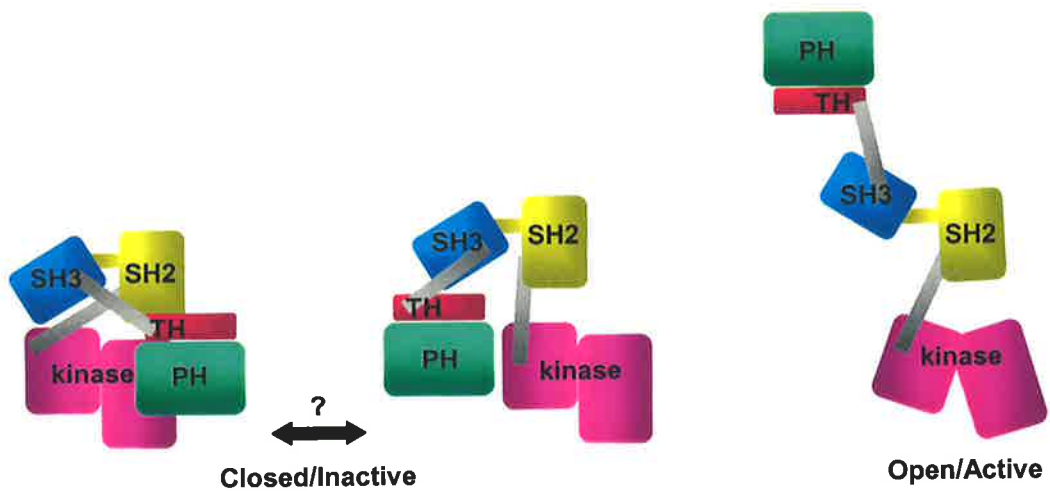
A



B



C



PI3K is thought to direct translocation of the PH domain-containing Tec-family protein to the cell membrane where it colocalises with its activation partners (Scharenberg *et al.*, 1998). Two subsequent tyrosine phosphorylation events and concomitant displacement of intramolecular interactions between the SH3 domain and PRR by exogenous ligands yields an active kinase domain (Park *et al.*, 1996, Rawlings *et al.*, 1996, Heyeck *et al.*, 1997, Moarefi *et al.*, 1997). Tyrosine phosphorylation in the kinase domain activation loop by a Src-family kinase is followed by autophosphorylation of a tyrosine residue in the SH3 domain.

Interestingly, the unique regions of the Src- and Tec-family proteins provide similar functions during kinase activity regulation. Intramolecular interactions between the SH2 domain and C-terminal phosphotyrosine of Src kinases, or the TH and SH3 domains of Tec kinases, can stabilise the kinase domain in a closed (inactive) conformation (see Figure 1.3 B and C; Cooper and Howell, 1993, Sicheri and Kuriyan, 1997, Xu *et al.*, 1997, Moarefi *et al.*, 1997, Patel *et al.*, 1997, Andreotti *et al.*, 1997, Pursglove *et al.*, 2002). In Itk, the SH2 domain can bind the adjacent SH3 domain and this may displace the TH-SH3 interaction to promote kinase activation upon membrane attachment (Brazin *et al.*, 2000). Furthermore, myristoylation and the phospholipid bound PH domain provide membrane tethering of Src- and Tec-family kinases, respectively, a step required for kinase activation (Pawson, 1997, August *et al.*, 1997).

The active conformation of the non-receptor PTK is expected to endorse the binding of a different repertoire of ligands to the various modular domains and to permit phosphorylation of substrates (see Figure 1.3B and C). Since the PH and TH domains are a unique feature of Tec-family PTKs, ligands of these domains may provide an additional means of kinase activity regulation, as well as links to unique functions for Tec PTKs compared with other PTKs.

The steps involved in the inactivation of Tec kinases are currently being elucidated. Recent evidence suggests that Btk activity is downregulated by PKC-mediated serine phosphorylation in the TH domain (Kang *et al.*, 2001). Potentially, Tec kinases can be inactivated directly by phosphatases or by disrupting the PI3K pathway. Phosphatase SHP-1 was shown to suppress the phosphorylation of Syk and Btk, although it is not known if Syk and Btk are substrates of SHP-1 (Maeda *et al.*, 1999). The lipid phosphatases SHIP and PTEN negatively regulate production of PI 3,4-P₂ and PI 3,4,5-P₃ (reviewed in Marshall *et al.*, 2000). Dephosphorylation of PI 3,4,5-P₃ by SHIP results in the dissociation of Tec kinases

from the cell membrane and their inactivation (Bolland *et al.*, 1998, Scharenberg *et al.*, 1998). PTEN was similarly shown to regulate the membrane localisation of Itk (Shan *et al.*, 2000).

1.4 Modular Domains

The modular protein-protein interaction domains of Tec-family kinases function to allosterically regulate the kinase activity through binding both intra- and inter- molecular ligands. It is well established that SH3 domains bind proline-rich peptides and SH2 domains bind phosphotyrosine-containing peptides. PH domains generally function to target the protein to the correct cellular site, such as the cytoskeleton or the cell membrane.

1.4.1 Pleckstrin Homology and Tec Homology Domains

PH domains are regulatory modules that are present in a variety of proteins involved in signal transduction, such as kinases, phospholipases, GTP exchange proteins, and adapter proteins (Musacchio *et al.*, 1993, Shaw, 1993, Ingley and Hemmings, 1994, Rameh *et al.*, 1997). They have also been discovered in constituents of the cytoskeleton including dynamin, β -spectrin, syntrophin, *Caenorhabditis elegans* unc-104/kinesin and yeast NUM1 (Musacchio *et al.*, 1993, Gibson *et al.*, 1994, Farkasovsky and Kuntzel, 1995). The PH domain was first noted as a duplicated region in pleckstrin, the major substrate of protein kinase C (PKC) in platelets (Tyers *et al.*, 1988), and defined upon identification of regions of sequence similarity in several other proteins (Mayer *et al.*, 1993). PH domain primary sequences are loosely conserved and approximately 100 residues in length.

The three dimensional structure of several PH domains has been solved with and without inositol ligands. Several groups have determined the structure of the PH domain of dynamin, β -spectrin and Sos (reviewed in Blomberg *et al.*, 1999). Soisson *et al.*, 1998, determined the structure of the Dbl homology and PH domain pair from Sos. The structure of the N-terminal PH domain of pleckstrin (Yoon *et al.*, 1994), the PH domain and Btk motif Btk (Hyvonen and Saraste, 1997), and the PH domains of and β -adrenergic receptor kinase-1 (β -ARK1; Fushman *et al.*, 1998) and unc-89 (Blomberg *et al.*, 2000) have also been solved.

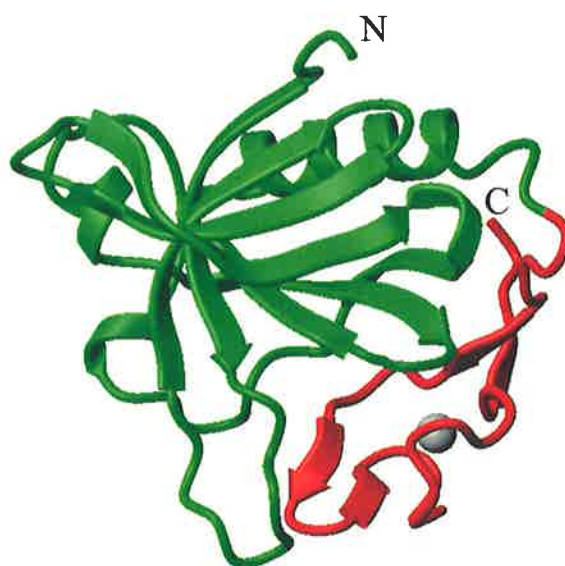
Despite the lack of sequence conservation in PH domains all known cases have a common structure consisting of two perpendicular anti-parallel β -sheets, followed by a C-terminal amphipathic helix. The structure of the Btk PH domain and Btk motif is illustrated in Figure 1.4A and B in ribbons diagram and electrostatic polarisation surface diagram format, respectively. The lengths of the loops connecting the β -strands provide the biggest

Figure 1.4 PHTH Domain Structure

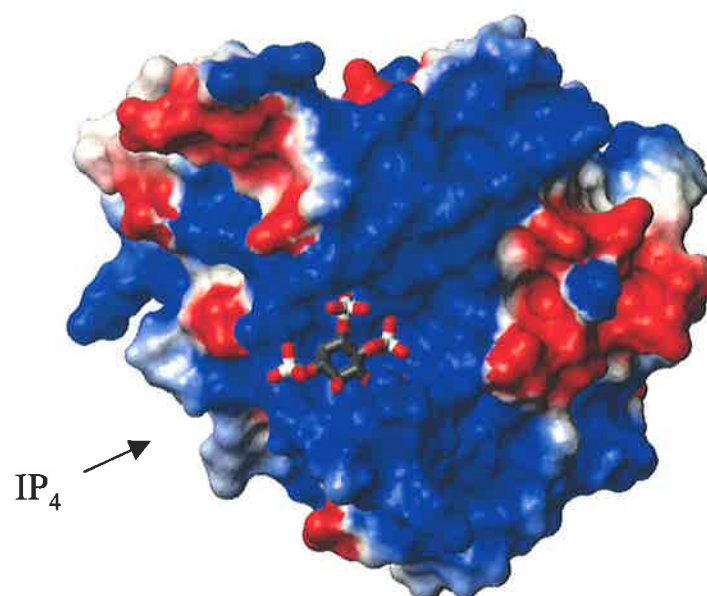
- A Ribbons diagram showing the tertiary structure of the pleckstrin homology domain, shown in green, and Btk motif, shown in red, of Btk protein tyrosine kinase. The Zn^{2+} ion is shown in grey and is coordinated by the sidechains of four residues of the Btk motif: H143, C154, C155 and C165. The positions of the amino-terminus (N) and carboxyl-terminus (C) of the molecule are indicated. PDB code: 1btk (Hyvonen and Saraste, 1997).
- B Electrostatic polarisation surface diagram of the Btk pleckstrin homology domain and Btk motif, shown in the same orientation as in part (A), with bound inositol (1,3,4,5)-tetrphosphate ligand (IP_4 ; shown in ball-and-stick format). Basic residues are shown in blue and acidic residues are shown in red. PDB code: 1b55 (Baraldi *et al.*, 1999).

Figures were generated using MOLMOL software (Koradi *et al.*, 1996) by Dr Kasper Kowalski.

A



B



differences when comparing PH domains. PH domains have strong polarisation of charges and the overall electrostatic effects are expected to be a major determinant in ligand binding (Blomberg *et al.*, 1999).

The face of the PH domain that interacts with inositol phosphate is surrounded by a strong positive potential (see Figure 1.4B; Blomberg *et al.*, 1999). Structures of β -spectrin PH domain and soluble inositol phosphates (Hyvonen *et al.*, 1995), phospholipase C (PLC) PH domain and inositol 1,4,5- P_3 (Ferguson *et al.*, 1995) and Btk PH domain in complex with inositol 1,3,4,5- P_4 (Figure 1.4B; Baraldi *et al.*, 1999) were elucidated. The contacts between the molecules define the specificity and high affinity of the phospholipid interactions. The inositol 1,3,4,5- P_4 -binding sites in PLC- δ and Btk are similar (Baraldi *et al.*, 1999). In Btk, ligand binding involves the $\beta 1$ - $\beta 2$ and $\beta 3$ - $\beta 4$ loops and key residues K12 and R28 (Baraldi *et al.*, 1999).

Many naturally occurring mutations in Btk that cause the human immunodeficiency disease X-linked agammaglobulinemia (XLA) map to the PH domain and disrupt PI 3,4,5 P_3 -binding. The mutations are classified as structural mutants that disrupt the overall fold of the domain or functional mutants that alter ligand-binding sites (Hyvonen and Saraste, 1997). Substitution of R28C is manifest by an inability to bind the 3'-phosphate of PI 3,4,5- P_3 due to removal of the positive charge. Other substitutions, such as K12R and S14F, which introduce larger amino acids, are likely to sterically hinder ligand binding. Phospholipid-binding is, thus, crucial for the proper function of Btk.

In the Tec-family kinases, the N-terminal PH domain is abutted by a Btk motif, which is held together by a zinc ion that coordinates four conserved residues: one histidine and three cysteines (see Figures 1.2A and 3.3; Hyvonen and Saraste, 1997). In Btk, the globular 27-residue Btk motif packs against β -strands 5 and 7 of the PH domain. The combination of Btk motif and proline-rich region makes up the TH domain, which is approximately 80 residues in total and precedes the SH3 domain (Vihinen *et al.*, 1994b). Intramolecular interactions have been detected between the PRR and the adjacent SH3 domain in Itk, Btk and Tec (Andreotti *et al.*, 1997, Hansson *et al.*, 2001a, Pursglove *et al.*, 2002). The extensive contacts between the PH domain and Btk motif of the TH domain suggest that they form a functional unit. Collectively, this unit is described in this thesis as the PHTH domain. The ribbons diagram structure of the Btk PHTH domain is shown in Figure 1.4 and illustrates the apposition of the PH domain and zinc binding Btk motif.

The structures of the Tec-family PTH domains were modelled on that of Btk (Okoh and Vihinen, 1999). Each domain was predicted to have similar scaffolding and electrostatic polarisation and therefore, suggested to have related but not identical properties and functions (Okoh and Vihinen, 1999). Some differences in the binding regions were predicted and this helps to explain differences in ligand-specificity and regulation of Tec-family kinase members. There is higher sequence conservation in PH domains of Tec-family members (up to 50%; see Figure 1.2D) than is generally seen between other PH domains (some less than 10%).

PH domains are characterised as targeting domains as well as allosteric regulators of kinase activity. Recent studies have linked Tec-family kinases to cytoskeletal components through the PTH domain (Yao *et al.*, 1999, Chen *et al.*, 2001) and identified an inhibitory effect of the PH domain on activation of the tyrosine kinase domain (Saito *et al.*, 2001). Thus, the PTH domain plays an essential role in regulation and function of the Tec-family proteins.

1.4.2 Src Homology Domains: SH3, SH2 and Kinase

SH3 and SH2 domains are well documented as protein-protein interaction modules. They are found in diverse proteins of various functions, including intracellular signalling and cytoskeletal rearrangement (Schlessinger, 1994). The kinase domain provides the catalytic activity and converts specific tyrosine residue(s) of substrate molecules into phosphotyrosine(s).

SH3 domains are 50-75-residue modules that bind proline-rich motifs (Ren *et al.*, 1993). The topology of SH3 domains consists of two short antiparallel β -sheets packed almost perpendicularly to each other in a sandwich-like fold and often includes a single turn of a 3_{10} helix located at the C-terminal end (Booker *et al.*, 1993). The ribbons diagram structure of the Abl SH3 domain is shown in Figure 1.5A (Musacchio *et al.*, 1994). The corresponding ligand-bound electrostatic polarisation surface diagram is shown in Figure 1.5B.

The binding preference of SH3 domains is a stretch of 9-10 hydrophobic residues very rich in prolines that adopts a poly-proline type II helix conformation. Due to the pseudo-symmetric nature of this helix there are two orientations of SH3 domain ligand binding (Ren *et al.*, 1993, Saraste and Musacchio, 1994, Yu *et al.*, 1994). In the class I orientation the consensus sequence for binding is +XXPXXP, where + is an amino acid with a positive charge (lysine or arginine), X is any amino acid and P is a proline. Class II peptides,

Figure 1.5 SH3 Domain Structure

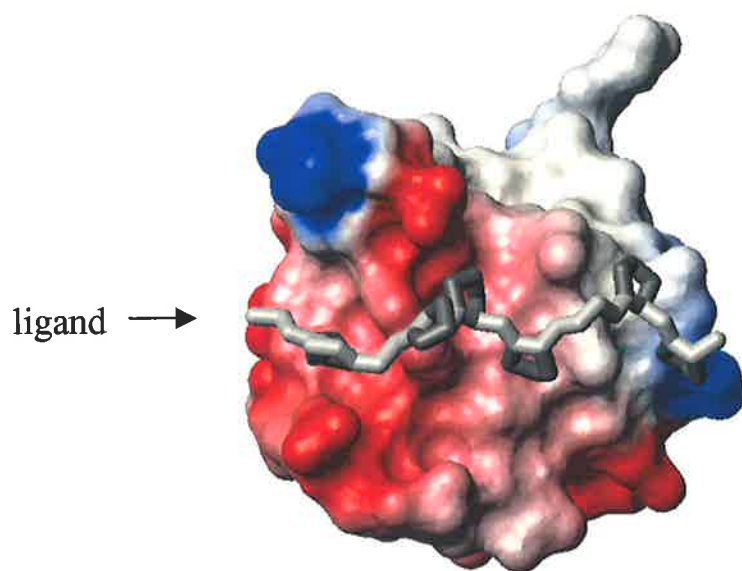
- A Ribbons diagram showing the tertiary structure of the Src homology-3 (SH3) domain from Abl protein tyrosine kinase. The positions of the amino-terminus (N) and carboxyl-terminus (C) of the molecule are indicated.
- B Electrostatic polarisation surface diagram of the Abl SH3 domain, shown in the same orientation as in part (A), complexed with a ten-residue proline-rich peptide ligand shown in grey in ball-and-stick format. The ligand was derived from the SH3-binding proteins 3BP-1 and 3BP-2 and interacts with three major sites on the SH3 molecule by both hydrogen-bonding and van der Waals contacts (Musacchio *et al.*, 1994).

Figures were generated using MOLMOL software (Koradi *et al.*, 1996) by Dr Kasper Kowalski. PDB code: 1abo(Musacchio *et al.*, 1994).

A



B



in comparison, bind in the reverse orientation and have a consensus sequence of PXXPX+ (Yu *et al.*, 1994). In Btk, the side chain of Y223, which becomes autophosphorylated upon activation of Btk, is exposed within the potential SH3 ligand-binding site (Hansson *et al.*, 1998). Therefore, phosphorylation of this residue is expected to hinder the binding of SH3 domain ligands and, instead, attract phosphotyrosine binding ligands.

Deletion of the Tec SH3 domain was shown to result in constitutive Tec kinase activation (Yamashita *et al.*, 1996). Deletion of the C-terminal 21 residues of Btk SH3 domain, which provided a variant of Btk equivalent to the Type 3 isoform of Tec, yielded Btk that caused XLA (Zhu *et al.*, 1994).

SH2 domains are approximately 100-residue modules that bind specific phosphotyrosine-containing sequences (Songyang *et al.*, 1993). Their structures consist of two β -sheets surrounded by two α -helices, with a well-conserved hydrophobic core and phosphotyrosyl-peptide-binding site (see Figure 1.6A and B; Booker *et al.*, 1992, Vihinen *et al.*, 1994a, Narula *et al.*, 1995). They provide for phosphorylation-dependent assembly of receptor signalling complexes in a sequence-specific context (Schlessinger, 1994). The SH2 domain of Btk is essential for the activation of PLC- γ in DT40 cells (Takata and Kurosaki, 1996).

The kinase domain consists of about 250 residues and comprises N- and C-terminal lobes that each contain conserved sites (reviewed in Vihinen and Smith, 1996). Adenosine triphosphate (ATP) is bound in a cleft between the two lobes. In Src, K303 binds a phosphate group of the ATP molecule and is equivalent to K397 in Tec. The substrate interacts mainly with the C-terminal lobe. During activation, a tyrosine residue in the activation loop is phosphorylated and there is rotation of the N-terminal lobe, which locks the position of the ATP molecule.

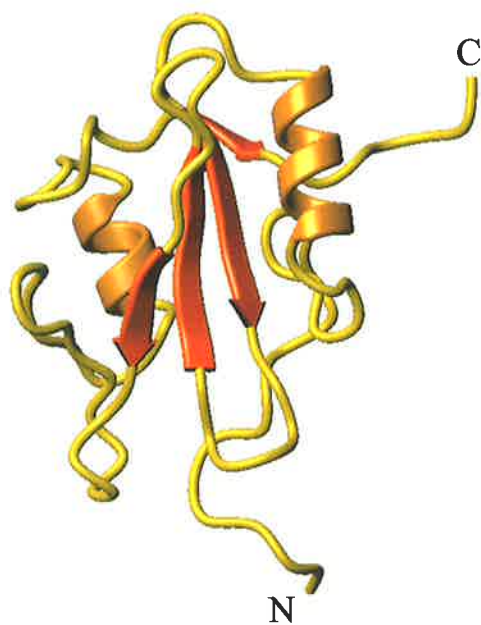
The crystal structure of the SH3, SH2 and kinase domains of inactive Src and Hck kinases revealed two intramolecular interactions that prevent full kinase activation of these proteins (see Figure 1.3B left panel; Xu *et al.*, 1997, Sicheri *et al.*, 1997). The first interaction was between the SH3 domain and the sequence connecting the SH2 and kinase domains, and the second was between the SH2 domain and the C-terminal regulatory phosphotyrosine. The compact domain organisation constrained by these interactions pushes the two lobes of the catalytic domain close together in a conformation that disables the active site (Figure 1.3B). Although different intramolecular interactions were identified for Tec-family kinase SH3

Figure 1.6 SH2 Domain Structure

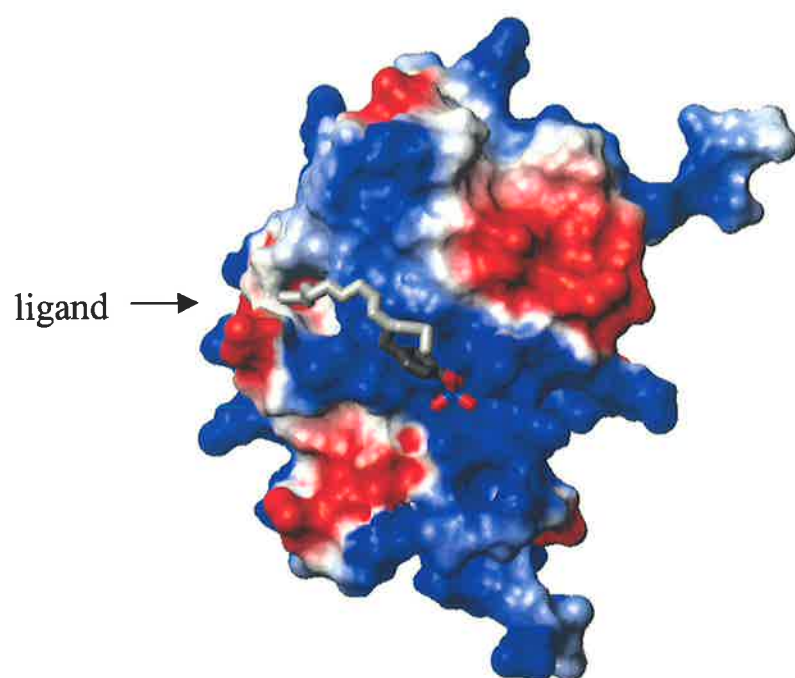
- A Ribbons diagram showing the tertiary structure of the carboxyl-terminal Src homology-2 (SH2) domain from Syk protein tyrosine kinase. The positions of the amino-terminus (N) and carboxyl-terminus (C) of the molecule are indicated.
- B Electrostatic polarisation surface diagram of the Syk SH2 domain, shown in the same orientation as in part (A), complexed with a phosphotyrosine pentapeptide ligand shown in grey in ball-and-stick format. The ligand was derived from the γ -subunit of the IgE receptor. The phosphotyrosine and leucine residues of the peptide ligand interact with pockets on the protein (Narula *et al.*, 1995).

Figures were generated using MOLMOL software (Koradi *et al.*, 1996) by Dr Kasper Kowalski. PDB code: 1csy (Narula *et al.*, 1995).

A



B



domains, they were also suggested to control kinase activity (Andreotti *et al.*, 1997, Pursglove *et al.*, 2002, Hansson *et al.*, 2001b); they are depicted in Figure 1.3C.

SH3 domain displacement is a prerequisite for activation of Src kinases (Moarefi *et al.*, 1997). Furthermore, mutations in the SH3 domain of Src can result in a constitutively active protein through destabilising the inactive protein conformation (Xu *et al.*, 1997). Therefore, in addition to targeting, SH3 and SH2 domains are required for proper regulation of kinase activity in Src-family and Tec-family kinases through an allosteric mechanism (Xu *et al.*, 1997, Sicheri and Kuriyan, 1997, Andreotti *et al.*, 1997).

1.5 Function of Tec-family Kinases

1.5.1 Receptor Initiated Signalling Pathways of Tec Kinases

Tec-family kinases are involved in multiple cellular processes including cell proliferation, apoptosis, differentiation and migration (reviewed in Qiu and Kung, 2000). One or more Tec-family kinases are involved in signalling downstream of a variety of cell surface receptors. These include antigen receptors such as the B and T cell receptors (BCR and TCR, respectively) and Fc-gamma receptor (Fc- γ R), cytokine receptors, receptor tyrosine kinases, G-protein coupled receptors and integrin adhesion receptors (reviewed in Qiu and Kung, 2000). The signalling involves tyrosine phosphorylation of a subset of proteins including Src-family-, Syk-family- and Tec-family kinases, and alterations in cellular phospholipid and calcium levels. Associated with these events is the formation of multicomponent signalling complexes that contain structural proteins, signalling molecules and adaptor molecules. Most of the current knowledge of Tec-family signalling has been gained from studies on Btk and Itk, and their involvement in signalling from the BCR and TCR, respectively (see Figure 1.7).

Btk functions in signalling from the BCR and is required for the maturation of B lymphocytes (Satterthwaite *et al.*, 1998). Lack of functional Btk causes XLA in humans and the related, but less severe X-linked immunodeficiency (Xid) phenotype in mice (Rawlings *et al.*, 1993, Thomas *et al.*, 1993). In humans, the disease is characterised by a decrease in the number of circulating B cells and immunoglobulins and impaired BCR signalling. The model of Btk activation is described in Section 1.8.

Itk signalling downstream of the TCR is analogous to the role of Btk in BCR signalling (Liao and Littman, 1995). Recently, similar roles for Tec in Fc- γ R signalling (Atmosukarto, 2001) and Bmx/Etk in integrin signalling (Chen *et al.*, 2001) were identified.

Figure 1.7 Signalling Pathways Involving Tec Family Kinases

Tec kinases and the antigen receptor signalosome (reproduced from Figure 2 in Lewis *et al.*, 2001).

- A T cell receptor (TCR) signalling. Calcium mobilization in T cells is initiated upon TCR ligation, which activates Lck. Lck, in turn, phosphorylates Tec kinases, as well as immunoreceptor tyrosine activation motifs (ITAMs) on the TCR invariant chains, which leads to the recruitment, phosphorylation and activation of ZAP-70. Kinase active ZAP-70 subsequently phosphorylates adaptor proteins including LAT and SLP-76. This results in a complex that forms around LAT and contains GADS, SLP-76, Itk and PLC- γ . Phosphotyrosines on LAT can interact with SH2 domains of PLC- γ , GADS, Grb-2 and SLP-76. The SH2 domains of Tec kinases and Vav can then bind phosphorylated SLP-76 to participate in this complex. Formation of this complex is essential for the activation of PLC- γ , although what kinases specifically phosphorylate PLC- γ are not certain. Complete activation of PLC- γ and the Tec kinase Itk is also dependent on interactions of the PH domains with PIP₃, a product of active PI3K (activated in T cells both by the TCR (not shown) and by the costimulatory molecule CD28). PTEN can attenuate this activation by hydrolysing PIP₃ to produce PIP₂. Activated PLC- γ then leads to the production of IP₃ and DAG from PIP₂. The molecule IP₃ binds to receptors that initialise mobilization of intracellular endosomal stores of Ca²⁺. This emptying of intracellular Ca²⁺ stores subsequently initiates calcium entry through calcium release activated channels (CRACs) in the plasma membrane. DAG separately activates PKC and RasGRP, both of which may contribute to the activation of ERK 1/2. Not detailed in this figure is the activation of MAPKs downstream of Grb-2-SOS-Ras, DAG-RasGRP and DAG-PKC mediated pathways.
- B B cell receptor (BCR) signalling. Calcium mobilization in B cells is initiated upon BCR ligation, which activates Src family kinases that phosphorylate Syk, Btk and ITAMs in Ig α and Ig β . Kinase active Syk subsequently phosphorylates proteins including BLNK/SLP-65. Protein interactions result in a complex containing BLNK/SLP-65, Btk and PLC- γ . Formation of this complex is essential for the production of IP₃ and DAG by active PLC- γ . Complete activation of PLC- γ and Btk is dependent on interactions of the PH domains with PIP₃, the product of active PI3K. SHIP1 can attenuate this activation through the hydrolysis of PIP₃. The molecule IP₃ initiates Ca²⁺ mobilization from intracellular endosomal stores, which subsequently initiates Ca²⁺ mobilization through CRACs in the plasma membrane. Vav is also recruited to this complex and contributes to Ca²⁺ mobilization via Rac-mediated activation of phosphatidylinositol 4 phosphate 5 kinase (PIP5K), a phosphoinositide kinase that generates the substrate of PLC- γ , PIP₂. Vav also contributes to these pathways in both B and T cells via regulation of the actin cytoskeleton.

Y's:	phosphotyrosine
round indentations:	SH2 domains
rectangular indentations:	PH domains.

1.5.2 Signalling Pathways That Involve Tec

In signalling pathways of some receptor systems, more than one Tec-family member is involved. For example, Tec also has roles in BCR and TCR signalling during B and T cell activation, respectively (Ellmeier *et al.*, 2000, Yang *et al.*, 1999). However, Tec also has distinct roles to Btk and Itk in these cells. Upon activation of the BCR, Tec can phosphorylate BRDG1 whereas Btk cannot (Ohya *et al.*, 1999). Similarly, Tec can phosphorylate Dok-1 upon activation of the CD28 receptor, whereas Itk cannot (Yang *et al.*, 1999). The distinct roles are confirmed by genetic studies in which slightly different phenotypes are identified in mice with single compared with double gene knockouts of Tec-family members (Ellmeier *et al.*, 2000, Schaeffer and Schwartzberg, 2000).

Tec has been implicated in numerous other signalling pathways, many of which pertain to haematopoietic cells. Early experiments identified the involvement of Tec in c-Kit receptor signalling: upon stem cell factor binding to c-Kit, Tec was found to be tyrosine phosphorylated and activated (Tang *et al.*, 1994). Tec is involved in signalling pathways activated by Interleukin-3 (IL-3) and Erythropoietin (EPO), which induce association of Vav with Tec (Machide *et al.*, 1995). Granulocyte colony-stimulating factor (G-CSF) stimulation led to tyrosine phosphorylation and activation of Tec and association with Vav in mouse cell lines that grow or differentiate in response to G-CSF (Miyazato *et al.*, 1996). Thrombopoietin similarly induced phosphorylation and activation of Tec, although Tec was found to be constitutively associated with Vav in a human thrombopoietin-dependent cell line (Yamashita *et al.*, 1997).

Activation of Tec was induced by the stimulation of gp130 by IL-6 or soluble IL-6 receptor- α , and Tec was found to associate with the gp130 subunit of the IL-6 receptor independent of IL-6 stimulation (Matsuda *et al.*, 1995). Tec is tyrosine-phosphorylated and activated by granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation in a human GM-CSF-dependent cell line and was found to be directly involved in the regulation of c-fos transcription (Yamashita *et al.*, 1998).

Tec was rapidly tyrosine phosphorylated in response to platelet agonists which activate G-protein coupled receptors and Tec translocation to the cytoskeleton was dependent on platelet aggregation, suggesting that Tec can be a component of integrin-mediated signalling (Hamazaki *et al.*, 1998). Tec was implicated in prolactin receptor signalling in conjunction with Vav and was shown to enhance the guanine nucleotide exchange factor

activity of Vav (Kline *et al.*, 2001). Tec was thus suggested to act in concert with Vav in regulation of Rho-family-mediated cytoskeletal alterations (Kline *et al.*, 2001).

Propagation of these signalling pathways involves specific and regulated protein interactions and, indeed, numerous ligands have been identified for Tec-family kinases.

1.6 Ligands of Tec-Family Kinases

The modular protein domains mediate interactions with cellular proteins and factors and promote the formation of signal complexes. The precise interactions mediated with a Tec-family kinase at any one time is likely to depend on the host cell type and cellular compartment within which it resides as well as the activation state of the cell.

1.6.1 PHTH Domain Ligands

The PHTH domains of Tec-family kinases bind phospholipids as well as proteins. Phospholipid binding is thought to play a key role in membrane targeting during the activation of Tec kinases. Tec kinases bind 3' phosphorylated inositol lipids preferentially and this suggests they act downstream of PI3K. This constraint was demonstrated by PI3K activity-dependent membrane translocation of the Btk PH domain fused to green fluorescent protein (GFP) (Varnai *et al.*, 1999). *In vitro* experiments demonstrated that the Btk PH domain directly binds PI 3,4,5-P₃ and this required R28, a residue that is mutated in some X-linked agammaglobulinemia patients (Salim *et al.*, 1996, Rameh *et al.*, 1997). PI 3,4,5-P₃ was recently shown to directly regulate Btk signalling by relieving the normal suppression on kinase activity and access to substrates imposed by the PH domain (Saito *et al.*, 2001). The Tec PH domain also binds PI 3,4,5-P₃ (Shirai *et al.*, 1998).

In addition to phospholipids, Tec-family PH domains bind several cellular proteins. Both the α and $\beta\gamma$ subunits of heterotrimeric G-proteins have been shown to bind the PHTH domain of Btk (Tsukada *et al.*, 1994b, Jiang *et al.*, 1998). The binding sites are located in the C-terminal part of the PH domain and in the Btk motif of the TH domain and overlap for α and $\beta\gamma$ subunits of G-proteins. Tec and Bmx were shown to be involved in G α 12/13-induced, Rho-mediated activation of serum response factor (Mao *et al.*, 1998). Therefore, there is strong evidence to expect protein ligands for Tec PHTH domain. Recent studies that identified novel protein ligands for Btk and Bmx PHTH domains complement the prospect of identifying protein ligands for the Tec PHTH domain.

Btk was shown to bind filamentous actin (F-actin) and cytoskeletal translocation was shown to be dependent on the PH domain (Yao *et al.*, 1999). The actin-binding site was mapped to a 10-residue region of the N-terminal region of Btk PH domain that is rich in basic residues (Yao *et al.*, 1999). This region is conserved in Tec and, thus, Tec is expected to bind actin.

Yao and co-workers also demonstrated that PKC binds to the second and third β -strands of Btk PH domain (Yao *et al.*, 1997), that Btk is a substrate of PKC and its enzymatic activity is down-regulated by PKC-mediated phosphorylation (Yao *et al.*, 1994). The PKC-binding site overlaps with the phospholipid-binding site, therefore, competitive binding of these molecules may regulate membrane targeting of Tec kinases. On the basis of sequence homology to Btk, it appears reasonable that Tec kinase would also bind to PKC. Recent evidence suggests that PKC- β acts as a feedback loop inhibitor of Btk activation and this may involve serine phosphorylation in a short linker within the Tec homology domain of Btk by PKC- β (Kang *et al.*, 2001).

The PH domain of Bmx/Etk is involved in binding to the focal adhesion kinase (FAK), which is a key mediator of integrin signalling (Chen *et al.*, 2001). An interaction between Bmx and the tyrosine phosphatase PTPD1, which required the PH domain of Bmx, was shown to stimulate the kinase activity of Bmx and resulted in increased tyrosine phosphorylation of both proteins (Jui *et al.*, 2000). Tec was also activated by PTPD1 (Jui *et al.*, 2000). In contrast, a recently identified protein ligand of the Btk PH domain, IBtk, downregulated Btk kinase activity, Btk-mediated calcium mobilisation and NF- κ B-driven transcription (Liu *et al.*, 2001).

The Bmx PH domain also binds the transcription factor Stat3, which leads to tyrosine phosphorylation and activation of Stat3 (Tsai *et al.*, 2000). The transcription factor BAP-135/TFII-I was similarly shown to physically associate with the PH domain of Btk and be tyrosine phosphorylated by Btk (Yang and Desiderio, 1997, Novina *et al.*, 1999). Fas binds to the PH domain and kinase domain of Btk and this interaction inhibits the pro-apoptotic effects of Fas ligation in several B-lineage lymphoid cell lines (Vassilev *et al.*, 1999).

Immunoprecipitation experiments demonstrated that Tec kinase binds to Vav upon EPO and IL-3 stimulation and that binding involves the TH domain (Machide *et al.*, 1995). Other studies have shown that Vav also binds to the SH2 domain of Tec (Takahashi-Tezuka *et al.*, 1997). Therefore, there may be multiple contacts between these molecules.

The PRR region in the TH domain has been identified as a binding site for Src-family kinase SH3 domains (Mano *et al.*, 1996, Rawlings *et al.*, 1996). This leads to Src-mediated phosphorylation and activation of Tec kinases. The PRR of Itk also binds the adaptor protein Grb-2 and this is involved in the formation of signalling complexes in T cell receptor-initiated signalling (Bunnell *et al.*, 2000).

1.6.2 SH3 Domain Ligands

SH3 domains bind proline-rich motifs using intramolecular or intermolecular interactions. External ligands can compete with intramolecular interactions (Moarefi *et al.*, 1997). Target recognition is thought to be coupled with conformational activation of the kinase domain (Nguyen and Lim, 1997). The Tec SH3 domain binds to the proline-rich motifs of the CD28 receptor in an activation-dependent manner during T cell signalling (Yang *et al.*, 1999).

Phosphorylation of the tyrosine residue in the ligand-binding site alters the binding preference of the Btk SH3 domain. Syk kinase preferentially interacts with the phosphorylated SH3 domain, whereas this significantly diminishes binding of WASP, a protein that associates with the cytoskeleton (Morrogh *et al.*, 1999). Syk belongs to the Syk/ZAP-70-family of tyrosine kinases and is required for the BCR-induced PLC- γ 2 activation (Takata and Kurosaki, 1996).

Binding of SAB adaptor to the Btk SH3 domain inhibits Btk activity and reduces BCR-mediated calcium mobilisation (Yamadori *et al.*, 1999). Interactions with Cbl have also been reported for Btk and Itk SH3 domains (Cory *et al.*, 1995, Bunnell *et al.*, 1996).

1.6.3 SH2 Domain Ligands

The SH2 domain acts to recruit Tec kinases to signalling complexes that facilitate their activation. Adapter proteins of the SLP/BLNK-family link Tec kinases to activation of PLC- γ using phosphotyrosine/SH2 interactions (Hashimoto *et al.*, 1999, Su *et al.*, 1999). The Itk SH2 domain is essential for the formation of TCR-inducible Itk-LAT complexes that are vital for Itk activation and function (Ching *et al.*, 2000). Furthermore, the Itk SH2 domain interacts with Syk-phosphorylated SLP-76. This recruits Itk to LAT-nucleated signalling complexes and facilitates the activation of LAT-associated PLC- γ 1 by Itk (Bunnell *et al.*, 2000). Similarly, B-cell linker protein (BLNK) was identified as a major Btk-SH2 domain-binding

protein in B cells and the interaction was shown to contribute to PLC- γ activation (Hashimoto *et al.*, 1999).

The adaptor protein Dok-1 was demonstrated to bind the SH2 domain of Tec and be a substrate of Tec kinase domain (Yang *et al.*, 1999, Yoshida *et al.*, 2000). Activation and phosphorylation were shown to be PI3K-dependent (van Dijk *et al.*, 2000). Dok-1 has been suggested to function as a scaffold molecule in c-Kit-mediated signalling (van Dijk *et al.*, 2000) and as a negative regulator of Ras (Yoshida *et al.*, 2000).

1.6.4 Kinase Domain Ligands

New data suggests that activated Btk phosphorylates PLC- γ 2, leading to its activation (Kurosaki *et al.*, 2000). Four tyrosine residues of PLC- γ were identified as Btk substrates using *in vitro* kinase assays (Rodriguez *et al.*, 2001, Watanabe *et al.*, 2001). These residues include the major phosphorylation sites upon BCR engagement, as substitution of all four tyrosine residues with phenylalanines almost completely eliminated the BCR-induced PLC- γ 2 phosphorylation.

Tec and Itk have also been implicated in PLC- γ activation (Bunnell *et al.*, 2000, Bony *et al.*, 2001). Tec was suggested to work in concert with a Src-family kinase and PI3K- γ to fully activate PLC- γ in ATP-stimulated cardiac cells (Bony *et al.*, 2001).

Apart from PLC- γ and Dok-1, the docking protein BRDG1, the adaptor Grb10/GrbIR and PI3K subunits have been identified as substrates of Tec kinase domain. The tyrosine residue in the SH3 domain ligand-binding site is expected, by analogy to Btk, to also be a substrate of Tec kinase domain (Hansson *et al.*, 1998). Autophosphorylation is expected to be mediated in trans upon contact of two molecules.

BRDG1 and Grb10/GrbIR were identified as binding proteins of Tec kinase domain using yeast two-hybrid studies and were shown to be tyrosine phosphorylated when co-expressed in 293 cells with Tec (Mano *et al.*, 1998, Ohya *et al.*, 1999). Expression of Grb10/GrbIR was shown to suppress the cytokine-driven and Tec-driven activation of the c-fos promoter.

The adaptor protein BCAP is a substrate of Btk. Syk- and Btk-mediated tyrosine phosphorylation of BCAP was shown to provide binding site(s) for the p85 subunit of PI3K and, thus, regulate PI3K localisation and bridge BCR-associated kinases to the PI3K pathway (Okada *et al.*, 2000). Recruitment of PI3K to glycolipid-enriched microdomains was significantly attenuated in the absence of BCAP (Okada *et al.*, 2000). The p85 and p55PIK

subunits of PI3K were also shown to directly bind the SH2-kinase domain of Tec using yeast two-hybrid studies (Takahashi-Tezuka *et al.*, 1997); the interaction was found to be dependent on Tec kinase activity.

Recent studies have established that transcription factors are targets of the Btk kinase domain. Phosphorylation of BAP/TFII-I by Btk may link engagement of receptors such as surface immunoglobulin to modulation of gene expression (Egloff and Desiderio, 2001). Bmx induces the tyrosine phosphorylation and DNA-binding activity of Stat1, Stat3, and Stat5 and, thus, can function as an activator of the Stat signalling pathway (Saharinen *et al.*, 1997). Anti-IgM (or BCR) stimulation results in enhanced tyrosine phosphorylation of STAT5A in Btk-competent B cells from wildtype mice but not in Btk-deficient B cells from Xid mice (Mahajan *et al.*, 2001). Btk transmits signals from the BCR to the transcription factor NF- κ B (Petro and Khan, 2001). Btk-dependent activation of NF- κ B is essential for reprogramming the expression of genes that control B cell survival and proliferation (Khan, 2001).

1.7 Downstream Effectors of Tec-family Kinases

Tec kinases impact multiple signal pathways and generate pleiotropic effects (reviewed in Qiu and Kung, 2000). Downstream effects involve calcium influx, changes in gene expression, apoptosis and, as has been recently recognised, cytoskeletal reorganisation. These are all important events in the function, differentiation and homeostasis of haematopoietic cells.

The most well characterised downstream effector of Tec kinases is PLC- γ . This leads to induction of sustained calcium influx, which may be important in the regulation of transcriptional events essential for cell growth or survival (Rawlings, 1999). The activated PLC- γ 2 converts PI 4,5-P₂ into the second messenger inositol 1,4,5-P₃ (IP₃) which regulates intracellular calcium mobilisation, and diacylglycerol, which leads to membrane localisation and activation of a subset of PKC isoforms. Binding of IP₃ to the IP₃ receptors located on the endoplasmic reticulum is essential for triggering a calcium release from the endoplasmic reticulum and consequent entry of extracellular calcium (Kurosaki *et al.*, 2000).

Transcription factor activation and gene expression changes have been demonstrated downstream of Tec kinases. The Tec-family kinases are required for activation of transcription factors that are essential for numerous immune cell responses, including NF-AT, c-fos, NF- κ B, GATA-3, TFII-I and Stats (Yamashita *et al.*, 1998, Novina *et al.*, 1999, Mahajan *et al.*, 2001, Schaeffer *et al.*, 2001, Yamamoto *et al.*, 2001). Presumably, the

induction of genes upon cytokine stimulation of haematopoietic cells reflects the *in vivo* function of these cells in responding to environmental signals to combat infection.

Both pro-apoptotic and anti-apoptotic roles have been demonstrated for Btk. It promotes radiation-induced apoptosis but inhibits Fas-activated apoptosis in B cells (Uckun, 1998). Therefore, Btk may be a modulator of the apoptotic signal, which is regulated by cell surface receptors and cytoplasmic and nuclear regulatory molecules (Islam and Smith, 2000). The final outcome might depend on the phenotype of the cell with its fate determined by a cell type-specific biological response.

Mitogen activated protein kinases (MAPKs) or extracellular signal-regulated protein kinases (ERKs) are ubiquitously expressed and play roles in most signal transduction pathways (Karandikar and Cobb, 1999). The Ras/MAPK signalling pathway is activated by Tec kinase signalling pathways (Wan *et al.*, 1997, Deng *et al.*, 1998, Jiang *et al.*, 1998, van Leeuwen and Samelson, 1999).

Recently, the cytoskeleton has been defined as a downstream target of Tec kinases. The involvement of Vav in Tec-mediated signalling pathways can lead to potential activation of Rho/Cdc42/Rac pathways. Rho-family GTPases are known for their regulation of the actin cytoskeleton structure. Tec and Bmx were shown to enhance G-protein coupled receptor activation of RhoA in fibroblasts (Mao *et al.*, 1998). Cytoskeletal links of Tec kinases were further established by identification of F-actin-binding to the Btk PH domain (Yao *et al.*, 1999). Possible tyrosine kinase regulation of cytoskeletal components was consolidated by connecting Bmx with FAK in integrin signalling and cell migration (Chen *et al.*, 2001), Itk in integrin-mediated T cell adhesion (Woods *et al.*, 2001) and Tec and Btk in integrin-mediated platelet activation (Laffargue *et al.*, 1997, Hamazaki *et al.*, 1998, Laffargue *et al.*, 1999).

1.8 Model of Tec-family Kinase Activation

The current model of Tec-family kinase activation is based on results from biochemical and genetic studies of Tec-family kinase signalling combined with structural studies on the domains of Src- and Tec-family members (see Figure 1.7 reproduced from Lewis *et al.*, 2001).

Upon antigen binding to the B or T cell receptor, Src kinases are activated and phosphorylate the cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) on the antigen receptor invariant chains (reviewed in Schaeffer and Schwartzberg, 2000). The phosphotyrosines provide SH2 domain docking sites and serve to recruit Syk or ZAP-70.

Src-family kinases phosphorylate and activate Syk and ZAP-70 kinases, which in turn, phosphorylate the adaptor molecules BLNK in B cells or LAT and SLP-76 in T cells (Hashimoto *et al.*, 1999, van Leeuwen and Samelson, 1999).

PI3Ks are concomitantly activated by receptor engagement and produce PI 3,4-P₂ and PI 3,4,5-P₃. Local accumulation of PI 3,4,5-P₃ facilitates translocation of the PH domain-containing Tec-family protein to the cell membrane (Scharenberg *et al.*, 1998). The PH domain is indispensable for association of the kinase with the cell membrane (Ching *et al.*, 1999). Recently, the Btk-PI 3,4,5-P₃ interaction was also proposed to provide an allosteric role in kinase domain activation, as the PH domain was suggested to hinder access of the kinase domain to substrates and normally act to suppress Btk kinase activity (Saito *et al.*, 2001). Allosteric roles for other PH domains have been described in the literature.

PI3K-dependent membrane translocation of Tec-family kinases also facilitates their colocalisation with activation partners at a multiprotein signalling complex and promotes their binding to the scaffold protein BLNK or SLP-76. Src kinases phosphorylate a tyrosine residue in the kinase domain activation loop (Y551 for Btk) and this is expected to regulate the transition from the closed (inactive) to open (active) state by induced conformational change (Rawlings *et al.*, 1996). Full activation is achieved upon autophosphorylation in the SH3 domain (Y223 for Btk) (Park *et al.*, 1996).

Phosphorylation of the adapter molecules BLNK and SLP-76 by Syk or ZAP-70, provides neighbouring docking sites for SH2 domains of both Tec kinases and PLC- γ , thus bringing them into close proximity (Su *et al.*, 1999, Kurosaki *et al.*, 2000, Rodriguez *et al.*, 2001). The activated Tec-family kinase then phosphorylates PLC- γ , leading to its activation, IP₃ production, calcium release from the endoplasmic reticulum and subsequent entry of extracellular calcium. Thus, Tec kinases are required for full calcium mobilisation in lymphocytes (Fluckiger *et al.*, 1998, Schaeffer and Schwartzberg, 2000). The protein phosphatases SHIP, SHP-1 and SHP-2 balance these activation signals in the PLC- γ -calcium pathway and set threshold levels for activation signals as well as terminate activation responses (Kurosaki *et al.*, 2000).

1.9 Null Phenotypes of Tec-family Kinases

Several studies have provided cells devoid of, or expressing mutant versions of, the genes encoding Tec-family kinases. This has revealed a degree of redundancy in the function of Tec kinases. Not surprisingly, knockout mice devoid of signalling proteins in the same

signalling pathways as Btk have similar phenotype to Btk^{-/-} cells (Fruman *et al.*, 1999). The problems are manifest as immune signalling defects and result from impaired IP₃ production and calcium mobilisation due to reductions in PLC- γ phosphorylation.

Naturally occurring mutations in Btk result in the B cell immunodeficiencies XLA in humans and Xid in mice (Satterthwaite *et al.*, 1998). These diseases are characterised by decreased B cell numbers and markedly impaired B cell responses to BCR, or surface IgM, engagement; B cells lacking Btk fail to migrate. XLA causing mutations have been detected in all five domains of BTK (Vihinen *et al.*, 1999). Btk knockout mice confirmed that Xid is caused by loss of Btk function (Kerner *et al.*, 1995, Khan *et al.*, 1995). Although Xid resembles XLA, the phenotype is not as severe.

Although Tec knockout mice were viable and displayed no obvious defects, Tec and Btk double deficient mice had a more severe phenotype than Btk null mice. Btk can compensate for Tec, but Tec can only partially compensate for Btk (Ellmeier *et al.*, 2000). Myeloid cell function is yet to be investigated in the Tec^{-/-} mice.

The defects in TCR signalling in Itk deficient or Itk/Txk double deficient cells include impaired proliferation and cytokine production (Liao and Littman, 1995, Liu *et al.*, 1998, Schaeffer *et al.*, 1999). These defects are analogous to the BCR signalling defects in Btk deficient cells. However, mice null for Txk exhibit no major defects in T cell functional responses and this may be due to compensation by Itk or even Tec (Schaeffer *et al.*, 1999).

PI3K α mutant cells have a similar phenotype to Xid mice (Fruman *et al.*, 1999). Furthermore, activation of PLC- γ is blocked by mutation of either Syk or BLNK in B cells or ZAP-70 in T cells, and calcium mobilisation is blocked in T cells harbouring mutant LAT or SLP-76 (Shan and Wange, 1999, Kurosaki, 2000, Kurosaki and Tsukada, 2000, Schaeffer and Schwartzberg, 2000). Therefore, formation of signalling complexes is critical for the activation of PLC- γ and downstream signalling. These mutations generally lead to more profound defects in calcium mobilisation than those caused by mutation of Tec-family kinases. It was, therefore, recently suggested that the critical role of Tec kinases might be to regulate full PLC- γ activation and modulate the strength or duration of the antigen driven responses (Lewis *et al.*, 2001).

1.10 Yeast Two-Hybrid

The yeast two-hybrid interaction trap is routinely used to detect protein-protein interactions. The system is based on the modular nature of the eukaryote transcriptional activator complex, in which juxtaposition of separate DNA-binding and activation domains leads to transcription activation. The two domains are brought into close proximity when their fusion partners interact and the resulting reporter gene expression can be assayed. This system can be used to test interaction of known proteins or to screen a library of unknown proteins with a known bait protein. A comprehensive overview of the Clontech Matchmaker LexA Two-Hybrid System (CLONTECH Laboratories, 1996) used in the studies presented in this thesis is described in Section 3.3.2.

There are a number of advantages of using the yeast two-hybrid assay to screen for protein ligands over methods such as affinity column purification or ligand overlay assay. These include:

- i) It is a sensitive method for detecting weak and transient protein-protein interactions.
- ii) The proteins are more likely to be in their native conformation as the environment more accurately mimics *in vivo* conditions.
- iii) The fusion proteins are expressed at high levels.
- iv) The reporter gene expression is high as the cloned promoters amplify the intensity of even weak signals.
- v) The use of a nutritional marker as a reporter gene is a sound selection tool.

Most importantly, this approach provides direct access to the complementary DNA (cDNA) of the unknown binding partner and therefore enables further characterisation of the protein-protein interaction. Deletion and substitution mutants of the ligand can be created to map the binding site. A range of biochemical techniques can be used to dissect the binding determinants.

The limitations of this method include: the requirement for a good quality cDNA library to screen; the inability to detect lipid interactions (which may bind cooperatively with protein ligands) and the inability to test for proteins that require cooperative interactions with other parts of Tec without increasing the total scope of ligands.

1.11 Aims

Little was understood about the role of Tec-family kinases in intracellular signalling pathways when this PhD project was begun. Furthermore, ligands of the PHTH domain of Tec had not yet been identified. The aim of this project was to identify protein partners of Tec PH and TH domains and to characterise the interactions to better understand the function of PH and TH domains and their role in Tec signalling pathways.

The specific objectives included:

- i) Screening a human liver cDNA library with LexA-PHTH fusion protein as bait in a yeast two-hybrid assay to identify PHTH interacting proteins.
- ii) Characterise the interaction of the proteins by:
 - a. Mapping the interacting residues by testing truncated peptides and substitution mutants in the yeast two-hybrid assay
 - b. Protein expression and colocalisation studies in mammalian cells using Western blot and immunofluorescence experiments, respectively.
 - c. Binding analysis in cultured cells using co-immunoprecipitation experiments
 - d. Identifying a physiological function relevant to the interaction.
 - e. *In vitro* binding analysis using glutathione-S-transferase- (GST)-pulldown experiments and surface plasmon resonance experiments.
 - f. Visualising the 3D structure of the interacting protein with and without PHTH domain protein ligand using NMR spectroscopy.

It is anticipated that protein partners isolated and characterised by this study will reveal factors that influence the function and regulation of PHTH domains and Tec kinase.

CHAPTER

2

Materials And Methods

2.1 Materials

The materials that were used and their main supplier are listed.

2.1.1 Sheet Materials

3MM chromatography paper	Whatman Ltd
Biomax™ MR X-ray film	Kodak/Integrated Sciences
Hybond™-C (nitrocellulose membrane)	Amersham
Photographic slide film	Kodak
X-ray film	Konica, AGFA

2.1.2 Other Materials

Electroporation cuvettes	BioRad
Bioassay plates, 500 cm ²	Nunc
Centricon concentrators, 3 kDa molecular weight cut-off	Amicon/Millipore
CryoTube™ vials	Nunc
Filters	Millipore
Minisart 0.45 µm filters, syringe top	Sartorius
Needles	Becton Dickinson
PD10 Columns	Pharmacia
Prefilters	Millipore
Scalpel blades	Swann Morton
Syringes	Becton Dickinson
Tissue culture plastic: T75 and T175 flasks, 6-well trays and 10cm dishes	Falcon/Becton Dickinson

2.1.3 Chemicals and Reagents

All chemicals and reagents were of analytical grade, or of the highest purity available. The major sources of chemicals are listed below.

Acetic acid	BDH-AnalaR
Acrylamide (Acryl/bis 29:1)	Astral Scientific
Agarose, DNA grade	Progen
Amino acids	Sigma
Ampicillin	Sigma
Ammonium persulphate (APS)	Sigma

Adenosine triphosphate (ATP)	Sigma
Bacto-agar	Difco
Bacto-tryptone	Difco
β -mercaptoethanol	Sigma
Bovine serum albumin (BSA)	Sigma
Bradford reagent	Biorad
Bromophenol blue	Sigma
Cesium chloride	Cabot
Chloroform	BDH-AnalaR
Cloned Pfu Polymerase Buffer, 10 $\times$	Stratagene
Coomassie Brilliant Blue R	Sigma
D ₂ O	Aldrich
Deoxynucleotide triphosphates (dNTP's)	Sigma
Dimethyl fluoride (DMF)	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Dithioerythritol (DTE)	Sigma
Dithiothreitol (DTT)	Diagnostic Chemicals
DOTAP TM	Boehringer Mannheim
Ethanol	BDH-AnalaR
Ethidium bromide	Sigma
Ethylene diamine tetraacetic acid (EDTA)	Sigma
Ethylene glycol tetraacetic acid (EGTA)	Sigma
FUGENE-6 TM	Roche
γ -globulins, human	Sigma
Glacial acetic acid	Sigma
Glass beads	Sigma
Glutathione, reduced form	Sigma
Glutathione agarose	Zymatrobe
Hydrochloric acid	BDH-AnalaR
Imidazole	Sigma
Isopropyl- β -D-thiogalactopyranoside (IPTG)	Progen
Kanamycin	Sigma
KCl	BDH-AnalaR
L-Glutamine	Sigma
LipofectAMINE TM 2000	Gibco BRL
Lithium acetate	Sigma
Luminol	Sigma
Methanol	BDH-AnalaR
Mineral oil	Sigma
NaCl	BDH-AnalaR

Nickel-IDA agarose	Zymatrobe
Normal Donkey serum	Jackson ImmunoResearch
NP40	BDH Chemicals Ltd.
p-Courmaric acid	Sigma
p-Phenyldiamine (PPD)	Sigma
Paraformaldehyde (PFA)	Merck
Phenol	BDH Chemicals Ltd.
Phenol red	CSL
Phenylmethylsulfonylfluoride (PMSF)	Sigma
Polyethylene-glycol-4000 (PEG-4000)	BDH Chemicals Ltd.
Propan-2-ol (Isopropanol)	BDH-AnalaR
Protease inhibitor cocktail	Sigma
Protein-A Sepharose CL4B	Sigma
Protein-G plus agarose	Santa Cruz
Raffinose	Sigma
RPMI 1640	Gibco BRL
Salmon sperm DNA	Sigma
Sephadex G50	Pharmacia
Skim milk powder	Diploma
Sodium azide	Sigma
Sodium dodecyl sulfate (SDS)	Sigma
Sorbitol	Sigma
Spermidine	Sigma
Superdex 75	Pharmacia
Temed	BIORAD
Triton X-100	Sigma
Tween-20 (polyoxyethylene-sorbitan monolaurate)	Sigma
XGAL (5-bromo-4-chloro-3-indoyl-galactopyranoside, BCIG)	Progen
Xylene cyanol FF	Sigma
Yeast nitrogen base	Difco
Zymosan A	Sigma

2.1.4 Solutions

All buffers and solutions were made up in Milli-Q[®] water and sterilised by autoclaving or filtering with 0.45 µm filter. Tissue culture solutions were filtered in-vacuo using Corning disposable bottle top filters (0.22 µm pore size). The following solutions were used:

Anode Buffer:	0.2 M Tris (pH 8.9)
Blocking solution:	PBS, 0.1% (v/v) Tween-20, 4% (w/v) skim milk

BU salts (pH 7.0) [10×]: 7% (w/v) $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 3% (w/v) NaH_2PO_4 . Adjust pH to 7.0, autoclave, store at room temperature.

Cathode Buffer:	0.1M Tris (pH 8.25), 1% SDS, 1.0M Tricine
Coomassie blue stain:	0.1% (w/v) Coomassie brilliant blue, 30% (v/v) methanol, 10% (v/v) acetic acid
Cracking solution:	20 mM NaOH, 0.5% SDS, 5 mM EDTA, 0.02% bromophenol blue, 10% glycerol
Cytoskeletal lysis buffer:	30 mM HEPES (pH 7.4), 60 mM KCl, 0.5% Triton X-100, 2 mM EDTA, 2 mM EGTA, 5 mM MgCl_2 and freshly added 2 mM PMSF, 1 mM Na_3VO_4 , 50 mM NaF and 1× Protease inhibitor cocktail.
Destain:	50% (v/v) methanol, 5% (v/v) glacial acetic acid
ECL solution 1:	2.5 mM Luminol, 0.4 mM coumaric acid, 100 mM Tris (pH 8.5)
ECL solution 2:	0.0192% H_2O_2 , 100 mM Tris (pH 8.5)
Freezing Solution:	10% DMSO, 90% fetal bovine serum (FBS)
Gel Buffer [3×]:	3.0 M Tris base, 0.3% (w/v) SDS
GLB [10×]:	50% (v/v) glycerol, 0.05% (w/v) bromophenol blue, xylene cyanol 0.05% (w/v)
IP binding buffer:	10 mM Tris (pH 7.5), 0.1% Triton-X-100, 150 mM KCl, 2 mM EDTA, 1 mM PMSF
IP wash buffer:	10 mM Tris (pH 7.5), 0.1% Triton-X-100, 150 mM KCl, 2 mM EDTA, 1 mM PMSF, 0.2% skim milk
Ligase Buffer [10×]:	500 mM Tris-HCl pH7.6, 100 mM MgCl_2 , 100 mM DTT, 500 $\text{mg} \cdot \text{mL}^{-1}$ BSA
Megadeath solution:	0.1M NaOH, 0.5% (w/v) SDS, 10 mM Tris pH8.0, 1 mM EDTA pH8.0
Midiprep Solution 1:	50 mM Glucose, 25 mM Tris pH8.0, 10 mM EDTA (pH 8.0)
Midiprep Solution 2:	0.2 M NaOH, 1% (w/v) SDS
Midiprep Solution 3:	5M Potassium acetate, 11.5% (v/v) glacial acetic acid
Ni-IDA binding buffer:	500 mM NaCl, 5 mM imidazole, 20 mM Tris (pH 7.9)
Ni-IDA wash buffer:	500 mM NaCl, 60 mM imidazole, 20 mM Tris (pH 7.9)
Ni-IDA elute buffer:	500 mM NaCl, 1M imidazole, 20 mM Tris (pH 7.9)
Ni-IDA strip buffer:	100 mM EDTA, 250 mM NaCl, 20 mM Tris (pH 7.9)
Ni-IDA charge buffer:	50 mM NiSO_4
PBS:	8% (w/v) NaCl, 0.02% (w/v) KCl, 0.02% (w/v) KH_2PO_4 , 0.115% (w/v) Na_2HPO_4 .
PBST:	1× PBS, 0.1% (v/v) Tween-20
PEG/LiAc solution:	40% PEG4000, 10 mM Tris pH7.5, 1 mM EDTA, 100 mM LiAc
Plate Solution:	40% PEG4000, 100 mM LiAc, 10 mM Tris pH7.5, 1 mM EDTA
PMA:	1 $\text{mg} \cdot \text{mL}^{-1}$ in DMSO (= 1.62 M)
PPD/PBS/glycerol:	9.3 mM PPD, 0.1 X PBS, 80% glycerol (pH 9.0). Stored in the dark at -20°C .
RNase A buffer:	10 mM Tris-Cl (pH 7.5), 15 mM NaCl
Super Duper buffer [10×]:	330 mM Tris pH8.3, 625 mM KAc, 100 mM MgAc, 40 mM spermidine, 5 mM DTE
SDS-PAGE load buffer:	0.05M Tris (pH 6.8), 4% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) β -mercaptoethanol, 0.01% (w/v) Coomassie Brilliant Blue
Sonication buffer:	50 mM Tris (pH 8.0), 0.02% Triton-X-100, 1 mM EGTA and freshly added 2 mM PMSF, 1 mM NaVO_4 , 50 mM NaF, 1× Protease inhibitor cocktail.
Stripping Buffer:	100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7)

TAE:	40 mM Tris, 20 mM NaAc, 10 mM EDTA (pH 8.2)
TBS:	25 mM Tris pH7.4, 137 mM NaCl, 2.7 mM KCl
TE:	10 mM TrisHCl (pH 7.5), 1 mM EDTA
TEN:	10 mM TrisCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl
TTBS:	TBS, 0.1% Triton-X-100
WCE buffer:	20 mM HEPES, 420 mM NaCl, 0.5% NP-40, 25% Glycerol, 0.2 mM EDTA, 1.5 mM MgCl ₂ and freshly added 1 mM PMSF and 1× Protease inhibitor cocktail
XGAL:	20 mg.mL ⁻¹ in DMF, stored at -20°C
XGAL overlay solution:	0.5M Na phosphate pH7.0 (made by mixing 39 mL 1 M NaH ₂ PO ₄ with 61 mL 1 M Na ₂ HPO ₄ , check pH 7.0), 0.1% SDS, 0.5 mg.mL ⁻¹ XGAL, 0.5% agarose

2.1.5 Bacterial Strains

Stock cultures of these *Escherichia coli* strains (and transformants) were stored as glycerol stocks at -80°C.

DH5α:	The <i>E. coli</i> DH5α strain was used in transformations and was a host for all recombinant plasmids. DH5α: supE44, DlacU169 (phi80,lacZ,DM15), hsdR17, recA1, endA1, gyrrA96, thi1, relA1.
BL21(DE3):	The <i>E. coli</i> BL21(DE3) strain was used in transformations and was a host for recombinant protein expression plasmids. BL21(DE3): hsdS, gal (λcIts857, ind1, Sam7, nin5, lacUV5-T7gene1)
KC8:	The <i>E. coli</i> KC8 strain was used in transformations to rescue plasmids from yeast. KC8: hsdR, leuB600, trpC9830, pyrF::Tn5, hisB463, lac-Δ-X74, strA, galU, K. KC8 is kanamycin resistant.

2.1.6 Yeast Strain

Stock cultures of this strain and transformants were stored as glycerol stocks at -80°C.

EGY48:	The <i>Saccharomyces cerevisiae</i> EGY48[p8opLacZ] strain (Estojak <i>et al.</i> , 1995) was used in transformations and was a host for recombinant plasmids in yeast-two-hybrid assay controls and screens. The EGY48[p8opLacZ] reporter host strain carries a wildtype LEU2 gene under control of LexA operators and a p8opLacZ 10.3 kb reporter plasmid that encodes a LacZ gene under the control of LexA operators (CLONTECH Laboratories, 1996, p14-15). EGY48: MATα, his3, trp1, ura3, LexA _{op(x6)} -Leu2.
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2.1.7 Mammalian Cell Lines

The following cell lines have been used throughout the course of this study:

- COS1: COS 1 monkey kidney fibroblast cells (Gluzman, 1981) were obtained from American-Type Tissue Culture Collection (ATCC). This cell line is a suitable host for transfection, especially for vectors requiring expression of the SV40 T antigen.
- MCF-7: MCF-7 human breast adenocarcinoma cells (Soule *et al.*, 1973) were obtained from F. John Ballard, Human Nutrition, CSIRO, Adelaide.
- U937: U937 human histiocytic lymphoma cells (Sundstrom and Nilsson, 1976) were obtained from ATCC. The cells bear receptors for Fc and C3, phagocytose antibody-coated erythrocytes and latex beads and can be induced to terminal monocytic differentiation by various agents including phorbol ester (taken from ATCC catalogue).
- HepG2: HepG2 human liver hepatocellular carcinoma cells (Aden *et al.*, 1979) were obtained from ATCC.
- Myc 1-9E10.2: Myc 1-9E10.2 mouse hybridoma cells (Evan *et al.*, 1985) were obtained from ATCC. The cells secrete monoclonal antibody (IgG1) that recognises human c-myc protein and the Myc epitope-tag (GEQKLISEEDLN) by Western blot analysis.

2.1.8 Bacterial Growth Media

Growth media were prepared using Milli-Q[®] water and were sterilised by autoclaving. When required, Ampicillin (Amp, final 100 $\mu\text{g.mL}^{-1}$) or Kanamycin (Kan, final 25 $\mu\text{g.mL}^{-1}$) was added after the media solution had cooled to 50°C.

- Luria broth (LB): 1% (w/v) bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl. The pH was adjusted to 7.0 with NaOH.
- LB agar plate: LB medium supplemented with 1.5% (w/v) bacto-agar.
- MinA: 60 mM K_2HPO_4 , 33 mM KH_2PO_4 , 1.7 mM $\text{Na}_3\text{Citrate}$, 15 mM NH_4Cl . Autoclave. Then add 0.005% (w/v) thiamine, 0.2% (w/v) glucose, 0.8 mM MgSO_4 . (Miller, 1972)
- M9/-Trp/Amp plates: 1.5% bacto-agar, 0.1 mM CaCl_2 , 1 mM MgSO_4 , 10 $\mu\text{g.mL}^{-1}$ Thiamine, 0.005% casamino acids, 0.2% glucose, 1× M9 salts, 100 $\mu\text{g.mL}^{-1}$ Amp

SOC medium:	2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl ₂ , 10 mM MgSO ₄ , 20 mM glucose
YENB medium:	0.75% bacto yeast extract, 0.8% bacto nutrient broth

2.1.9 Yeast Growth Media

Synthetic-dropout selection (or stock) media contains yeast-nitrogen-base (final 0.8% w/v), carbon source (glucose: final 2% w/v) and synthetic-dropout amino acids (final 1×). In synthetic-dropout induction (or screen) media, the carbon source is galactose (final 2% w/v) supplemented with raffinose (final 1% w/v; raffinose improves growth properties of the cells; CLONTECH Laboratories, 1996, p23). Solid media is prepared from liquid media supplemented with 2% w/v Bacto-Agar. During yeast two-hybrid screening the synthetic-dropout/galactose/raffinose induction plates are supplemented with 10× BU salts (final 1×) and XGAL (final 80mg.L⁻¹). Note: If medium is too hot (>55°C) when BU salts added they will precipitate; XGAL is thermolabile and will be destroyed if added to hot medium.

2.1.10 Synthetic-Dropout Amino Acid Mix

Synthetic-dropout media is made with omission of appropriate amino acids in the 10× stocks that contain the following ingredients per 100 mL:

20 mg each of arginine, histidine (omitted for selection of plasmid pLexA) and methionine.

30 mg each of isoleucine, lysine and tyrosine.

50 mg each of phenylalanine, leucine (omitted for selection of protein-protein interaction in screen) and tryptophan (omitted for selection of plasmid pB42AD).

100 mg of uracil (omitted for selection of plasmid p8opLacZ).

150 mg each of valine and adenine.

200 mg of threonine.

10× amino acid stock is prepared by dissolving all of the above ingredients in Milli-Q[®] water at 70-80°C, with stirring and sterilised by filtration through a Sartorius Minisart 0.45 µm filter. Stocks are stored at 4°C. Adenine and Uracil are added with the amino acids, instead of separately, for convenience.

2.1.11 Mammalian Cell Growth Media

The following tissue culture media components were purchased from the listed suppliers.

Dulbecco's modified eagles medium (DMEM)	Gibco BRL
Fetal bovine serum (FBS)	JRH
L-Glutamine	Gibco BRL
RPMI 1640	Gibco BRL

2.1.12 Kits

UltraClean TM plasmid DNA preparation	Geneworks, MoBio
UltraClean TM DNA purification	Geneworks, MoBio
Quikchange TM Mutagenesis	Stratagene
Matchmaker LexA Two-Hybrid System	CLONTECH Labs, Inc

2.1.13 Enzymes

Enzymes were obtained from the following listed suppliers:

Restriction endonucleases	AP Biotech, Geneworks
Calf intestinal phosphatase (CIP)	Boehringer Mannheim
T4 DNA ligase	Geneworks
<i>E. coli</i> DNA polymerase I (klenow fragment)	Geneworks
Turbo Pfu DNA polymerase	Stratagene
Ribonuclease A (RNaseA)	Sigma
Thrombin, bovine plasma	Sigma
Trypsin	Gibco BRL
Enterokinase	Sigma

2.1.14 DNA Molecular Weight Standards

The following DNA markers were purchased from Geneworks:

SPP1:	<i>Eco</i> RI digested SPP1 DNA markers. Band sizes (bp): 8557, 7427, 6106, 4899, 3639, 2799, 1953, 1882, 1515, 1412, 1164, 992, 710, 492, 359, 81.
pUC19:	<i>Hpa</i> II digested pUC19 DNA markers. Band sizes (bp): 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 34, and 26.

2.1.15 Protein Molecular Weight Standards

The following protein markers were purchased from Sigma:

- SDS-7: Contains a mixture of 7 proteins. Approximate MW (kDa): 66, 45, 36, 29, 24, 20.1 and 14.2.
- C3437: Wide Range Colour Markers contains a mixture of 8 coloured proteins. Attached dye colour and approximate MW (kDa): blue 280, turquoise 126, pink 83, yellow 48, orange 28, green 22, purple 15 and blue 9.

The following protein markers were purchased from Gibco:

- BenchmarkTM Prestained Protein Ladder molecular weight markers: Contains a mixture of 10 proteins stained blue except 67 kDa stained pink. Approximate MW (kDa): 221, 133, 93, 67, 56, 42, 28, 23, 17, and 11.

2.1.16 Cloning Vectors and Bacterial Protein Expression Vectors

- pBluescriptII-KS⁺, pBluescriptII-SK⁺ (Stratagene): 2961 bp cloning vector.
- pGem-T EASY (Promega): 3015 bp cloning vector for cloning PCR products.
- pGex2T, pGex4T2, pGex6P1 (AP Biotech): 4900 bp cloning and bacterial expression vector; used to generate fusions of a known protein with glutathione-S-transferase (GST) protein (26 kDa).
- pET32a⁺ (Novagen): 5900 bp cloning and bacterial expression vector; used to generate fusions of a known protein with Thioredoxin (Trx) (109aa) and 6-His tag.

2.1.17 Yeast Two-Hybrid Vectors (CLONTECH Laboratories, 1996)

- pLexA: 10.2 kb cloning and yeast expression vector (Gyuris, 1993); used to generate fusions of the target protein with the LexA protein (amino acids 1-202).
- pB42AD: 6.45 kb cloning and inducible yeast expression vector (Gyuris, 1993); used to generate fusions of a known protein (or a collection of library-encoded proteins) with the B42 AD and HA epitope-tag (YPYDVPDYAS).
- p8opLacZ: 10.3 kb reporter plasmid; encodes a LacZ gene under control of LexA operators (Estojak *et al.*, 1995).

pLexA-53:	11.1 kb positive control plasmid; encodes lexA/murine p53 (amino acids 72-390) fusion protein in pLexA.
pB42AD-T:	8.5 kb positive control plasmid; encodes an AD/SV40 large T-antigen (amino acids 87-708) fusion protein in pB42AD.
pLexA-POS:	13.5 kb positive control plasmid; encodes and expresses a LexA/GAL4 AD fusion protein.
pLexA-Lam:	10.6 kb false positive detection plasmid; encodes a LexA/human laminC (amino acids 66-230) fusion protein in pLexA.

2.1.18 Mammalian Cell Expression Vectors

pEGFP-C2, pEGFP-N2 (CLONTECH):	4700 bp cloning and mammalian cell expression vector; used to generate fusions of a known protein with the enhanced green fluorescent protein (EGFP) protein (240aa). C2 is used to express fusions to the C-terminus of EGFP. N2 is used to express fusions to the amino terminus of EGFP with the inserted gene including the initiating ATG codon.
pXMT2:	5129 bp cloning and mammalian cell expression vector (PDR lab). Used to express proteins from the adenovirus major late promoter.

2.1.19 Cloned Tec DNA Sequences

Mouse Tec2A was a kind gift from Dr James Ihle (St Judes Hospital, Tennessee).

Mouse Tec2B was a kind gift from Dr James Ihle (St Judes Hospital, Tennessee).

Mouse Tec4 was cloned from mouse Tec2B (above) in the lab of Dr Booker by I Atmosukarto and J Doumanis by removal of the 99 bp insertion in the kinase domain-encoding sequence.

Human Tec4 was a kind gift from Dr Hiroyuki Mano (Jichi Medical School, Tokyo).

2.1.20 Cloned Actinin-4 DNA Sequence

Human Actinin-4 was a kind gift from Dr Tesshi Yamada in the lab of Dr Setsuo Hirohashi (National Cancer Centre Research Institute, Tokyo).

2.1.21 Human Liver complementary DNA (cDNA) library

The human liver cDNA library in pB42AD vector was purchased from CLONTECH Laboratories, Inc.

2.1.22 Oligonucleotide Primers Used in Polymerase Chain Reactions

The following primers, listed in Table 2.1, were used in one or more of standard PCR, sequencing or mutagenesis experiments. Their stock number, terminal encoded residue or substitution mutation encoded residue(s), restriction site used for subcloning and nucleotide sequence are listed.

2.1.23 Primary Antibodies

All antibodies were diluted according to the manufacturer's recommendation.

The following antibodies were obtained from these sources:

anti-Actinin-4, raised in rabbit was a kind gift from Dr Tesshi Yamada (National Cancer Centre Research Institute, Tokyo).

anti-Actinin-4 (NCC-Lu632) monoclonal antibody, raised in mouse was a kind gift from Dr Tesshi Yamada (National Cancer Centre Research Institute, Tokyo).

anti-green fluorescent protein (GFP), raised in rabbit was a kind gift from Dr Pamela Silver (Dana-Farber Cancer Institute, Boston, MA).

anti-GFP(I-16) (sc-5385), raised in goat from Santa Cruz Biotechnology (Santa Cruz, CA).

anti-6HIS (12CA5) monoclonal antibody, raised in mouse was provided by Joe Wrin (Adelaide University).

anti-LexA, raised in rabbit was a kind gift from Dr Rob Saint (Adelaide University).

anti-LexA, raised in mouse was a kind gift from Dr Rob Saint (Adelaide University).

anti-Myc (Myc 1-9E10.2) monoclonal antibody, raised in mouse was prepared from hybridoma cells (Section 2.3.4.2).

anti-pTyr monoclonal antibody, raised in mouse, was a kind gift of Dr Shaun McColl (Adelaide University).

anti-Tec kinase (sc-1109), raised in goat from Santa Cruz Biotechnology (Santa Cruz, CA)

Table 2.1 Oligonucleotide Primers

Number	Encoded	RE Site	Nucleotide Sequence
1	S152	<i>EcoRI</i>	GGCAGGGGAATTCTTAAGTACTC
2	M1	<i>BamHI</i>	CAACCAGGGATCCGAGATGAATTTC
5	M117	<i>BamHI</i>	CAATAGGATCCATGATTAAATAC
6	K107	<i>EcoRI</i>	GTTCTTGAATTCTTATTTTAAC
7	-	-	GTAAACGACGGCCAGT
8	-	-	CACACAGGAAACAGCTATGACCATG
9	-	-	GGGCTGGCAAGCCACGTTTGGTG
10	-	-	CCGGGAGCTGCATGTGTCAGAGG
48	-	-	CGAACGCCAGCAGCATGGACA
59	S224	<i>EcoRI</i>	CCGGAATTCGAGTGAAGGATATATC
60	E263	<i>BamHI</i>	CGCGGATCCTTCCGTTCTGAG
62	V344	<i>BamHI</i>	GCGGGGATCCGTCAGTACAAAGGGG
63	R630	<i>EcoRI</i>	GCCGAATTCAGTGGGGCCACC
80	-	-	CGCCCGAATTAGCTTGCTG
81	-	-	ATTTCTGGCAAGGTAGACAAGCCG
87	S152	<i>BamHI</i>	ATGGATCCAGTATAAGAAAGACC
136	-	-	CCAGCCTCTGTGAGTGGAGATG
137	-	-	CGTCAGCAGAGCTTACCATTG
140	M1	<i>EcoRI</i>	CAGGAATTCGAGATGAATTTTAACAC
141	R631	<i>EcoRI</i>	GAGGAATTCGTCACACATCACTTATC
142	N114	<i>EcoRI</i>	AAGGAATTCATAATATCATGATTAAATACCATCC
143	I498	<i>EcoRI</i>	TGCGAATTCATCTGTGACCAAGTGGGACGC
156	K518	<i>EcoRI</i>	GGAATTCAAAACAGAGAAGCAGCTGGAG
157	A538	<i>EcoRI</i>	GGAATTCGCCCCCTTCAACAAGTGG
160	A658	<i>XhoI</i>	CCGCTCGAGTTAGGCTGGCTGGCGAAGTGG
161	E678	<i>XhoI</i>	CCGCTCGAGTTACTCAATGGAGATGCGCC
162	S696	<i>XhoI</i>	CCGCTCGAGTTAGCTGCGTTCATACTGCTTCAG
163	L638	<i>XhoI</i>	CCGCTCGAGTTACAGGAGGGCATGGTCC
164	A602/A603/A604	<i>NotI</i>	CGCTGAGAGCAACGCGGCCGCGTGTGCGGCAG
165	A602/A603/A604	<i>NotI</i>	CTGCCCCGACAGCGCGGCCGCGTGTCTCAGCG
166	A18/A19/A20	<i>PstI</i>	GGTCACAGCAGGCTGCAGCGACATCGCCCTTAA
167	A18/A19/A20	<i>PstI</i>	TTTAAGGGCGATGTCGCTGCAGCCTGCTGTGACC
170	T519	<i>BamHI</i>	GGGATCCACAGAGAAGCAGCTGGAG
171	Q645	<i>XhoI</i>	GAGCTCGAGTTACTGCTGCTTGTCTGCTG
172	E187	<i>NcoI</i>	CGTTGTAGCCATGGAGATTTCGAAG
173	E187	<i>NcoI</i>	CTTGGAATCCTCCATGGCTACAACG
176	A271	<i>EcoRI, XbaI</i>	CGAATTCGCGACCATGGTTTCTAGAGCGCAGAAGGCTG
177	L911	<i>EcoRI</i>	GGAATTCCTCACAGGTCGCTCTCGCCATACAAG
178	M1	<i>EcoRI</i>	GGCGAATTCGACCATGAATTTCAACACTAT
179	I153	<i>BamHI</i>	GACGGATCCCTCTCGGTACTATACTACTCTCAAAAAGATTG
185	N403	<i>EcoRI</i>	TGGGAATTCATGAGATCCGCAGGC
186	D758	<i>XhoI</i>	GAGCTCGAGTTAGTCGCGGTGAGGATCT
215	-	-	CGTCGCCGTCAGCTCGACCAG
225	C29	-	CTTAAACTACAAGGAGTGCCTTTTGTACTTAC
226	C29	-	GTAAGTACAAAAAGGCACTCCTTGTAGTTTAAAG
249	E397	<i>PvuI</i>	CCAGTACAAAGTGGCGATCGAGGCTATCCGGG
250	E397	<i>PvuI</i>	CCCGGATAGCCTCGATCGCCACTTTGTACTGG
253	M1	<i>EcoRI</i>	CCTGAATTCGCCACCATGAATTTCAACACTATCC
254	R630	<i>BamHI</i>	CCCGGATCCCTCTTCCAAAAGTTCTTCACATTC

2.1.24 Secondary Antibodies

All antibodies were diluted according to the manufacturer's recommendation.

The following antibodies were obtained from these sources:

anti-goat-Cy3 conjugate, raised in rabbit	Sigma (St. Louis, MO)
anti-goat-HRP conjugate, raised in rabbit	Sigma (St. Louis, MO)
anti-goat IgG-FITC conjugate, raised in rabbit	Sigma (St. Louis, MO)
anti-goat IgG-(Fab ₂ fragment)-FITC conjugate, raised in donkey	Jackson ImmunoResearch
anti-goat IgG-TRITC conjugate, raised in rabbit	Sigma (St. Louis, MO)
anti-mouse-AP conjugate, raised in rabbit	Sigma (St. Louis, MO)
anti-mouse-HRP conjugate, raised in sheep	Sigma (St. Louis, MO)
anti-mouse IgM-FITC conjugate, raised in goat	Sigma (St. Louis, MO)
anti-mouse-(Fab ₂ fragment)-Rhodamine-Red-X conjugate, raised in donkey	Jackson IR
anti-rabbit-HRP conjugate, raised in donkey	Rockland (Gilbertsville)
anti-rabbit IgG-FITC conjugate, raised in goat	Sigma (St. Louis, MO)

2.2 Electronic Resources

2.2.1 Internet Databases

The following internet databases were accessed at these internet addresses.

GenBank:	www.ncbi.nlm.nih.gov/Genbank/Genbanksearch.html
SwissProt:	http://au.expasy.org/sprot
PubMed:	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

2.2.2 Internet Software

The following software was accessed using the internet to analyse DNA and peptide sequences.

BLAST:	Basic Local Alignment Search Tool (BLAST) was used to search the GenBank database for sequence that matches the input sequence (Altschul <i>et al.</i> , 1990). This is helpful to verify the identity and fidelity of cloned DNA sequences. Address: http://www.ncbi.nlm.nih.gov/BLAST/
ClustalW:	ClustalW was used to align DNA or protein sequences to identify homologous or conserved regions or residues (Thompson <i>et al.</i> , 1994). It produces biologically meaningful multiple sequence alignments of

divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen.

Address: <http://www2.ebi.ac.uk/clustalw/>

MULTALIN: MULTALIN was used to align multiple protein sequences to identify conserved residues (Corpet, 1988).

Address: <http://prodes.toulouse.inra.fr/multalin/multalin.html>

pI/MW: pI/MW was used to predict the pI and molecular weight of input peptide sequences for protein size estimation (Bjellqvist *et al.*, 1993). This is important for recombinant protein analysis such as SDS-PAGE and Western blot, and particularly when designing purification experiments to separate two protein fragments after protease cleavage.

Address: http://au.expasy.org/tools/pi_tool.html

SwissModel: SwissModel was used to model the 3D image of a protein domain by using the PDB coordinates of an experimentally determined structure as a template and threading through it the sequence of a related protein domain (Guex and Peitsch, 1997).

Address: <http://www.expasy.ch/swissmod/SWISS-MODEL.html>

2.2.3 DNA Sequence Analysis

The following software was used to analyse DNA sequence.

DNASIS: DNASIS software (Hitachi Software Engineering Co) was used to edit DNA sequence data, create DNA sequence contigs, search for restriction sites and determine encoded peptide sequences.

OligoTM: OligoTM software (Wojciech Rychlik) was used to help design DNA primers for PCR reactions. Primer sequence suitability was judged on calculated parameters including: unique 3' end/false priming, self annealing, hairpin-loop formation, % GC content, predicted melting temperature at which half maximal binding occurs and amplification parameters.

2.3 Methods

2.3.1 Molecular Biology Techniques

2.3.1.1 Primer Design

Primers for polymerase chain reaction (PCR) reactions were designed with the aid of OligoTM software. Generally, primers were >17 bp long, terminated in a G/C residue, had >40% G/C content, unique 3' end of 7 nucleotides (compared to the template) and did not self anneal. Primer pairs had similar melting temperature at which half maximal binding occurs. Primers designed to amplify sequences for cloning incorporated a restriction site at the beginning of the coding sequence (generally *EcoRI*) and a different restriction site (generally *XhoI*) and stop codon at the end of the coding sequence. Primers designed for mutagenesis reactions incorporated nucleotide mismatch(es) in the centre flanked on both sides by at least 10 matched nucleotides. Where possible, mutagenesis primers introduced or removed a restriction site for later ease of screening for mutants.

2.3.1.2 Polymerase Chain Reaction (PCR)

PCR reactions had final concentrations of 1× reaction buffer, 1 mM dNTPs, 5 ng.μL⁻¹ each primer, 0.8 ng.μL⁻¹ template DNA and 0.02 U.μL⁻¹ Pfu DNA polymerase in a total volume of either 25 μL or 50 μL. Reactions proceeded in a PTC-100 Programmable Thermal Cycler (MJ Research Inc). Initial denaturation (94°C) for 5 min was followed by 30 cycles of denaturation (94°C) for 1 min; annealing (50°C) for 1 min; and extension (72°C) for 1 min. After final extension (72°C) for 10 min, samples were stored at 4°C until further processing.

2.3.1.3 Site-Directed Mutagenesis

The QuikchangeTM mutagenesis kit (Stratagene) was used for mutagenesis PCR reactions according to the manufacturers recommendation. Pfu DNA polymerase was used to replicate both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers, which are each complementary to opposite strands of the vector (Stratagene). Briefly, each 50 μL reaction contained 1× reaction buffer, 1 mM dNTPs, 2.5 ng.μL⁻¹ each primer, 2.5 U Pfu DNA polymerase and 0-50 ng of template DNA. Negative control reaction contained no Pfu DNA polymerase. Initial denaturation (94°C) for 5 min was followed by 16-20 cycles of denaturation (94°C) for 1 min; annealing (50-60°C) for 1 min; and extension (68°C) for 9 min, in a PTC-100 Programmable Thermal Cycler (MJ Research Inc). Samples were stored at 4°C until *DpnI* digestion, which removed parental DNA. Reactions were precipitated in

NaAc/EtOH, resuspended in Tris-EDTA (TE) and transformed in to DH5 α competent cells using heat shock or electroporation. Mutants were identified, where possible, using restriction digest when mutagenesis primers introduced or removed a restriction site; otherwise, they were identified by DNA sequence analysis. The DNA sequence of the mutant clones was verified by automated DNA sequence analysis.

2.3.1.4 Agarose Gel Electrophoresis

Horizontal minigels were prepared by pouring 10-12 mL of melted 0.8-2.5% agarose/1 $\times$ TAE gel solution onto a 5.0 cm x 7.5 cm glass microscope slide with a comb inserted to form wells. Agarose gels were submerged in 1 $\times$ TAE buffer and DNA solution samples were mixed with gel load buffer (final 1 $\times$ GLB), loaded into a well and electrophoresed at 80 V for 30-60 min. The migration of DNA was visualised by staining with ethidium bromide (EtBr, 5 $\mu\text{g}.\text{mL}^{-1}$) followed with exposure to short wavelength ultra violet (UV) light on a Chromato-Vue transilluminator (Ultra-Violet Products Inc, San Gabriel, CA). Images were captured on photoprint paper with a Mitsubishi Video Copy Processor (Mitsubishi Electric Corporation). To avoid UV light-induced damage to DNA, preparative gels were visualised using long wave UV light and the desired DNA fragments were isolated using a fresh scalpel blade and processed as described in Section 2.3.1.8.

2.3.1.5 Restriction Endonuclease Digestion of Plasmid DNA

4-6 μg of plasmid DNA was digested with 2-4 units of restriction enzyme per 1 μg of DNA, in a 20 μL reaction at 37°C for 2-6 h in 1 $\times$ Super Duper buffer. The extent of digestion was assayed by agarose gel electrophoresis (Section 2.3.1.4).

2.3.1.6 Removal of 5' Phosphate from Linear DNA Fragments

To prevent recircularisation of the parental vector, the 5' termini of vector DNA fragments to be used in non-directional cloning reactions were dephosphorylated by calf intestinal phosphatase (CIP, Boehringer Mannheim Biochemicals). For termini with 5' overhang, 1 unit of CIP was added to 20 μL restriction enzyme digest reactions and incubated at 37°C for 30 min followed by addition of an extra 1 μL CIP and incubation at 37°C for 15 min. For blunt termini or 3' overhang termini, 2 units of CIP were added to 20 μL restriction enzyme digest reactions and incubated at 37°C for 15 min followed by incubation at 55°C for 30 min.

2.3.1.7 Endfill Reaction

Up to 5 µg of DNA with 5' overhangs, to be used in blunt end ligation reactions, was endfilled with reactions containing 1× Klenow reaction buffer (100 mM Tris-HCl (pH 7.5), 500ng DNA, 100 MgCl₂, 100 mM NaCl), 0.05 mM dNTPs and 3U Klenow fragment in a total volume of 20 µL at 37°C for 30 min.

2.3.1.8 Purification of Linear DNA Fragments

To purify DNA fragments for use in ligation reactions, restriction fragments were separated by agarose gel electrophoresis as described in Section 2.3.1.4. DNA fragments were extracted from agarose/TAE minigels using the Ultraclean™ (MoBio) DNA purification kits according to the manufacturers instructions. Briefly, the agarose containing the DNA was excised from the gel with a sterile scalpel blade, melted in salt solution at 55°C for 5 min and mixed with DNA-binding silica beads at room temperature for 5 min. The beads were harvested by centrifugation and washed. DNA was eluted with TE at 55°C for 3-5 min and collected after centrifugation.

2.3.1.9 Ligation Reactions

Ligation reactions typically contained a 3:1 molar ratio of insert to vector DNA fragments and were carried out in 1× Ligase Buffer with 1 mM ATP and 1U of T4 DNA ligase in a 20 µL volume. Reactions were allowed to proceed at 16°C for 2 h-overnight.

2.3.1.10 Preparation of Calcium Chloride Competent Cells

Competent cells used to transform plasmid DNA or ligation reactions were prepared using the following method. A small-scale bacterial culture was set up in LB medium from a single *E. coli* colony (DH5α, BL21DE3 or KC8 strain) and grown overnight at 37°C with shaking. The following morning, a 1% subculture was made in 500 mL of LB medium and the culture was expanded to an optical density at 600nm wavelength (OD_{600nm}) of 0.4-0.5. The culture was chilled on ice for 10 min and bacterial cells were collected by centrifugation at 2990 x g (4°C) for 15 min. Pellets were then washed in 100 mL ice cold CaCl₂ solution (0.06 M CaCl₂, 15% v/v glycerol), centrifuged at 2990 x g (4°C) for 15 min and resuspended in 20 mL ice cold CaCl₂ solution. Approximately 200 µL aliquots in Eppendorf tubes were snap frozen in a dry ice/ethanol bath and stored at -80°C for up to six months.

2.3.1.11 Preparation of Electro-Competent Cells

Competent cells used to transform plasmid DNA or site-directed mutagenesis reactions were prepared using the following method. A small-scale bacterial culture was set up in LB medium from a single *E. coli* DH5 α colony and grown overnight at 37°C, shaking. The following morning, a 1% subculture was made in 1 L of YENB medium (Section 2.1.8) and the culture was expanded until to an OD_{600nm} of 0.5-0.7. The culture was chilled on ice for 5 min and bacterial cells were collected by centrifugation at 4000 x g (4°C) for 10 min. Pellets were then washed twice in 100 mL ice cold water, once in 20 mL ice cold 10% glycerol with centrifuging as before and resuspended in 2 mL cold 10% glycerol. 40 μ L aliquots in Eppendorf tubes were snap frozen in a dry ice/ethanol bath and stored at -80°C for up to 2 years.

2.3.1.12 Heat Shock Transformation of Competent Cells

Competent cells were transformed with circular plasmids by the heat shock or electroporation method as follows. Plasmid DNA or 20 μ L ligation reaction was mixed with 200 μ L CaCl₂ competent cells and incubated on ice for 20 min before heat shock at 42°C for 2 min and then incubation on ice for a further 10 min. Cells were then plated onto LB agar plates with appropriate selection and incubated overnight at 37°C.

2.3.1.13 Electroporation Transformation of Competent Cells

1-5 μ L DNA in TE was mixed with 40 μ L electrocompetent cells and incubated on ice for 1 min before transfer to a cold 0.2cm electroporation cuvette (BIORAD). Cells were electroporated in a Bio-Rad Gene Pulser according to the manufacturers instructions. The cuvette was removed from the chamber and 1 mL of SOC medium (Section 2.1.8) was immediately added to the cells which were transferred into a 10 mL yellow cap tube and incubated in a rolling drum for 1 h before centrifuging 300 x g and plating onto LB agar plates with appropriate selection and incubated overnight at 37°C.

2.3.1.14 Making Glycerol Stocks

A small-scale bacterial culture was set up in selective LB medium from a single *E. coli* transformant colony and grown overnight at 37°C, shaking. The following morning, quadruplicate 500 μ L samples were diluted 1:1 with 80% glycerol, mixed and stored at -80°C.

2.3.1.15 Colony Cracking

Colony cracking was used to identify transformant colonies from ligation reactions that contain plasmid with insert. A transformant colony was picked with a yellow tip, dipped in LB medium (100 μ L) and incubated in 15 μ L cracking solution at 65°C for 15 min, expressed from the tip and further incubated for 10 min at 65°C with Eppendorf tube lid shut. The sample was dry loaded onto a 1% agarose gel next to control plasmid (uncut parental plasmid) and electrophoresed at 30 V for 10 min followed by addition of extra TAE buffer and electrophoresis at 90 V for 40 min. Gels were stained with EtBr and plasmids with insert were identified under UV light by the presence of larger plasmid than control. The 100 μ L LB medium sample was used to inoculate overnight culture (with appropriate selection) for plasmid preparation and glycerol stock.

2.3.1.16 Determining cDNA Library Titre

As a preliminary step to amplify the cDNA library in bacteria, the titre was determined as according to the manufacturers recommendation. Titre is expressed as colony forming units (CFU) per mL of culture. Serial 1:1000 dilutions were made from a thawed library aliquot. Samples of these were plated onto LB agar plates containing 100 μ g.mL⁻¹ Amp that were incubated overnight at 37°C. The number of colonies on each plate was counted. The titre was calculated by multiplying the number of colonies by the dilution factor and dividing this by the volume plated, for each plate.

2.3.1.17 Megadeath Preparation of Plasmid DNA

DNA used in restriction digests to check cloning steps was prepared as follows. Approximately 20 μ g of plasmid DNA was typically extracted from 5 mL bacterial cultures, containing 100 μ g.mL⁻¹ Amp or 25 μ g.mL⁻¹ Kan, inoculated with a single transformant bacterial colony and incubated overnight at 37°C in a rotating drum. Bacterial cells were harvested by centrifugation at 1400 x g for 5 min in 10 mL yellow cap tubes. Cell pellets were subsequently resuspended in approximately 50 μ L of supernatant, transferred to Eppendorf tubes and lysed by mixing with 600 μ L of Megadeath solution. Cellular proteins and chromosomal DNA were precipitated with 300 μ L of 3M NaAc (pH 5.2) and removed by centrifugation at 20,800 x g for 3 min. 600 μ L of supernatant was cleaned by phenol/chloroform and chloroform extractions. Plasmid DNA was precipitated with 1 mL ice cold 95% ethanol, centrifuged for 5 min at 20,800 x g, washed with 70% ethanol, air dried

and resuspended in 20 μL of TE. Bacterial ribonucleic acid (RNA) was removed with RNaseA (final 0.5 mg.mL^{-1}) digestion at 37°C for 15 min.

2.3.1.18 Midiprep Preparation of Plasmid DNA

DNA used in cloning, sequencing and transfection was prepared as follows. Medium scale DNA preparations were obtained from 50 mL cultures of LB medium containing either 100 $\mu\text{g.mL}^{-1}$ Amp or 25 $\mu\text{g.mL}^{-1}$ Kan that were inoculated with a single colony and grown at 37°C overnight with shaking. Bacterial cultures were centrifuged at 2990 x g for 5 min and cell pellets were resuspended in 3 mL Midiprep Solution 1. Cells were lysed by mixing with 6 mL Midiprep Solution 2 and incubation on ice for 5 min. Cellular proteins and chromosomal DNA were precipitated by addition of 4.5 mL Midiprep Solution 3 and incubation on ice for 20 min, and removed by centrifugation at 20,200 x g for 15 min. Plasmid DNA was precipitated with 8 mL isopropanol (final 37% v/v) and harvested by centrifugation at 17,200 x g for 5 min before resuspension in 400 μL TE. Bacterial RNA was removed by digestion with RNaseA (final 0.05 mg.mL^{-1}) for 30 min at 37°C. DNA preparations were cleaned by phenol/chloroform and chloroform extractions. Plasmid DNA was precipitated with NaAc (final 0.2M) and ethanol (final 65%) and collected by centrifugation at 20,800 x g for 10 min. Pellets were washed with 70% ethanol and resuspend in 200 μL TE.

2.3.1.19 Megaprep Preparation of Plasmid DNA

Large-scale DNA preparations were obtained from 500 mL cultures of LB medium containing 100 $\mu\text{g.mL}^{-1}$ Amp or 25 $\mu\text{g.mL}^{-1}$ Kan that were inoculated with a single colony and grown at 37°C overnight with shaking. DNA was prepared as in Section 2.3.1.18 with volumes scaled up accordingly.

2.3.1.20 Small Scale Kit Preparation of Plasmid DNA

DNA used in cloning and sequencing was prepared as follows. Up to 5 mL of overnight culture was processed using the UltracleanTM miniprep kit according to the manufacturers recommendation. Briefly, cells were pelleted and all traces of media were removed before resuspension in UltracleanTM miniprep kit Solution 1. Cells were lysed by the addition of kit solution 2. After addition of kit solution 3 contaminants were removed by centrifugation at 20,800 x g for 1 min and filtration through a disposable column. Plasmid DNA attached to the column was washed with kit solution 4 by centrifugation and eluted in TE with centrifugation.

Approximately 10 µg of plasmid DNA suitable for DNA sequencing or mammalian cell transfection was obtained from each preparation.

2.3.1.21 Large Scale Kit Preparation of Plasmid DNA

DNA used in transfections was prepared as follows. 100 mL of overnight culture was processed using the QIAGEN Maxi prep kit according to the manufacturers recommendation. Briefly, cells were pelleted at 2,990 x g for 10 min and resuspended in QIAGEN kit Buffer P1 and lysed by the addition of buffer P2. After addition of buffer P3 contaminants were removed by filtration through a disposable column into a QIAGEN-tip prepared by washing with buffer QBT. Plasmid DNA attached to the tip was washed with buffer QC and eluted in buffer QF, precipitated with isopropanol, washed with 70% ethanol and resuspended in TE. Approximately 500 µg of plasmid DNA suitable for mammalian cell transfection was obtained from each preparation.

2.3.1.22 Cesium Chloride Purification of Plasmid DNA

65% (w/v) CsCl in TL-100 tubes (1.4 mL) was underlayered with plasmid DNA/CsCl/EtBr mix (450-500 µL: prepared by dissolving 0.63 g CsCl in 360 µL DNA and adding 60 µL of 10 mg.µL⁻¹ EtBr) using a 21G needle and 1 mL syringe. After heat-sealing, tubes were centrifuged at 100,000 rpm (356,160 x g) for 3 h at 20°C in a TL100.2 fixed angle Ultracentrifuge rotor (Beckman, USA). Plasmid DNA was harvested under UV light using a 21G needle, extracted 3× with H₂O saturated butan-1-ol, diluted with an equal volume of H₂O, precipitated with 2 volumes of ethanol at room temperature for 15 min and pelleted by centrifugation for 10 min. Pellets were washed with 400 µL 70% ethanol and resuspended in Milli-Q[®] water (50 µL). Concentration was measured using a Cary 3Bio UV-visible Spectrophotometer with Cary Win UV software and adjusted to 1 µg.µL⁻¹.

2.3.1.23 Sequencing of Plasmid DNA

DNA sequencing was performed with reactions containing 0.5-1.0 µg of plasmid DNA, 100ng sequencing primer (Section 2.1.22) and 6 µL DYETERM[™] or BIGDYE[™] ready reaction mix (AP Biotech) in a total volume of 15 µL. 26 rounds of PCR were performed with denaturation at 96°C for 30 sec, annealing at 50°C for 15 sec and extension at 60°C for 4 min. The PCR reaction was precipitated with ethanol (71% final) and NaAc (85 mM final) at -20°C for 15 min followed by centrifugation for 10 min. Pellets were washed with 200 µL

70% ethanol and dried before being processed at the IMVS DNA sequencing facility. Alternatively, the PCR reaction was precipitated with isopropanol (60% v/v final) at room temperature for 15 min followed by centrifugation for 20 min. Pellets were washed with 250 μ L 75% v/v isopropanol and dried before being processed at the IMVS DNA sequencing facility which uses an ABI sequencer (Applied Biosystems).

2.3.2 Protein Chemistry Techniques

2.3.2.1 Induction and Preparation of GST or Trx Fusion Proteins

Bacterial protein expression systems were used to prepare high yields of soluble protein for *in vitro* protein-protein interaction studies and protein structure studies. A small scale bacterial culture was prepared by inoculating LB medium containing 100 μ g.mL⁻¹ Amp with a single *E. coli* BL21(DE3) transformant colony and incubation overnight at 37°C with shaking. The following morning, a 1% subculture was made (small scale: 2 mL; large scale: 1 L) and expanded to an OD_{600nm} of 0.5-0.6. Fusion-protein expression was induced with IPTG (0.1-0.2 mM final) for 2-16 h at 37°C with shaking. Bacterial cultures were centrifuged at 2990 x *g* for 5 min (small scale) or 20 min (large scale) and cell pellets were resuspended in Triton-X-100 Tris buffered saline (TTBS, for GST fusions) or Ni-IDA Binding buffer (for Trx fusions) (small scale: 1 mL; large scale: 100 mL) and lysed by one (small scale) or four (large scale) 30 sec bursts of sonication in the presence of PMSF (1 mM final). Alternatively, large scale preps were lysed by 3 passes through the French press (at 1000 PSI) in the presence of PMSF (1 mM final). The lysate was separated into soluble and insoluble fractions by centrifugation at 17,200 x *g* for 20 min. The supernatant was filtered through a prefilter and 0.45 μ M filter and fusion proteins were purified by affinity chromatography. Samples were taken at various points to monitor the induction and purification of fusion proteins.

2.3.2.2 GST Fusion Protein Affinity Chromatography

Soluble, bacterial-expressed GST-fusion proteins were purified using glutathione-agarose affinity chromatography. After column equilibration in Tris buffered saline (TBS, 4 column volumes) and TTBS (4 column volumes), filtered lysate containing the GST fusion protein was loaded onto the column and the flow through was collected. The column, with bound fusion protein, was washed with TTBS and equilibrated in TBS before the GST fusion protein was eluted with fresh 10 mM reduced glutathione in TBS (pH 8.0). The column was stored in

20% ethanol. Columns were regenerated by washing in HPLC grade water, 6M Guanidine Hydrochloride, water, 70% ethanol, water and TBS.

2.3.2.3 Trx Fusion Protein Affinity Chromatography

Soluble, bacterial-expressed Trx-fusion proteins were purified using Ni-IDA-agarose affinity chromatography. After column equilibration in Ni-IDA Binding Buffer (4 column volumes), filtered lysate containing the Trx fusion protein was loaded onto the column and the flow through was collected. The column, with bound fusion protein, was washed with Ni-IDA Binding Buffer and Ni-IDA Wash Buffer before the Trx fusion protein was eluted with Ni-IDA Elute Buffer. The column was stored in 20% ethanol. Columns were regenerated by washing in HPLC grade water, Ni-IDA Strip Buffer, water, Ni-IDA Charge Buffer, water, 70% ethanol, water and Ni-IDA Binding Buffer.

2.3.2.4 Bradford Assay

Protein concentration was determined by Bradford assay (Bradford, 1976). 10 μL of various Bovine serum albumin (BSA) standards (0 – 0.75 $\text{mg}\cdot\text{mL}^{-1}$) and sample (neat or diluted) were aliquoted into wells of a 96 well tray. 200 μL of 25% Bradford reagent was added to each well and the $\text{OD}_{600\text{nm}}$ was measured. A standard curve was plotted and the sample concentration was measured from the graph.

2.3.2.5 SDS-PAGE

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate proteins prior to Coomassie blue staining or transfer to nylon membrane for Western blot. Denaturing Tris/Tricine/SDS/acrylamide gels were prepared using a Hoefer gel pouring apparatus. 0.75 mM thick 4-12.5% gels were prepared by mixing ingredients listed in Table 2.2. The resolving gel was allowed to polymerise with an overlay of water. The stacking gel was allowed to polymerise with 10-well comb inserted.

Samples were mixed with 5 $\times$ SDS load buffer to a final concentration of 2 $\times$ and heated to 100°C for 3 min before loading onto the gel. Gels were electrophoresed at 50mA per gel using a continuous buffering system with Anode Buffer and Cathode Buffer. The gels were run until the ion front reached the bottom of the gel and either Coomassie blue stained or blotted onto nylon membrane (Section 2.3.2.10). Coomassie blue stain was used to visualise protein content. Stain was removed by extensive washing in Destain. Digital images were

recorded using a Canon laser scanner and UMAX MagicscanII software. SDS-PAGE gels were dried under vacuum using a Slab Gel Dryer SE1160 (Hoefer Scientific Instruments, San Francisco, CA).

Table 2.2 Recipe for 4 SDS-PAGE gels

		Resolving gel			Stacking gel
		8%	10%	12.50%	4%
Acrylamide (40%)	mL	5.0	6.3	7.9	0.89
50% glycerol	mL	4.2	5.3	6.6	-
3X gel buffer	mL	6.6	8.3	10.4	1.95
Water	mL	4.1	5.1	6.4	5.1
Temed	uL	28	35	44	8
APS	uL	80	100	125	63
TOTAL	mL	20.0	25	31.3	7.9

2.3.2.6 Concentration and Buffer Exchange of Recombinant Proteins

Recombinant proteins were concentrated with an Amicon stirred cell under pressure (<450 kPa) from bottled liquid N₂ (BOC Gases). 3 kDa or 10 kDa molecular weight cut-off (MWCO) membranes were used. Smaller volumes were concentrated by centrifugation in Centricon filters at 4,300 x g at 4°C. To exchange the buffer a protein was dissolved in, the protein was serially concentrated and diluted 10-fold in the new buffer up to 4 times. Sometimes PD10 columns (Pharmacia) were used to buffer exchange proteins according to the manufacturers directions.

2.3.2.7 Thrombin Cleavage of Fusion Proteins

To separate recombinant protein from its fusion partner, thrombin cleavage was used. The fusion protein was digested with 9-25 U Thrombin per mg protein in TBS with 2.5 mM CaCl₂ at 37°C for 1-16 h. Reactions were stopped by addition of PMSF to 2 mM and storage on ice.

2.3.2.8 Size Exclusion Chromatography

The products of thrombin cleavage reactions were separated by size exclusion chromatography using a Superdex-G75 column (Pharmacia) with bed volume of 200 mL connected to a Pharmacia pump P-50, lamp LKB-UV-MII, GradiFrac and chart recorder LKB-REC102. The column was initially prepared by washing for 2 h with 0.5 M NaOH at a flow rate of 1 mL.min⁻¹ and then equilibrated extensively in the appropriate buffer. The column was standardised against a solution containing the proteins lysozyme (14 kDa), chymotrypsin (24 kDa), ovalbumin (45 kDa) and BSA (66 kDa) to generate a standard curve.

Thrombin digested GST-fusion proteins were loaded onto the column that was equilibrated with phosphate-buffered saline (PBS) containing 0.01% NaN₃ for 16 h at 1 mL.min⁻¹. The sample was loaded and run at 2 mL.min⁻¹. After loading the sample, proteins were eluted in PBS/0.01% NaN₃. A flow rate of 2 mL.min⁻¹ was used and 5 mL fractions were collected. Protein was detected by measuring A_{280nm}, and this was plotted by the chart recorder. Bradford assay (Section 2.3.2.4) and SDS-PAGE (Section 2.3.2.5) were used to identify fractions containing protein, quantify the amount of eluted protein and analyse the protein size(s). Samples corresponding to the protein of interest were pooled and stored at 4°C.

2.3.2.9 GST Pulldown

0-5 µg of GST-ACTN4 protein was mixed with 40 µg lysate containing EGFP or EGFP-Tec and incubated overnight at 4°C. 10 µL of glutathione agarose beads were added and the mix was incubated for 1 h at 4°C to pulldown GST-containing complexes. After washing the beads 4 times in lysis buffer at 4°C the GST-containing complexes were eluted with SDS-PAGE load buffer at 100°C for 5 min. EGFP- and GST-containing proteins were detected by Western Blotting of pulldown eluants.

2.3.2.10 Western Blot

Briefly, proteins were separated using 8%, 10% or 12.5% Tris/Tricine/SDS/acrylamide gels with a Hoefer gel cast system and transferred to Hybond-C (Amersham) membrane at 40mA/gel for 90 min in 1× WTB containing 20% ethanol using a Hoefer SemiPhor Western Transfer apparatus (Pharmacia Biotech). A Hoefer 500 V/400 mA/200 W DC power supply was used. Membranes were blocked overnight in blocking solution washed where appropriate in wash solution and probed with antibody diluted in blocking solution. Primary antibodies: anti-GFP, anti-GST, anti-ACTN4 (polyclonal) and anti-Tec were used at 1:4000, anti-Myc was used at 1:4; secondary antibodies: anti-rabbit-HRP, anti-mouse-HRP and anti-goat-HRP were used at 1:4000. Enhanced chemiluminescence (ECL) detection was used as follows. Membranes were developed for 1 min in a freshly combined mixture of equal volumes of ECL solutions 1 and 2 and exposed to X-ray film that was developed using a CURIX 60 X-Ray developer. Digital images were recorded using a Canon laser scanner and UMAX MagicscanII software. To strip Western blots for reuse, they were submerged in Stripping buffer at 50°C for 30 min with occasional agitation. After washing twice in wash solution, blots were blocked overnight in blocking solution.

2.3.2.11 NMR Sample Preparation

For NMR spectroscopy analysis, protein was purified away from fusion partner, concentrated, buffer exchanged into PBS, 0.1% NaN₃ and further concentrated. Finally, the pH was adjusted with 1M HCl (to pH6.3) and the sample was supplemented with D₂O (10%, v/v). For samples in 100% (v/v) D₂O, the sample was lyophilised overnight using a Savant Speed Vac SC110 with refrigerated Vapor Trap RVT4104, resuspended in 100% D₂O, lyophilised a second time and finally resuspended in 100% D₂O from a fresh ampule.

NMR tubes were cleaned for NMR experiments by soaking overnight in a 10 M nitric acid solution. Tubes were then washed ten times in distilled water and then ten times in Milli-Q[®] water. The tubes were dried in a 60°C oven overnight, sealed and stored until required.

2.3.2.12 NMR Spectroscopy

NMR experiments were conducted on a Varian Inova 600 spectrometer equipped with a 5-mm inverse broadband triple resonance probehead and z-axis pulsed field gradients. Homonuclear 1D and 2D NMR experiments performed on Rpt3 are listed in Table 2.3.

Solvent suppression was achieved through the use of either on-resonance presaturation during the relaxation delay between scans, or pulsed-field gradients. A spectral width of 8000 Hz was used for ¹H. All experiments were carried out at 20°C.

NMR data were processed on Sun Microsystems workstations. Spectra were processed using the VNMR software package (Varian). The ¹H frequency scale of all spectra was directly referenced to the H₂O reference at 4.7 parts per million (ppm).

Table 2.3 NMR experiments performed on Rpt3

Experiment	Mixing Time (ms)	Scans per t1 increment
1D	n/a	512.0
DQF-COSY	n/a	32.0
TOCSY	50	96.0
NOESY	150	96.0

2.3.3 Yeast Two-Hybrid Assay Techniques

2.3.3.1 Quick Yeast Transformation (QYT)

EGY48[p8opLacZ] yeast were transformed with various combinations of LexA and B42AD expression plasmids to perform the yeast two-hybrid assay controls and experiments. These are denoted EGY48[p8op-LacZ, pLexA-#, pB42AD-#] where # indicates the insert in the

plasmid. For small-scale transformation of yeast, the quick yeast transformation was performed. Yeast cells were streaked onto appropriate synthetic-dropout plates and grown at 30°C for 2-3 days. Plasmid DNA (1 µg) and carrier DNA (0.1 mg) were mixed with a scraping of yeast cells (about 10^7 - 10^8 cells) in an Eppendorf tube by briefly vortexing. Cells and DNA were gently resuspended in 500 µL Plate solution and incubated overnight at room temperature. The bottom 50 µL, containing settled cells, was plated onto appropriate synthetic-dropout plates and incubated at 30°C for 3-4 days until transformants appeared.

2.3.3.2 Yeast Transformation by Electroporation

Large-scale transformation of yeast was performed using electroporation. 200 mL of fresh media was inoculated with overnight culture to an OD_{600nm} of 0.4 (approx. 10 mL) and incubated at 30°C with shaking until an OD_{600nm} of 0.8. Cells were pelleted by centrifugation (1300 rpm 300 x g for 5 min) washed twice in ice cold Milli-Q[®] water (100 mL), washed in ice-cold 1M sorbitol (10 mL) and resuspended in ice-cold 1M sorbitol (0.5 mL). For each electroporation, 100 µL of cell suspension was incubated with 1-5 µg DNA in pre-chilled tubes for 10 min on ice before transfer to a pre-chilled cuvette (Bio-Rad). After electroporation (Bio-Rad Genepulser, 1.5kV, 200 Ohms, 25 µF) ice-cold 1M sorbitol (750 µL) was added to the cells, which were plated onto appropriate synthetic-dropout plates and grown at 30°C.

2.3.3.3 Lithium Acetate Method of Yeast Transformation

Lithium acetate prepared competent cells were used for large-scale transformation of yeast with the cDNA library in pB42AD vector prior to screening, according to the MATCHMAKER LexA Two-Hybrid System User Manual (CLONTECH Laboratories, 1996). Briefly, a 50 mL yeast culture was set up in synthetic-dropout -His/-Ura/glucose medium from several EGY48p8opLacZ, pLexA-PHTH colonies and grown overnight at 30°C with shaking. This was used to inoculate a 300 mL culture to an OD_{600nm} of 0.2-0.3, which was grown for 3 h at 30°C until an OD_{600nm} of 0.5 ± 0.1 . The yeast were harvested by centrifugation at 1000 x g for 5 min at room temperature, washed in Milli-Q[®] water and resuspended in TE/100 mM LiAc (pH 7.5). The yeast were mixed with plasmid DNA and salmon sperm carrier DNA, resuspended in PEG/LiAc solution and incubated at 30°C for 30 min, shaking. DMSO was added (to 10% v/v) and cells were heat shocked at 42°C for 15 min before chilling on ice for 2 min and harvest by centrifugation at 1000 x g for 5 min. The yeast

were resuspended in TE, plated onto stock medium (synthetic-dropout -His/-Trp/-Ura/glucose) for amplification, grown at 30°C for 4 days, harvested, stored as glycerol stocks and titred.

2.3.3.4 Yeast Protein Extraction

To prepare samples for Western blot detection of LexA- or B42AD- fusion protein expression in yeast, yeast protein extracts were prepared. 100 mL of fresh media was inoculated with 1:10 dilution from overnight culture and incubated at 30°C with shaking to an OD_{600nm} of 0.8. Cells were pelleted by centrifugation (290 x g for 5 min) washed twice in ice cold Milli-Q[®] water (100 mL), washed in ice cold lysis buffer (20 mL) and resuspended in 0.6 mL ice cold lysis buffer with PMSF (1 mM final). Cells were mixed with ice-cold glass beads (0.5 mL) in a screw cap Eppendorf and broken open using the large bead beater. Three cycles of beat (30 sec) and incubation on ice (1 min) were followed by centrifugation for 10 min at 4°C. 200 µL of supernatant was harvested and 25 µL aliquots were snap frozen and stored at -80°C until further use.

2.3.3.5 Yeast Two-Hybrid Library Screening

The amplified yeast cotransformants were plated onto screen medium (synthetic-dropout -His/-Trp/-Ura/-Leu/galactose/raffinose) at high density (2x10⁶ CFU per 14 cm diameter large plate) and incubated at 30°C for 5 days. Colonies were streaked onto fresh screen medium (synthetic-dropout -His/-Trp/-Ura/-Leu/galactose/raffinose/BU salts/XGAL) and grown for a further 3-5 days at 30°C. When XGAL was not included in the screen medium, XGAL overlay assay was performed.

2.3.3.6 XGAL Overlay Assay

Screen plates with yeast colonies were warmed to 30°C and overlaid with molten (50°C) XGAL overlay solution (20 mL per 14 cm diameter large plate). Plates were incubated for 2 h at room temperature, stored at 4°C overnight and analysed for blue colour formation.

2.3.3.7 Recovering Plasmids From Yeast

Library plasmids encoding unknown proteins that enabled growth of yeast on screen medium were isolated using this method. Yeast from 5 mL of overnight culture was harvested by centrifugation and resuspended in 1 M sorbitol, 0.1 M EDTA and treated with lyticase (0.09

mg.mL⁻¹) at 37°C 1 h before resuspension in 50 mM Tris pH7.4, 20 mM EDTA and lysis of sphaeroblasts with SDS (1% w/v) at 65°C for 30 min. After addition of KAc (1.3M) and incubation on ice 1 h, the mixture was centrifuged and the supernatant cleaned by phenol/chloroform and chloroform extraction. The nucleic acid was precipitated by isopropanol and resuspended in TE. After RNase A digestion, plasmid DNA was precipitated with isopropanol, resuspended in TE, transformed into *E. coli* KC8 competent cells and plated onto M9/-Trp/Amp plates and incubated overnight at 37°C.

2.3.3.8 Yeast Colony PCR

The library insert in plasmids encoding unknown proteins that enabled growth of yeast on screen medium were amplified by PCR using yeast colony as template. A small portion of the yeast colony was picked, boiled for 5 min at 100°C in 125 µL NP40 (0.2%), and spun at 20,800 x g for 30 sec. 10 µL of 1% dilution of the supernatant was used as template in a 25 µL PCR reaction with 1 µL of Taq : Pfu polymerase mix (1 U.µL⁻¹ : 0.0125 U.µL⁻¹, respectively). PCR reactions were carried out at final concentrations of 1× reaction buffer, 1 mM dNTPs and 4 ng.µL⁻¹ each primer, which flank the pB42AD insert cloning site, in a PTC-100 Programmable Thermal Cycler (MJ Research Inc). Initial denaturation (94°C) for 4 min was followed by 30 cycles of denaturation (94°C) for 1 min; annealing (58°C) for 1 min; and extension (72°C) for 2 min. After final extension (72°C) for 10 min, samples were stored at 4°C until further processing.

2.3.4 Tissue Culture Techniques

2.3.4.1 Culture of Mammalian Cells

Standard tissue culture techniques were used in the culture of mammalian cells. These included thawing, subculturing, and freezing which are briefly described as follows. Cells in a vial stored in liquid N₂ were thawed rapidly in a 37°C water bath before dilution in 10 mL media, centrifugation (290 x g, 2 min) and resuspension in fresh medium and cultured. To passage cells, adherent cell monolayers were washed twice with PBS and treated with trypsin (1 mL per 75 cm²) for 5 min at room temperature. Detached cells were resuspended in medium containing 10% fetal bovine serum (FBS), centrifuged (290 x g, 3 min) and resuspended in fresh medium. A haemocytometer grid was used to estimate cell concentration prior to subculturing. Non-adherent cells were centrifuged, resuspended in fresh medium and counted before subculturing. Generally, cells were cultured in medium consisting of DMEM

supplemented with 10% FBS. U937 cells were cultured in RPMI1640 supplemented with 10% FBS. To freeze cells, healthy cells in exponential growth phase were washed, harvested and resuspended in freezing medium before immediate transfer into vials and storage at -80°C .

2.3.4.2 Harvesting Antibodies From Hybridoma Cell Cultures

Standard tissue culture techniques were used to culture hybridoma cells. Cells in exponential growth phase were subcultured (2%) in RPMI1640/10% FBS and grown to confluence plus one extra day, generally 5-7 days. Cells/debris were removed by centrifugation at $2990 \times g$ for 15 min at 4°C . The supernatant containing the secreted mouse monoclonal antibody was supplemented with 0.01% NaN_3 and stored at 4°C .

2.3.4.3 Transfection of Mammalian Cells

Transient transfections required for immunohistochemical analysis were carried out in 6 well plates. Mammalian cells were transfected with various reagents according to manufacturers recommendation. DOTAPTM and Fugene6TM were used initially, though LipofectAMINE2000TM (LF2000) was used in most experiments. Briefly, the reagent (LF2000) and plasmid DNA were each mixed with DMEM, then combined and incubated for 20 min at room temperature before adding to COS-1 cells plated onto petri dishes or coverslips the previous day. Cells were approximately 90% confluent at time of transfection. The transfected cells were incubated at 37°C , 5% CO_2 for 18-48 h before harvesting for lysis or fixing and staining.

2.3.4.4 Lysis of Mammalian Cells

Mammalian cells were lysed for protein expression analysis or protein-protein interaction experiments. Adherent cells in a 10 cm dish were washed twice in PBS, harvested by scraping into 1 mL TEN buffer, washed once in ice cold PBS and lysed by chemical lysis or sonication. For chemical lysis, cells were resuspended in 150 μL whole cell extract (WCE) buffer or 1 mL Cytoskeletal lysis buffer and lysed for 30 min at 4°C with mixing on the nutator. Alternatively, cells were resuspended in 1 mL sonication buffer and sonicated for 20 sec. Lysates were cleared by centrifugation at $20,800 \times g$ for 20 min at 4°C . Protein concentration was determined using 1 μL lysate in Bradford assay.

2.3.4.5 Immunoprecipitation of Proteins Expressed in Mammalian Cells

Lysates of cells transfected with plasmids encoding various truncated Actinin-4 or Tec fusion proteins were used in immunoprecipitation (IP) reactions to study Tec-Actinin protein-protein interactions. 200 μg of Myc-A4 plus EGFP- or EGFP-Tec-containing lysate was combined with anti-Myc or anti-GFP antibody in a total of 1 mL of IP binding buffer with protease inhibitors and incubated at 4°C for 1 h. 20 μL of protein-G or protein-A agarose beads were added and the mix was incubated for 1 h at 4°C to precipitate Myc- or EGFP-containing complexes. After washing the beads 3 times in IP wash buffer with inhibitors at 4°C the Myc- or EGFP-containing complexes were eluted with SDS-PAGE load buffer at 100°C for 5 min. Western Blotting of anti-Myc immunoprecipitates was used to detect immunoprecipitated Myc-containing protein as well as associated EGFP-containing proteins, and vice versa for anti-GFP immunoprecipitates.

2.3.4.6 Preparation of Phagocytic Target For Phagocytosis Assay

Zymosan A diluted in PBS (pH 7.2) to 5 mg.mL^{-1} was autoclaved for 10 min and opsonised by incubation with human γ -globulins (20 mg.mL^{-1}) at 37°C for 30 min. IgG-opsonised Zymosan A was washed 4 times with PBS and stored at 4°C until use. A particle:cell ratio of approximately 10:1 is achieved by mixing cells (to $3\text{--}4 \times 10^6 \text{ cells.mL}^{-1}$) with Zymosan A (to 0.5 mg.mL^{-1}). $3.5 \times 10^6 \text{ cells.mL}^{-1} = 5 \times 10^5 \text{ cells per } 143 \mu\text{L}$.

2.3.4.7 Preparation of Phagocytic Cells For Phagocytosis Assay

U937 cells were differentiated into phagocytic cells by treatment with 10 ng.mL^{-1} phorbol myristate acetate (PMA) (16.2 nM) in culture medium for 48 h at 37°C, 5% CO_2 . PMA-U937 are adherent and were harvested with trypsin, resuspended in medium and kept in suspension for 30 min prior to the experiment.

2.3.4.8 Phagocytosis by Adherent PMA-U937 Cells

5×10^5 cells were allowed to adhere to cellular fibronectin (cFn, 1 $\mu\text{g.cm}^{-2}$) or poly-L-lysine (PLL, 10 $\mu\text{g.cm}^{-2}$) coated coverslips for 10 min at room temperature before washing, chilling and exposing to cold IgG-opsonised Zymosan A (140 μL of 0.5 mg.mL^{-1} , per coverslip) on ice for 20 min to allow particle binding. Coverslips were transferred to 2 mL prewarmed media for 5-10 min to allow internalisation of IgG-opsonised Zymosan A particles, then washed twice with PBS and fixed and immunostained. Alternatively, cells allowed to engulf

IgG-opsonised Zymosan A were washed in PBS, harvested in TEN, resuspended in 1 mL sonication buffer and lysed by sonication for 20 sec. The cell lysate was cleared by centrifugation at $20,800 \times g$ for 20 min at 4°C . Protein concentration was determined using 1 μL lysate in Bradford assay and IP reactions were performed on the supernatant.

2.3.4.9 Phagocytosis by Non-Adherent PMA-U937 Cells

5×10^5 PMA-U937 were chilled on ice, exposed to 140 μL cold IgG-opsonised Zymosan A in PBS (0.5 mg.mL^{-1}) on ice for 20 min, supplemented with 2 mL prewarmed media, plunged into a 37°C water bath for 5 min to allow internalisation of IgG-opsonised Zymosan A particles then spun $290 \times g$ for 3 min, washed in TEN, resuspended in 1 mL sonication buffer and lysed by sonication for 20 sec. Unbound IgG-opsonised Zymosan A was removed by centrifugation at $290 \times g$ for 4 min and the supernatant containing the cell lysate was cleared by centrifugation at $20,800 \times g$ for 20 min at 4°C . Protein concentration was determined using 1 μL lysate in Bradford assay and IP reactions were performed on the supernatant.

2.3.4.10 Fixing and Immunostaining Cells

$3 \times 10^5 \text{ cells.well}^{-1}$ were cultured on coverslips in a 6-well tray for 24 h. Cells were washed twice with PBS, fixed with ice cold methanol for 2 min, or ice cold 4% w/v paraformaldehyde (PFA) in PBS for 20 min, rehydrated in PBS for 15 min, permeabilised in PBS/0.1% Triton X-100 for 10 min and washed 3 times in PBS. Coverslips were transferred to a fresh dry well, and cells were blocked in blocking solution (PBS/5% normal serum (pH 7.2)) for 1 h, washed where appropriate three times for 5 min in PBS/0.1% Tween-20 and probed with antibody diluted in PBS (pH 7.2) for 1 h to overnight for primary antibody and 1-2 h for secondary antibody. Primary antibodies: anti-ACTN4 monoclonal antibody, anti-Tec polyclonal antibody were used at 1:120; secondary antibodies: anti-mouse-IgM-FITC and anti-goat-FITC or anti-goat-Cy3 were used at 1:100 to 1:1000. Cells were kept in the dark during and after incubation with secondary antibody. Nuclei were stained with Hoechst 33258 (1:10,000) or propidium iodide (1:20) for 30 sec. After washing, coverslips were mounted onto microscope slides with antifade reagent p-Phenyldiamine in PPD/PBS/glycerol (pH 9.5) solution, before cell staining was analysed by fluorescence microscopy. Photographs of stained cells were recorded on 100ASA slide film (Kodak). Digital images were recorded from slide images using a Kodak Professional RFS3570 Film Scanner (Eastman Kodak Company, Rochester, NY) and UMAX MagicscanII software.

Non-adherent cells were processed in the same order, except cells were spun at 290 x *g* between steps involving changes of solution. To mount non-adherent cells, they were resuspended in 15 μ L PBS/glycerol, aliquoted onto a microscope slide, overlaid with a coverslip and sealed.

2.3.4.11 Fluorescence Microscopy

Cells were analysed at 60 $\times$ magnification or 100 $\times$ magnification under oil immersion. Several different microscopes were used to visualise the EGFP transfected cells or antibody stained cells. These include a Nikon inverted (Eclipse TE300) microscope and a BIORAD MRC-600 Laser Scanning Confocal Imaging System coupled to an Olympus IMT-2 Inverted Research Microscope. In some experiments, an Olympus Provus AX70 microscope (with filter sets: U-MNIBA for FITC; U-MWIG for TRITC/Cy3; and U-MWU for Hoechst 33258) attached to a Photometrix Cool Snap FX camera was used with V++ software.

2.3.5 Digital Imaging

For permanent records of Western blots, chromatography chart recordings, slides, photos and for illustration purposes, images of experimental results were scanned into the computer program Adobe Photoshop using a HP Scanjet 7400c Scanner (Hewlett Packard) and UMAX MagicscanII software (Kodak) or Scanwise software (Agfa). Images were cropped in Adobe Photoshop and imported into Microsoft PowerPoint for labelling.

2.3.6 Photography

Images of DNA bands separated by agarose gel electrophoresis and stained with EtBr were visualised by exposure to short wavelength UV light on a Chromato-Vue transilluminator (Ultra-Violet Products Inc, San Gabriel, CA) and recorded on photoprint paper with a Mitsubishi Video Copy Processor (Mitsubishi Electric Corporation) attached to a 16mm COSMICAR television lens camera (Ultra-Violet Products Inc, San Gabriel, CA). Images of yeast plates were recorded under bright light using the same system. Printouts from the camera were digitised where required and processed as in Section 2.3.5. Digital images were downloaded into Adobe Photoshop, cropped and imported into Microsoft PowerPoint for labelling.

CHAPTER

3

Yeast Two-Hybrid Assay With Tec PHTH Domain

3.1 Introduction: Tec-family PHTH Domain

Most members of the Tec kinase-family contain a PH domain and a TH domain near the N-terminus. This makes Tec-family kinases unique amongst known protein tyrosine kinase families. PH domains are characterised as targeting domains; they modulate protein associations with the cytoskeleton, cell membrane and proteins involved in signal transduction (Ferguson *et al.*, 1994, Tsukada *et al.*, 1994a, Blomberg and Nilges, 1997). The targeting function is thought to place the protein in close proximity to activators and substrates in order to propagate downstream signalling. In Tec-family kinases, PH domains have been shown to provide a reversible membrane tethering function, dependent on the availability of specific phosphatidylinositol ligands (Bolland *et al.*, 1998, Saito *et al.*, 2001). In Src-family kinases the corresponding region contains a myristoylated glycine residue that yields constitutive plasma membrane association (Kaplan *et al.*, 1990). Despite these differences in regulated versus constitutive membrane targeting, membrane association is a prerequisite for full activation of the catalytic domain for both families of kinases (Scharenberg and Kinet, 1998).

Combined with the knowledge that the PH domain and Btk motif of the TH domain, described here as the PHTH domain, is not constitutively membrane associated, the size and surface exposure of the PHTH domain immediately suggest its ability to interact with other factors such as proteins. Indeed, protein ligands have been described for Tec-family PHTH domains. They include protein kinase C isoforms (Yao *et al.*, 1994, Kawakami *et al.*, 1995, Yao *et al.*, 1997), $\beta\gamma$ subunits of heterotrimeric G proteins (Touhara *et al.*, 1994 Tsukada *et al.*, 1994b), BAP-135 (Yang and Desiderio, 1997), F-actin (Yao *et al.*, 1999) and FAK (Chen *et al.*, 2001). Putative interaction sites have been mapped to clusters of residues in the Btk PHTH domain. This is illustrated in the space-filling diagram shown in Figure 3.1. The structures for the PH domains of Tec, Itk and Bmx were modelled based on the Btk structure (Okoh and Vihinen, 1999); while the Tec-family PH domains have structural similarities, there are differences in their ligand-binding sites. Electrostatic polarisation suggests Tec-family PH domains have related, but not identical, properties and functions (Okoh and Vihinen, 1999).

PH domains have strong electrostatic polarisation and the positive pole contributes to the binding site of phosphatidylinositols. Conversely, acidic motifs have been implicated in the binding of PH domains to protein ligands (Burks *et al.*, 1998). Lipid ligands of Tec-family PH domains include PI 3,4,5-P₃, which is generated from PI 4,5-P₂ by PI3K (Salim *et al.*,

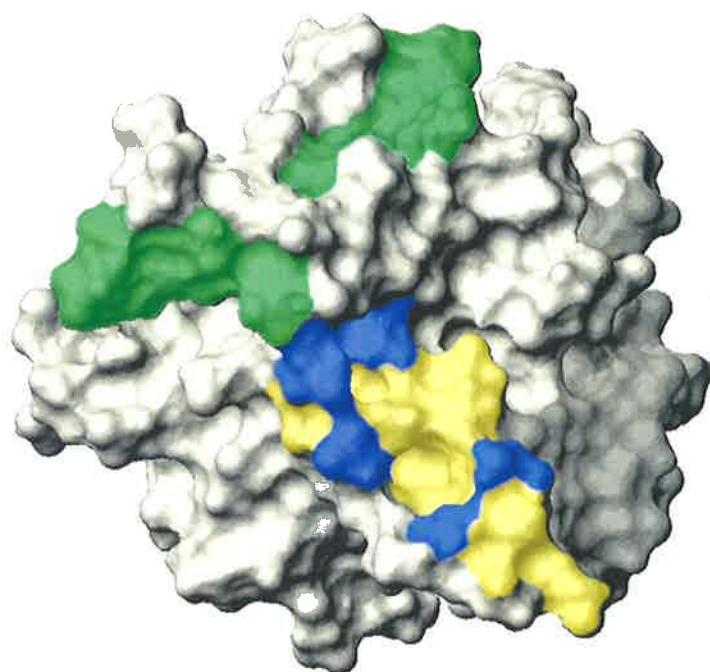
Figure 3.1 Putative Binding Sites of Btk PHTH domain

- A Electrostatic polarisation surface diagram of the Btk PH domain (light grey) and Btk motif (dark grey), shown in the same orientation as in Figure 1.4, with ligand binding sites highlighted in colour. Residues implicated in binding to F-actin (yellow), PIP₃/IP₄ (blue) and PKC (green) have been coloured. The residues implicated in binding to G-βγ are on the rear face of the molecule (see part B).
- B Rear view of the molecule shown in part (A) with the PH domain shown in light grey and the Btk motif shown in dark grey. Residues implicated in binding to G-βγ (shown in purple) extend diagonally across the rear face of the structure and include the carboxyl-terminal α-helix of the PH domain.

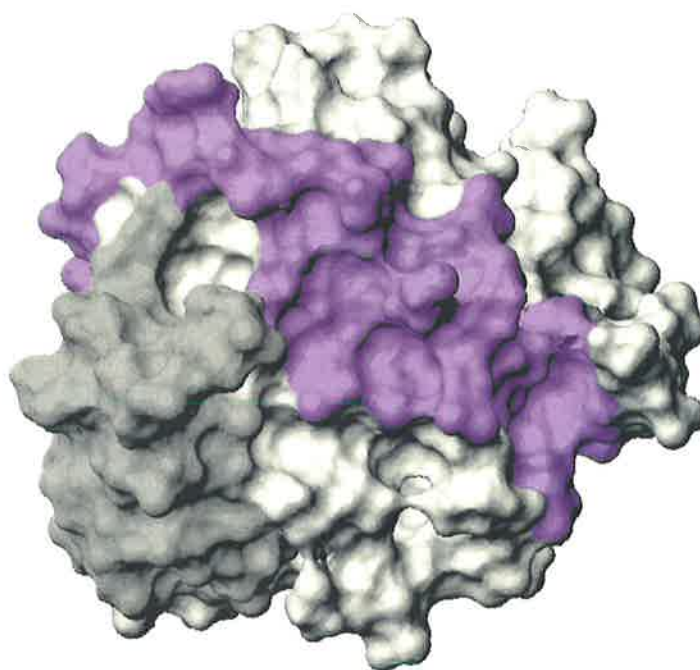
Figures were generated using MOLMOL software (Koradi *et al.*, 1996) by Dr Kasper Kowalski. PDB code: 1b55 (Baraldi *et al.*, 1999).

PH	pleckstrin homology
F-actin:	filamentous-actin
PIP ₃ :	phosphatidylinositol 3,4,5-trisphosphate
IP ₄ :	inositol 1,3,4,5-tetraphosphate
PKC:	protein kinase C
G-βγ:	βγ subunit of heterotrimeric G-protein

A



B



1996, Rameh *et al.*, 1997, Toker and Cantley, 1997, Yang *et al.*, 2001), and other 3'-phosphorylated lipids such as inositol 1,3,4,5-tetraphosphate (IP₄) and IP₆ (Fukuda *et al.*, 1996, Fukuda and Mikoshiba, 1997). Independently isolated XLA-causing mutations of Btk include K12E, K19E and R28C, which map to surfaces involved in binding PI 3,4,5-P₃ or IP₄ and alter the local electrostatic potential (Baraldi *et al.*, 1999).

At the outset of this PhD project, Tec was implicated in signalling events downstream of numerous cell surface receptors, but the signalling pathways and substrates of Tec were largely unknown.

3.2 Aims

The aim of this section of work was to identify protein ligands for the Tec PHTH domain and to begin characterisation of these protein-protein interactions. It was expected that previously described Tec-family PHTH domain ligands and novel ligands would be identified and that analysis of the interactions would contribute to the understanding of Tec function.

3.3 Approach

3.3.1 Overview Of Approach

The yeast two-hybrid assay was used to identify PHTH domain ligands from a library of potential clones. PCR-based experiments were used to alter the coding sequence of the interacting clone. Successive in frame truncations of the 5' and 3' coding sequence of the clone were created by PCR with nested primers and expressed as a deletion series in which the clone had N-terminal or C-terminal truncations. Site-directed mutagenesis was used to modify specific potential interaction site residues. Truncation and substitution mutants of the interacting protein were tested for binding to Tec PHTH domain in the yeast two-hybrid assay. Each of these components is described in more detail in the following sections.

3.3.2 Yeast Two-Hybrid System

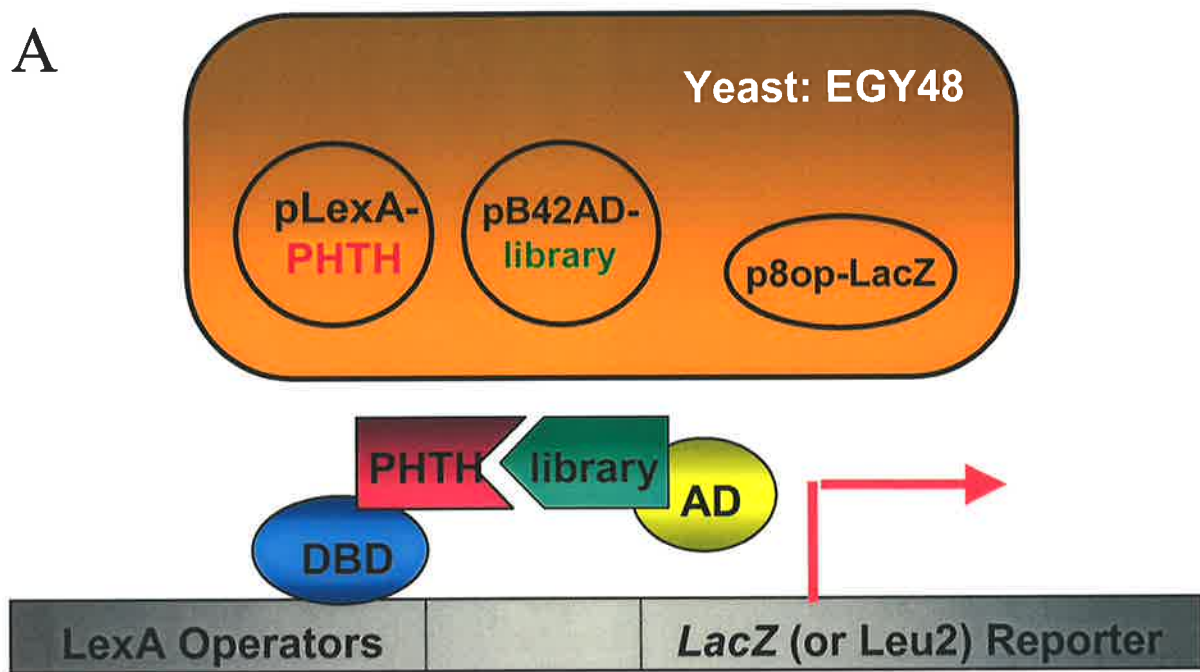
The yeast two-hybrid assay is an *in vivo*-based assay system for detection of protein-protein interactions. It is based on the fact that expression of two specific fusion proteins can activate reporter gene expression when the fusion proteins interact. An illustration of the CLONTECH MATCHMAKER LexA Two-Hybrid System is shown in Figure 3.2. It can be used to screen a cDNA library for genes that encode interacting proteins

Figure 3.2 Yeast Two-Hybrid Assay

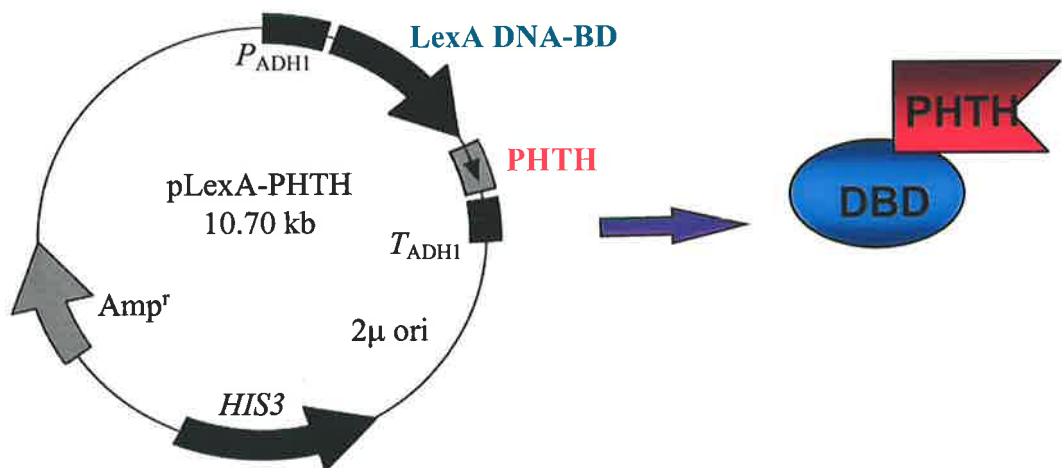
The CLONTECH MATCHMAKER LexA Two-Hybrid System (CLONTECH Laboratories, 1996) was used to identify protein ligands of Tec pleckstrin homology domain and Btk motif (PHTH domain). EGY48 strain yeast were transformed with a reporter plasmid, p8opLacZ, and two expression plasmids, pLexA and pB42AD, which encode hybrid proteins containing DNA binding domain (DBD) and bait (PHTH domain) or activation domain (AD) and library proteins, respectively. Protein-protein interaction between the two expressed hybrid proteins activates transcription of the reporter genes and is assayed by plating yeast on screen medium that contains XGAL. Screen medium is a synthetic dropout medium that lacks histidine, tryptophan, uracil and leucine, which are essential for yeast growth, and contains galactose (and no glucose) for induction of the AD library hybrid protein. Yeast only grow on screen medium when the leucine reporter gene is activated. This gene, LEU2, is chromosomally encoded and has six LexA operator sites in the promoter. XGAL is a substrate of LacZ, which converts clear XGAL into a blue product. The plasmid encoded LacZ reporter gene contains eight LexA operators at the promoter.

- A Diagrammatic representation of the yeast two-hybrid assay system showing yeast host and reporter and expression plasmids above a schematic diagram of reporter gene activation.
- B Vector map of the plasmid pLexA (Gyuris, 1993), which is used to generate fusions of the LexA DNA binding domain (DBD, 202 residues) with a bait protein (shown here as PHTH domain). DBD-PHTH hybrid protein expression is controlled by the strong yeast ADH1 promoter and in this assay was constitutive. The HIS3 transformation marker is used for selection in yeast. pLexA-PHTH was cloned by PCR amplification using primers #7 and #1 (Section 2.1.22) on the pSK+TecIIB template. The 583 bp PCR product was cleaved with *EcoRI* and the 474 bp PHTH domain-encoding region was ligated into *EcoRI*/CIP prepared pLexA plasmid. Clones with the insert in the correct orientation were identified by *AccI* restriction digestion and verified by automated DNA sequencing.
- C Vector map of the pB42AD-cDNA library plasmid purchased from CLONTECH. pB42AD plasmid (Gyuris, 1993) was used to generate fusions containing the SV40 nuclear localisation sequence, B42 acidic activation domain (AD, 88 residues) and hemagglutinin epitope tag (HA, 10 residues) with a library encoded protein. AD-library hybrid protein expression is controlled by the galactose inducible GAL1 promoter. The TRP1 transformation marker is used for selection in yeast. The human liver cDNA library was cloned into the unique *EcoRI* and *XhoI* restriction sites.

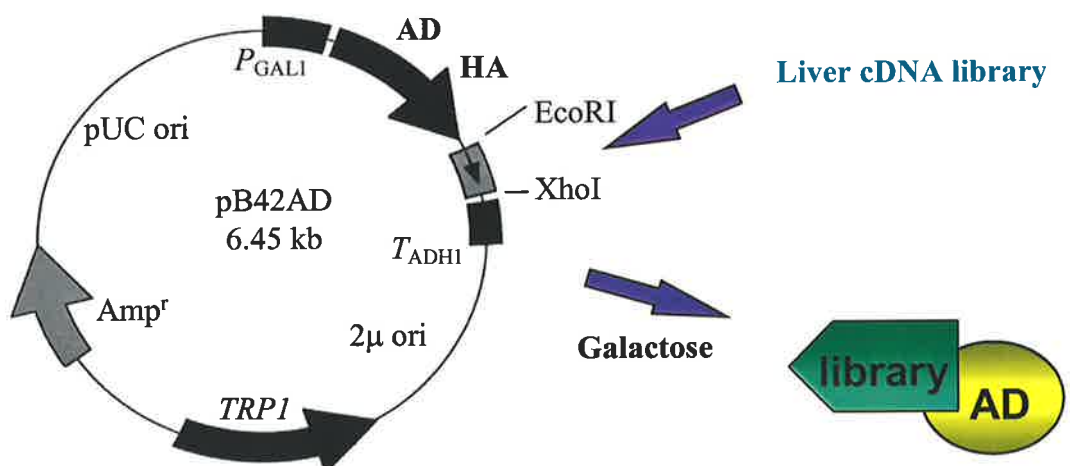
A



B



C



of a known protein of interest (bait). Most importantly, it directly provides the cDNA of the ligand for further studies.

3.3.3 Components of the Yeast Two-Hybrid System

The main components of the CLONTECH MATCHMAKER LexA Two-Hybrid System (CLONTECH Laboratories, 1996, Gyuris, 1993, Estojak *et al.*, 1995) are the yeast strain, the reporter genes and the two plasmids that express the fusion proteins (Figure 3.2A). The yeast strain EGY48 is auxotrophic for histidine, tryptophan and uracil. The chromosomal leucine gene (LEU2), one of the reporter genes, is under the control of six LexA operators. The LEU2 promoter is activated by a reconstituted transcription unit formed when the fusion protein containing the LexA DNA-binding domain (DBD-bait) interacts with the fusion protein containing the B42 activation domain (AD-library). The other reporter gene, p8opLacZ, is a plasmid-encoded LacZ gene under the control of eight LexA operators that is activated by the interaction of the two-hybrid proteins. The p8opLacZ plasmid contains a uracil marker gene. The two reporter genes have different sequences flanking the LexA operators to reduce false-positive activation and to confirm the positive two-hybrid interaction. The two plasmids that express the fusion proteins are pLexA and pB42AD, which contain histidine and tryptophan marker genes, respectively (Figure 3.2).

3.3.3.1 pLexA Vector and Control Vectors Derived From pLexA

The pLexA vector is used to generate fusions of the DNA-BD (the 202 residue LexA protein) with a bait protein (Figure 3.2B) (CLONTECH Laboratories, 1996, Gyuris, 1993). In the screen, LexA is fused with the PHTH domain (without the proline-rich region) of Tec. Several other LexA fusion proteins are used as controls in the yeast two-hybrid system and to verify the positive two-hybrid screen result. These include LexA-53 and LexA-POS, which are used in positive controls, and LexA-Lam and LexA alone, which are used in negative controls. The strong yeast ADH1 promoter controls constitutive LexA fusion protein expression.

3.3.3.2 pB42AD Vector and Derivatives

The pB42AD vector expresses cDNAs or other coding sequences inserted into the unique *EcoRI* and *XhoI* sites. These sequences are inserted as translational fusions to a cassette consisting of the SV40 nuclear localisation sequence (NLS), the 88-residue B42

acidic activator domain (AD), and the hemagglutinin (HA) epitope-tag (YPYDVPDYA)(Figure 3.2C) (CLONTECH Laboratories, 1996, Gyuris, 1993). Fusion protein expression is under the control of the galactose inducible promoter. The pB42AD-T plasmid encodes an AD/SV40 large T-antigen fusion protein, which, combined with LexA-53 provides a positive control for interacting proteins. A human liver cDNA library cloned in to pB42AD was used in the screen.

3.3.3.3 The cDNA Library

The CLONTECH cDNA library used in the screen was prepared from a male human liver. This liver cDNA library was chosen because Tec was first identified in liver and later found to be highly expressed in hepatocellular carcinoma cell lines (Mano *et al.*, 1990, Mano *et al.*, 1993). Subsequent work showed that the Tec3 isoform is the predominant isoform expressed in mouse liver tissue; reverse transcription PCR analysis showed that Tec4 is present (Merkel *et al.*, 1999).

The Product Analysis Certificate (CLONTECH Laboratories, Inc) states that the cDNA library in pB42AD vector contains 3.2×10^6 independent clones and estimates that only 85% of the clones contain insert in the vector. Amplification of the cDNA library is recommended before use. The recommended number of clones to amplify is 2-3 times the number of independent library clones, at a density of 275 CFU.cm^{-2} .

3.3.4 Preparation For Yeast Two-Hybrid Screen

In preparation for the screen, a number of yeast two-hybrid system requirements need to be verified. Once the bait is cloned into the pLexA vector, fusion protein expression in yeast needs to be confirmed by Western blot. This is to ensure that the protein is of the expected size and is not toxic to the cells or degraded. Secondly, the hybrid construct needs to be tested and found negative for autonomous reporter gene activation. Thirdly, controls need to be performed to demonstrate that the system is functional and that there is no leaky expression of the reporter genes.

3.3.5 Yeast Two-Hybrid Screen

The plasmid containing the cDNA library is transformed into EGY48 yeast containing the bait and reporter plasmids. The cotransformants are amplified on stock (-His/-Trp/-Ura, glucose-containing) medium to allow plasmids to reach maximum copy number. The screen

is performed by plating yeast onto screen medium that additionally lacks leucine and contains galactose instead of glucose (-His/-Trp/-Ura/-Leu, galactose-containing). Colonies are isolated with a sterile toothpick, restreaked onto screen medium containing XGAL and assayed for β -galactosidase activity.

3.3.6 Analysis and Verification of Positive Two-Hybrid Interactions

It is important to verify a positive yeast two-hybrid result as false positive results can arise, for example, when the AD/library hybrid directly activates reporter genes. In a false positive result, the hybrid proteins do not directly interact.

To begin verification of a positive result, the plasmid encoding the ligand is first rescued from yeast. The ability of the two-hybrid proteins to interact is then retested after transformation of the isolated AD/library plasmid into yeast containing the bait. A number of controls are also performed at this point. The AD/library plasmid is introduced into yeast containing plasmid expressing no hybrid (LexA alone) or a hybrid with a non-interacting protein (LexA-LAM) and tested for reporter gene activation. When a specific two-hybrid interaction occurs, only cotransformants containing the bait and candidate AD/library plasmid will yield a positive result (CLONTECH Laboratories, 1996).

To further verify positive interactions, the vectors encoding the bait and library proteins are switched before repeating the two-hybrid assay. This eliminates false positive results that rely on the relative position of the bait or library protein to the fusion partner.

Further analysis of the interaction is performed by creating deletion and substitution mutants of the bait and ligand. This is achieved using PCR once the DNA sequence of the library-encoded protein from the rescued plasmid is known. Finally, other biochemical methods, such as co-immunoprecipitation and affinity chromatography, are used to confirm the newly identified protein-protein interaction.

3.3.7 Site-Directed Mutagenesis

In vitro site-directed mutagenesis is a simple and rapid PCR based technique for modifying nucleotide sequences cloned into a vector (Section 2.3.1.3). It is an efficient method for creating specific amino acid substitutions to analyse potential ligand-binding residues. For example, mutants corresponding to those in Btk that cause XLA could be created, as some of these work by affecting the interaction of Btk with other molecules in the signalling pathway.

3.4 Results

3.4.1 LexA-PHTH Bait and Controls Were Expressed in Yeast

The PHTH domain-encoding region lacking the proline-rich region was cloned into the pLexA vector as described in Figure 3.2B. An *AccI* restriction digest was used to identify clones with an insert in the correct orientation and the sequence of clone #3 was verified by automated DNA sequencing (Section 2.3.1.23). Figure 3.3 shows the DNA sequence and corresponding amino acid sequence of the PHTH domain used in the yeast two-hybrid screen. The Tec DNA template used in the cloning process was obtained from J. Ihle (St Jude's Hospital, Tennessee). Comparison of the DNA sequence of the full template with its published sequence (Mano *et al.*, 1993) identified five nucleotide differences that result in amino acid substitutions. The sequence used in the yeast two-hybrid screen was consistent with the sequence identified in exons of genomic Tec DNA and encoded residues that are conserved in other Tec-family members (Merkel *et al.*, 1999). In the PHTH domain region the differences were P23 (not L), F31 (not C) and T34 (not P).

The pLexA-PHTH plasmid and control vectors were transformed into yeast using quick yeast transformation (Section 2.3.3.1). Transformant colonies that were grown on synthetic-dropout -His/-Ura/glucose plates were used to inoculate cultures from which soluble protein extracts (Section 2.3.3.4) were analysed by Western blot (Section 2.3.2.10) for expression of LexA-fusion proteins using anti-LexA monoclonal or polyclonal antibody. As shown in Figure 3.4, all the fusion proteins were of the expected size. When the rabbit anti-LexA polyclonal antibody was used in combination with anti-rabbit-HRP-conjugated secondary antibody and enhanced chemiluminescence detection, an approximately 25 kDa band was observed in all samples including untransformed control yeast indicating this was residual background (Figure 3.4A). This band was not present when mouse anti-LexA monoclonal antibody was used in combination with anti-mouse-AP and NBT-BCIP substrate detection (Figure 3.4C). In each Western blot experiment, loading controls were performed in which duplicate gels were stained with Coomassie blue (Figure 3.4B and D).

3.4.2 The Yeast Two-Hybrid System Was Functional

The nutritional requirements of the yeast host strain EGY48[p8opLacZ] were tested by plating the yeast onto various synthetic-dropout media. The yeast did not grow on media lacking histidine, tryptophan or leucine, as expected. LacZ reporter gene expression was tested by plating EGY48[p8op-LacZ, pLexA-POS] yeast onto non-induction

Figure 3.3 Nucleotide and Amino Acid Sequence of Mouse Tec PHTH Domain

The DNA sequence and corresponding amino acid sequence of the PH domain and Btk motif (PHTH domain) of Tec that was used as a bait in the yeast two-hybrid screen. The construct does not include the proline rich region of the TH domain.

The *Eco*RI restriction sites used to clone the PHTH domain encoding sequence into pLexA plasmid (and later into pEGFP-C2 plasmid) are boxed. The *Acc*I restriction site used to determine the orientation of the insert is boxed. Nucleotide differences to the sequence published by Mano *et al.*, (1990, 1993) that introduce amino acid substitutions (P23, C31, T34) are underlined. The sequence shown here is consistent with sequence identified in exons of genomic Tec sequence and encodes residues that are conserved in other Tec family members (Merkel *et al.*, 1999).

Residues are listed in single letter code (stop codon, *) and numbered according to the published translation of the full-length protein. PH domain residues are shown in green while Btk motif residues are shown in red. K18, K19 and K20 in the PH domain that were substituted with alanines in experiments described in Chapter 4 are numbered; the corresponding residues in the Btk PH domain are implicated in binding filamentous-actin (Yao *et al.*, 1999). The H121, C132, C133 and C143 residues of the Btk motif that are predicted to coordinate a Zn^{2+} ion, by analogy to residues of Btk (Hyvonen and Saraste, 1997), are indicated with (#).

PH: Pleckstrin homology
TH: Tec homology

EcoRI															
GAA	TTC	GAG	ATG	AAT	TTC	AAC	ACT	ATC	CTA	GAA	GAG	ATT	CTT	ATT	45
E	F	E	M	N	F	N	T	I	L	E	E	I	L	I	12
			1												
AAA	AGG	TCC	CAG	CAG	AAA	AAG	AAG	ACA	TCA	CCC	TTA	AAC	TAC	AAA	90
K	R	S	Q	Q	K	K	K	T	S	P	L	N	Y	K	27
					18	19	20								
GAG	AGA	CTT	TTT	GTA	CTT	ACA	AAA	TCC	GTG	TTG	AGC	TAC	TAT	GAG	135
E	R	L	F	V	L	T	K	S	V	L	S	Y	Y	E	42
			29												
GGT	CGA	GCG	GAG	AAG	AAA	TAC	AGA	AAG	GGC	GTC	ATT	GAT	ATT	TCC	180
G	R	A	E	K	K	Y	R	K	G	V	I	D	I	S	57
AAA	ATC	AAG	TGT	GTG	GAG	ATA	GTG	AAG	AAC	GAT	GAT	GGT	GTC	ATT	225
K	I	K	C	V	E	I	V	K	N	D	D	G	V	I	72
CCC	TGT	CAA	AAT	AAA	TTT	CCA	TTC	CAG	GTT	GTT	CAT	GAT	GCT	AAT	270
P	C	Q	N	K	F	P	F	Q	V	V	H	D	A	N	87
ACA	CTT	TAT	ATT	TTT	GCA	CCT	AGT	CCA	CAA	AGC	AGG	GAC	CGA	TGG	315
T	L	Y	I	F	A	P	S	P	Q	S	R	D	R	W	102
GTG	AAG	AAG	TTA	AAA	GAA	GAA	ATA	AAG	AAC	AAC	AAT	AAT	ATC	ATG	360
V	K	K	L	K	E	E	I	K	N	N	N	N	I	M	117
ATT	AAA	TAC	CAT	CCT	AAA	TTC	TGG	GCA	GAT	GGG	AGT	TAC	CAG	TGT	405
I	K	Y	H	P	K	F	W	A	D	G	S	Y	Q	C	132
			#												
AccI															
TGT	AGA	CAA	ACA	GAA	AAA	CTA	GCA	CCC	GGA	TGT	GAG	AAG	TAC	AAT	450
C	R	Q	T	E	K	L	A	P	G	C	E	K	Y	N	147
			#												
EcoRI															
CTT	TTT	GAG	AGT	AGT	TAA	GAA	TTC								474
L	F	E	S	S	*										
					152										

Figure 3.4 LexA Fusion Protein Expression in Yeast

Duplicate samples of yeast protein extracts obtained from yeast that were untransformed, transformed with pLexA empty plasmid or one of various pLexA-fusions were run on 12.5% Tris-tricine gels and Coomassie blue stained or transferred onto nitrocellulose and probed in a Western blot. Constitutive fusion protein expression from the pLexA plasmid is controlled by the strong yeast ADH1 promoter (CLONTECH Laboratories, 1996).

A Samples from four independent pLexA-PHTH yeast clones (1-4) were probed by Western blot with rabbit-anti-LexA primary antibody and anti-rabbit-HRP secondary antibody. LexA-PHTH protein (40 kDa) expression was detected in all four clones. Also present was a smaller protein band, which by comparison with the negative control untransformed (U) yeast is residual background. The 28 kDa protein in the Benchmark molecular weight markers (M) was also detected.

B Coomassie blue stained gel of duplicate samples that were tested in part (A).

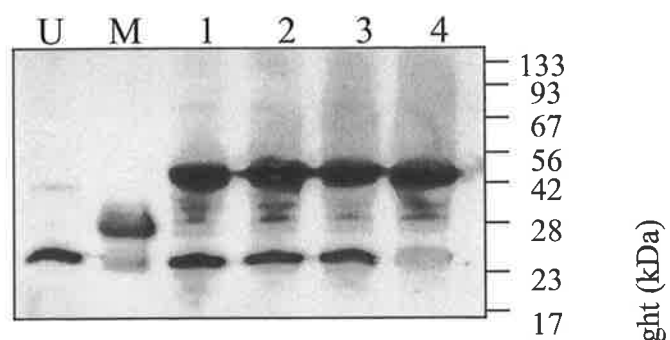
C Samples were probed by Western blot with mouse-anti-LexA primary antibody and anti-mouse-AP secondary antibody. All the fusion proteins were of expected molecular weight:

lane	1:	pLexA	22 kDa
	2:	pLexA-PHTH	40 kDa
	3:	pLexA-SH3	28 kDa
	4:	pLexA-53	57 kDa
	5:	pLexA-LAM	40 kDa
	6:	pLexA-POS	143 kDa

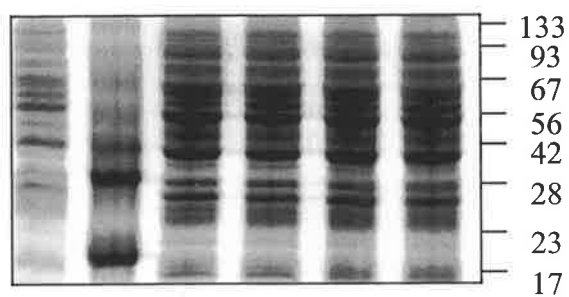
D Coomassie blue stained gel of duplicate samples that were tested in part (C).

Primary antibodies were used at 1/5,000 and 1/2,500 dilution for rabbit and mouse, respectively, while AP-conjugated secondary antibody was used at 1/30,000 dilution and HRP-conjugated secondary antibody was used at 1/20,000 dilution. Alkaline phosphatase (AP) conjugated secondary antibody was developed with NBT/BCIP. Horseradish peroxidase (HRP) conjugated secondary antibody was developed with enhanced chemiluminescence and exposed to X-ray film. Benchmark or Sigma Colour Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated. pLexA plasmid and derivatives used in control experiments were provided in the CLONTECH MATCHMAKER LexA Two-Hybrid System Kit (CLONTECH Laboratories, 1996). pLexA-SH3 was cloned and provided by Joanna Doumanis (Adelaide University).

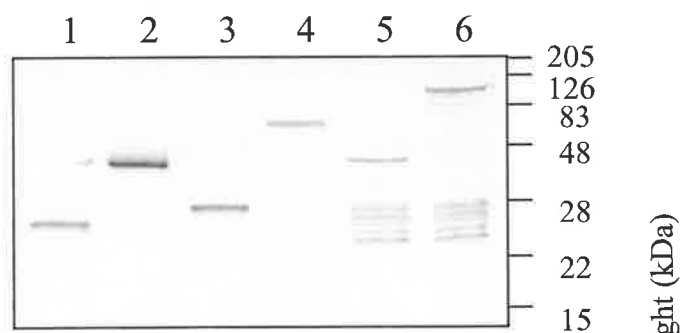
A



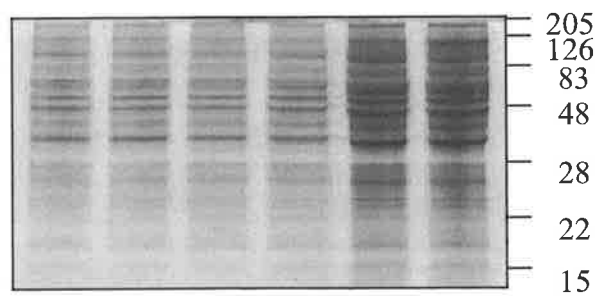
B



C



D



(glucose-containing) and induction (galactose-containing) medium, where yeast grew white and blue, respectively, as expected.

For positive and negative control interactions, respectively, pLexA-53 and pB42AD-T, or pLexA and pB42AD were cotransformed into yeast, grown on stock plates and tested on screen plates containing XGAL. As expected, blue yeast grew on the screen plate for the positive control and no yeast grew on the plate for the negative control. Another negative control, EGY48[p8op-LacZ, pLexA-Lam, pB42AD-T], was also tested and found negative, indicating the system was working as expected.

3.4.3 The Human Liver cDNA Library Was Amplified in Bacteria

In the absence of an available mouse liver cDNA library, a human liver cDNA library was used in the yeast two-hybrid screen. This was deemed acceptable since there is remarkable sequence identity between mouse and human Tec as shown in the sequence alignment in Figure 3.5. There are seven amino acid differences in the PHTH domain giving this region 95.4% identity across the two species. Five are conserved substitutions and one is a semi-conserved substitution and they are spread over the linear sequence of the PHTH domain. As shown in the ribbons and solvent exposed surface diagram models of Tec PHTH domain in Figure 3.6A and B, four of the substituted residues map to a cluster in the PH domain. The other three substituted residues are spread over the PHTH domain surface and are illustrated in Figure 3.6C.

Bacteria purchased from CLONTECH Laboratories containing the human liver cDNA library in pB42AD plasmid were titred and amplified. During the amplification experiment, the titre was measured at $2.4\text{--}2.5 \times 10^{10}$ CFU.mL⁻¹ (Section 2.3.1.16). This was 1.6-fold higher than the previous estimate of $1.6\text{--}1.9 \times 10^{10}$ CFU.mL⁻¹ that was measured by serial dilution and plating on selective Luria broth plates and used to plan the amplification step. Consequently, more clones were amplified (at a higher density) than expected. During the amplification step, 11×10^6 CFU on 48 bioassay dishes (Nunc) were plated at a density of 460 CFU.cm⁻².

3.4.4 The cDNA Library Has Characteristics of Human Liver Expression

To verify the origin of the cDNA library, plasmid DNA from 12 discrete clones was analysed by restriction digest (Section 2.3.1.5) and DNA sequencing (Section 2.3.1.23). The clones were isolated from a sample of amplified library plasmid that was transformed into

Figure 3.5 Amino Acid Sequence Alignment of Human and Mouse Tec

Multiple sequence alignment of residues of mouse and human Tec proteins produced using the ClustalW program (Section 2.2.2). Below the aligned sequence is the consensus line:

"*" = identical residues

":" = indicates conserved substitutions

"." = indicates semi-conserved substitutions.

The domains are coloured green (pleckstrin homology), red/grey (Btk motif/proline rich region of the Tec homology), blue (Src homology-3, SH3) orange (SH2) and pink (kinase). The linker between the SH2 and kinase domain is shown in black.

The amino acid sequences of mouse and human Tec4 were predicted from corresponding nucleotide sequences using DNASIS software (Section 2.2.3). Nucleotide sequences of mouse and human Tec4 cDNAs (Section 2.1.19) were obtained by automated DNA sequencing (Section 2.3.1.23).

Mouse	MNFNTILEEILIKRSQQKKKTSPLNYKERLFVLTKSVLSSYYEGRAEKKYRKGVIDISKIK	60
Human	MNFNTILEEILIKRSQQKKKTSPLNYKERLFVLTKSMLTYEGRAEKKYRKGFIDVSKIK	60
	*****:*.*****.***:***	
Mouse	CVEIVKNDDGVIPCQNKFPPQVVHDANTLYIFAPSPQSRDRWVKKLKEEIKNNNNIMIKY	120
Human	CVEIVKNDDGVIPCQNKYPFQVVHDANTLYIFAPSPQSRDLWVKKLKEEIKNNNNIMIKY	120
	*****:***** *****	
Mouse	HPKFWADGSYQCCRQTEKLAPGCEKYNLFESSIRKTLPPAPEIKKRRPPPIIPPEEE-NT	179
Human	HPKFWTDGSYQCCRQTEKLAPGCEKYNLFESSIRKALPPAPETKKRRPPPIPLEEEDNS	180
	*****:*****:***** ***** *	
Mouse	EEIVVAMYDFQATEAHDRLRLERGQEYIILEKNDLHWWRRARDKYGSEGYIPSNYVTGKKS	239
Human	EEIVVAMYDFQAAEGHDLRLERGQEYLILEKNDVHWWRRARDKYGNEGYIPSNYVTGKKS	240
	*****:*.*****:*****:*****.*****	
Mouse	NLDQYEWYCRNTNRSKAEQLLRTEDEGGFMVRDSSOPGLYTVSLYTKFGGEGSSGFRHY	299
Human	NLDQYEWYCRNMNRSKAEQLLRSEDEGGFMVRDSSOPGLYTVSLYTKFGGEGSSGFRHY	300
	***** *****:*****	
Mouse	HIKETATSPKKYYLAEKHAFGSIPELIEYHKHNAAGLVTRLRYPVSTKGKNAPTTAGFSY	359
Human	HIKETTTSPKKYYLAEKHAFGSIPELIEYHKHNAAGLVTRLRYPVSVKGKNAPTTAGFSY	360
	*****:*****.*****	
Mouse	DKWEINPSELTFMRELGSGLFGVVRGKWRAQYKVAIKAIREGAMCEEDFIEEAKVMMKL	419
Human	EKWEINPSELTFMRELGSGLFGVVRGKWRAQYKVAIKAIREGAMCEEDFIEEAKVMMKL	420
	:*****	
Mouse	THPKLVQLYGVCTQQKPIYIVTEFMERGCLLNFLRQRQGHFSRDMLLSMCQDVCEGMEYL	479
Human	THPKLVQLYGVCTQQKPIYIVTEFMERGCLLNFLRQRQGHFSRDVLLSMCQDVCEGMEYL	480
	*****:*****	
Mouse	ERNFSIHRDLAARNCLVNEAGVVKVSDFGMARYVLDDQYTSSSGAKFPVKWCPPEVFNYS	539
Human	ERNFSIHRDLAARNCLVSEAGVVKVSDFGMARYVLDDQYTSSSGAKFPVKWCPPEVFNYS	540
	*****.*****	
Mouse	RFSSKSDVWSFGVLMWEIFTEGRMPFEKNTNYEVVMTVTRGHRHLRHPKLASKYLYEVMLR	599
Human	RFSSKSDVWSFGVLMWEVFTGRMPFEKYTNIEVVMTVTRGHRLYQPKLASNYVYEVMLR	600
	*****:***** *****:*.*****:*.*****	
Mouse	CWQERPEGRPSFEDLLRTIDELVECEETFGR	630
Human	CWQEKPEGRPSFEDLLRTIDELVECEETFGR	631
	:**	

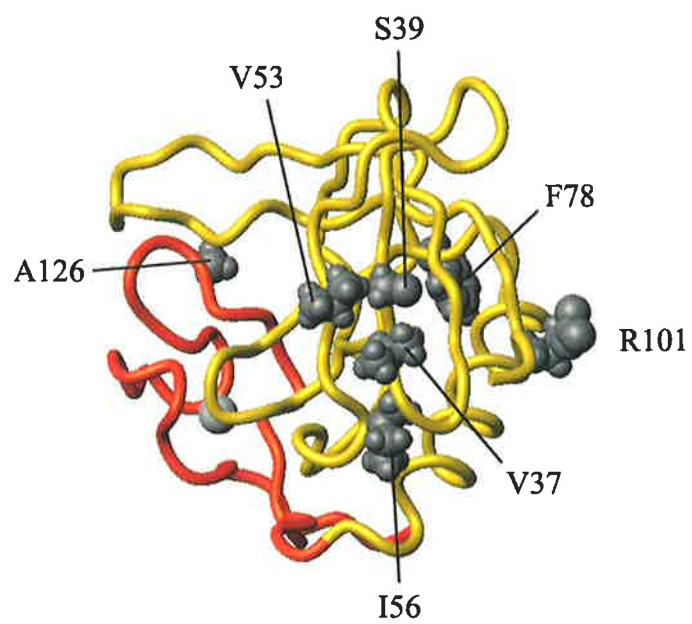
Figure 3.6 Mouse Tec PHTH Domain Model With Substitutions Highlighted

The structure of the mouse Tec PHTH domain was modelled on that of Btk (PDB code: 1btk; Hyvonen and Saraste, 1997) using SwissModel (Section 2.2.2) and is shown in ribbons and electrostatic polarisation surface diagrams. Residues that are different in human Tec are highlighted.

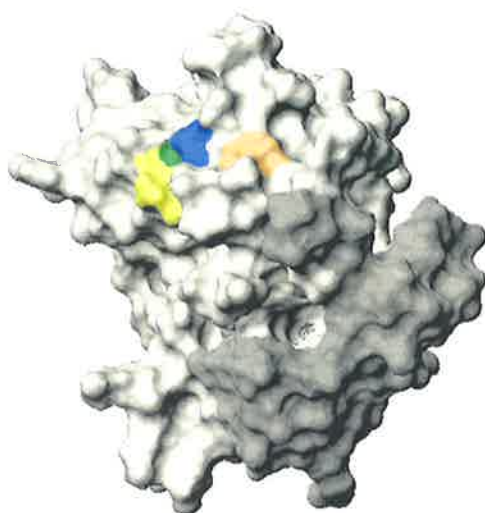
- A Ribbons diagram of the mouse Tec PH domain (yellow) and Btk motif (red), with residues that are substituted in the human Tec PHTH domain highlighted in grey and labelled.
- B Electrostatic polarisation surface diagram of the mouse Tec PH domain (light grey) and Btk motif (dark grey), with residues that are substituted in the human Tec PHTH domain highlighted in colour (V37:blue, S39:green, V53:yellow and I56:orange). These four residues map to a cluster on the surface of the Tec PHTH domain.
- C Rear view of the molecule shown in part (B) showing the remaining three residues that are substituted in the human Tec PHTH domain (F78:dark orange, R101:red and A126:purple). The figures in parts (B) and (C) are related by a 180 degree rotation about the vertical axis.

Figures were generated using MOLMOL software (Koradi *et al.*, 1996) by Dr Kasper Kowalski.

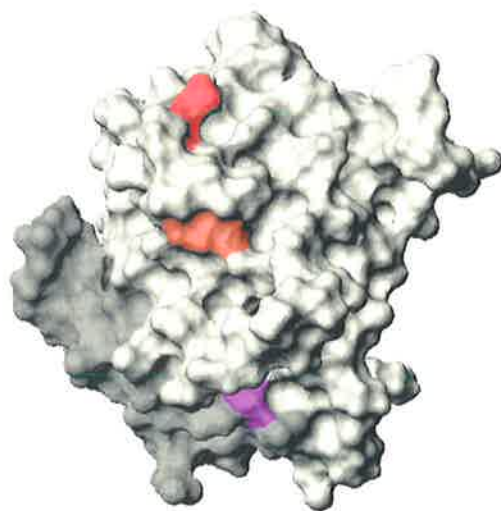
A



B



C



bacteria and plated at low density. Eight clones (75%) were found to contain an insert that was excised by *EcoRI/XhoI* restriction digestion and their characteristics are tabulated in Figure 3.7. The insert size ranged from 300-1500 bp and the average insert length was 690 bp. Several of the sequences contained Human Alu-like sequences, which are repetitive elements, unique to primates, and interspersed within the human genome with an average spacing of 4 kilobase pairs (Claverie and Makalowski, 1994). The sequence of the insert in cDNA clone 11 matched 285 bp of the human 7S L gene that has previously been identified in Human embryonic liver (Kleinert and Benecke, 1988). Therefore, the cDNA library displayed characteristics of human liver expression.

The presence of Human Tec DNA in the cDNA library was confirmed by PCR and is shown in Figure 3.7C. Primers #140 and #1 (see Section 2.1.22) were used to amplify the 485 bp hPHTH product in a PCR reaction. A band of the correct size was visualised by comparison with pUC19 molecular weight markers using agarose gel electrophoresis. This band was not present in a negative control reaction lacking cDNA library template DNA.

3.4.5 A Library of Yeast Transformants Was Created

The amplified cDNA library was transformed into yeast by large scale yeast transformation. Electroporation (Section 2.3.3.2) gave low transformation efficiency (20-115 CFU. μg^{-1} cDNA). The lithium acetate transformation method (Section 2.3.3.2) was used in two separate large scale transformation experiments after the transformation efficiency was optimised. The transformation efficiencies for the lithium acetate transformation experiments were 12,816 and 11,232 CFU. μg^{-1} cDNA, respectively. In these experiments, 6 mL of competent yeast cells in total were transformed with 600 μg cDNA, resuspended in 43.2 mL of plate solution and plated in 400 μL aliquots onto large plates.

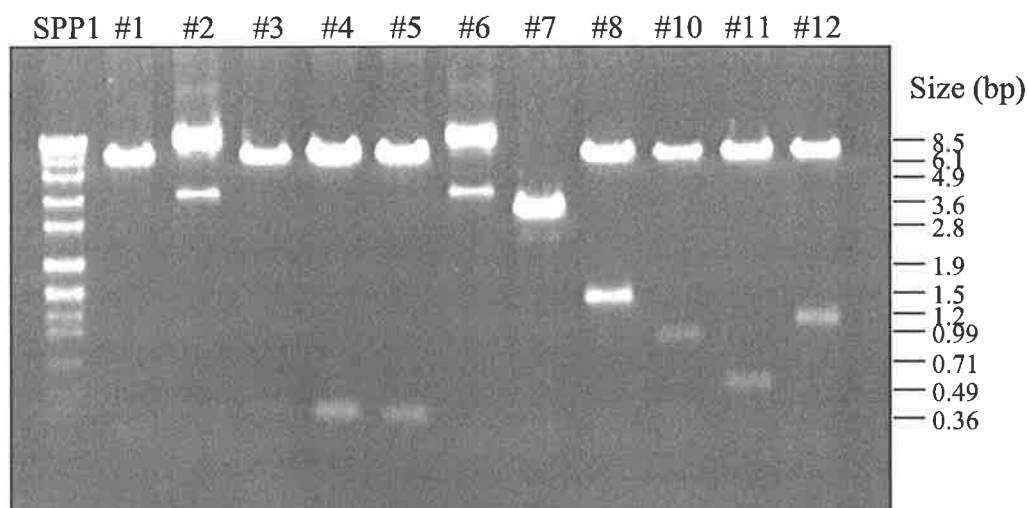
Transformant yeast colonies from the lithium acetate transformations were harvested in eight batches, pooled, prepared as glycerol stocks and titred. As calculated in Table 3.1, it was estimated that 7.0×10^6 yeast clones were amplified from the two independent transformations. An approximate titre of $5.2\text{--}8.0 \times 10^8$ CFU.mL $^{-1}$ was measured by serial dilution and plating on selective synthetic-dropout plates (stock plates: -His/-Trp/-Ura/glucose). Since there were approximately 3.2×10^6 independent clones in the library, each clone should be represented up to 160-250 times per millilitre of glycerol stock.

Figure 3.7 Characterisation of the cDNA Library

The human liver cDNA library in pB42AD plasmid, purchased from Clontech, had inserts cloned into the unique *EcoRI* and *XhoI* restriction sites in pB42AD plasmid (Figure 3.2C). The cDNA library was amplified and checked for adequate and variable insert size and characteristics of human liver expression.

- A A sample of amplified library plasmid was transformed into bacteria and plated at low density. Plasmid DNA was prepared from twelve random and discrete cDNA library clones in bacteria. No plasmid DNA was obtained from Clone #9. Library insert DNA was excised from pB42AD plasmid using *EcoRI/XhoI* double restriction enzyme digestion. Samples were run on a 1% agarose-TAE gel at 70 V for 40 min, stained with ethidium bromide and photographed under UV light. SPP1 markers were used for size comparison of DNA bands; the positions of molecular weight standards are indicated.
- B Characteristics of the cDNA library clones isolated in part (A) are tabulated. The insert size was estimated from the gel in part (A). No detectable insert is indicated with '-'. Clones with detectable insert were further analysed by automated DNA sequencing using pB42AD-5' and/or pB42AD-3' sequencing primers (#136 and #81; Section 2.1.22). The sequence of library insert DNA was compared against the GenBank database of clones using BLAST software (Section 2.2.2) to identify the gene origin of the library clones.
- C A sample of amplified library plasmid was used as a template in PCR reactions with primers (#140 and #1; Section 2.1.22) to amplify human Tec PHTH domain sequence (lanes 2 and 3). Negative control reaction (lane 1) contained no template DNA. Samples were run on a 2% agarose-TAE gel at 80 V for 40 min, stained with ethidium bromide and photographed under UV light. PUC19 markers were used for size comparison of DNA bands; the positions of molecular weight standards are indicated.

A



B

Clone Number	Insert Size (bp)	Sequence
1	300	<i>Homo sapiens</i> genomic DNA
2	-	-
3	380	Human orosomucoid1/ α -1 acid glycoprotein
4	360	mit. cytochrome c oxidase II subunit
5	350	Human Alu sequence
6	-	-
7	-	-
8	1500	<i>Homo sapiens</i> predicted mRNA
9	-	-
10	990	Human serum albumin
11	520	Human mRNA for 7SL RNA
12	1100	<i>Homo sapiens</i> genomic DNA

C

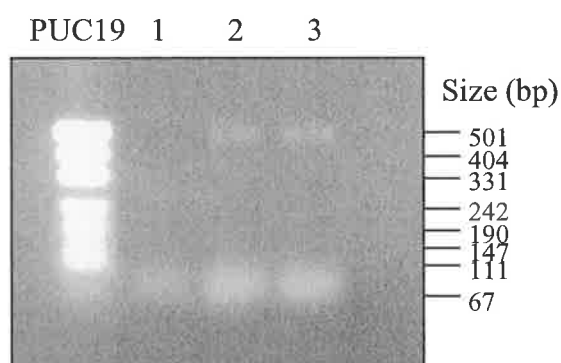


Table 3.1 Calculation of Total Number of Amplified Clones

Transformation Number	Colonies Per Plate	Number of Plates	Number of Clones
1	71,200	53	3.8×10^6
2	62,400	52	3.2×10^6
Total			7.0×10^6

3.4.6 Isolation of Tec PHTH Domain Potential Protein Ligands

To screen the cDNA library, 3.6×10^7 CFU of yeast containing bait and library plasmids were plated onto screen medium lacking XGAL at $0.5\text{--}2.0 \times 10^6$ CFU per plate (Section 2.3.3.5). 800 Leu⁺ colonies were assayed for β -galactosidase activity by restreaking onto screen medium containing XGAL, or were restreaked onto screen medium and subjected to an XGAL overlay assay (Section 2.3.3.6). Figure 3.8 contains pictures of two of the plates after XGAL overlay assay, illustrating potential positive clones that are blue in colour together with the positive control. Each plate included 50 unique colonies, as well as positive and negative controls; EGY48[p8op-LacZ, pLexA-53, pB42AD-T] and EGY48[p8op-LacZ, pLexA, pB42AD], respectively.

The screening of the 800 Leu⁺ colonies was performed in two parts. Four potential positive clones were isolated from the first screen. However, upon further analysis, these clones lost their ability to activate the LacZ reporter gene. These clones were put aside and the second screen was performed.

Colonies that gave the most intense blue colour from both screens were restreaked (R) onto stock medium and screen medium and these were denoted plates R5 and R6. Maintaining protein-protein interaction selective pressure, yeast clones from R6 were restreaked onto R7 and then R8. Some clones were a pale blue colour on plate R8 while others grew white. Glycerol stocks were prepared from clones on plate R8. The names of the cDNA library clones were designated pB42AD-R8##, where ## was the grid position of the colony.

The appropriate controls for the yeast two-hybrid interaction were performed and the potential positive clones were restreaked onto the summary plates shown in Figure 3.9. The screen plate shown in Part A and the stock plate in Part B were both replica-plated with a velvet stamp inoculated from a master stock plate. Patches 5-9 all contained pLexA-PHTH and pB42AD-R817, the clone that was extensively analysed during this work. As seen in the figure, interaction of PHTH with R817 yielded yeast that converted the clear XGAL substrate

Figure 3.8 Yeast Two-Hybrid Screen

Pictures of yeast two hybrid screen plates after XGAL overlay assay. Yeast transformed with pLexA-PHTH and pB42AD-library plasmids were plated onto screen medium without XGAL and grown at 30°C for 5 days. In total, 3.6×10^7 CFU were plated onto screen medium. 2.0×10^6 CFU (as measured on stock plates) yielded between 70-160 Leu⁺ colonies on each 14 cm diameter screen plate. Eight hundred average sized colonies were picked and streaked onto fresh screen plates and grown at 30°C for 3 days. Each plate had restreaks from 50 unique colonies and positive (+) and negative (-) control yeast clones. LacZ reporter gene activation was analysed by XGAL overlay assay and yeast were photographed. Potential positive clones that are blue in colour like the positive control are indicated with arrows.

A Plate #1 from the yeast two hybrid screen. Arrow is pointing to potential positive clone in patch 28.

B Plate #7 from the yeast two hybrid screen. Arrow is pointing to potential positive clone in patch 12.

Screen plates: synthetic-dropout -His/-Trp/-Ura/-Leu medium containing galactose, raffinose, XGAL and BU salts and lack glucose.

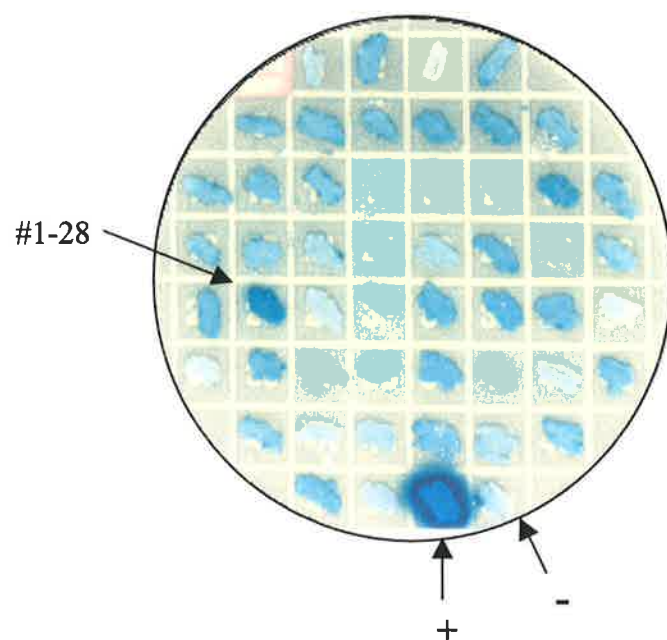
Stock plates: synthetic-dropout -His/-Trp/-Ura medium containing glucose. Enables growth of the yeast without requirement for interaction of the LexA and B42AD fusion proteins.

CFU: colony forming units

(+) control: EGY48[p8op-LacZ, pLexA-53, pB42AD-T]

(-) control: EGY48[p8op-LacZ, pLexA, pB42AD]

A



B

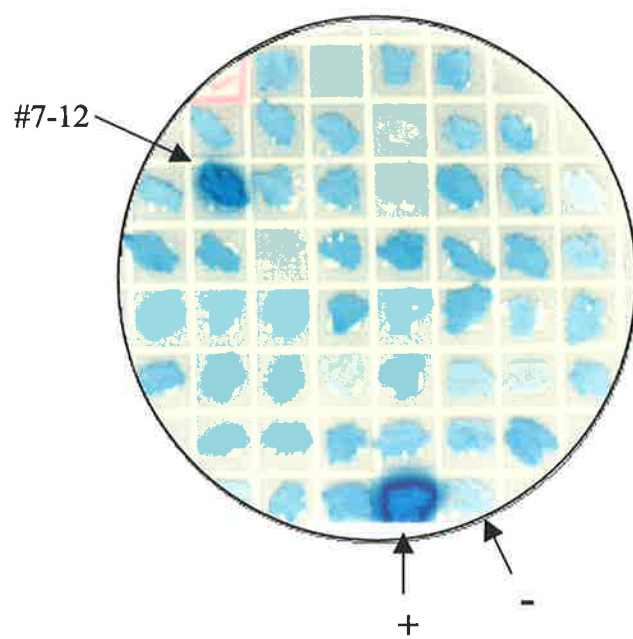


Figure 3.9 Summary of the Yeast Two-Hybrid Screen

Yeast clones that were isolated during the screening process were restreaked onto a summary stock plate, grown at 30°C for 3 days and used to inoculate a velvet stamp that was replica plated onto:

A a screen plate containing XGAL and

B a stock plate.

The two replica plates were grown at 30°C for 3 days and photographed. Each clone contains EGY48 yeast with the reporter plasmid p8opLacZ as well as pLexA- and pB42AD- plasmid with insert listed below. Clones are numbered according to the grid stuck on the bottom of the plate. List of clones on the plates:

Patches 5-9:	pLexA-PHTH	and	pB42AD-R817
Patches 12-16:	pB42AD-R817	and	pLexA-LAM (negative control)
Patch 20:	pLexA	and	pB42AD (negative control)
Patch 21:	pLexA-53	and	pB42AD (negative control)
Patch 22:	pLexA	and	pB42AD-T (negative control)
Patch 23:	pLexA-53	and	pB42AD-T (positive control)
Patch 24:	pLexA-LAM	and	pB42AD (negative control)
Patch 28:	pLexA-PHTH	and	pB42AD-7-12
Patch 29:	pLexA-PHTH	and	pB42AD-1-28
Patch 30:	pLexA-PHTH	and	pB42AD-2-14
Patch 31:	pLexA-PHTH	and	pB42AD-8-49
Patch 36:	pLexA-PHTH	and	pB42AD-R816
Patch 37:	pLexA-PHTH	and	pB42AD-R817
Patch 38:	pLexA-PHTH	and	pB42AD-R818
Patch 39:	pLexA-PHTH	and	pB42AD-R819
Patch 40:	pLexA-PHTH	and	pB42AD-R820
Patch 41:	pLexA-PHTH	and	pB42AD-R821
Patch 43:	pLexA-PHTH	and	pB42AD-R835
Patch 44:	pLexA-PHTH	and	pB42AD-R836
Patch 45:	pLexA-PHTH	and	pB42AD-R837
Patch 46:	pLexA-PHTH	and	pB42AD-R838
Patch 47:	pLexA-PHTH	and	pB42AD-R839
Patch 48:	pLexA-PHTH	and	pB42AD-R840
Patch 49:	pLexA-PHTH	and	pB42AD-R841
Patch 50:	pLexA-PHTH	and	pB42AD-R842
Patch 51:	same as Patch 23	(+, positive control)	
Patch 52:	same as Patch 20	(-, negative control)	

Screen plates: synthetic-dropout -His/-Trp/-Ura/-Leu medium containing galactose, raffinose, XGAL and BU salts and lack glucose.

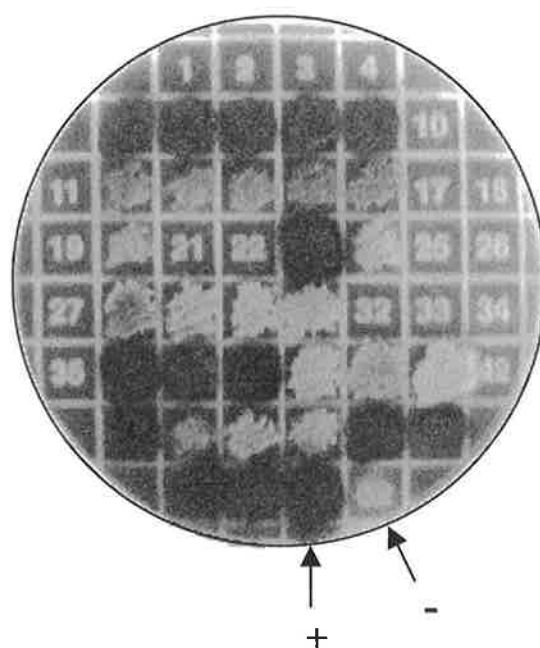
Stock plates: synthetic-dropout -His/-Trp/-Ura medium containing glucose. Enables growth of the yeast without requirement for interaction of the LexA and B42AD fusion proteins.

(+) control: EGY48[p8op-LacZ, pLexA-53, pB42AD-T]

(-) control: EGY48[p8op-LacZ, pLexA, pB42AD]

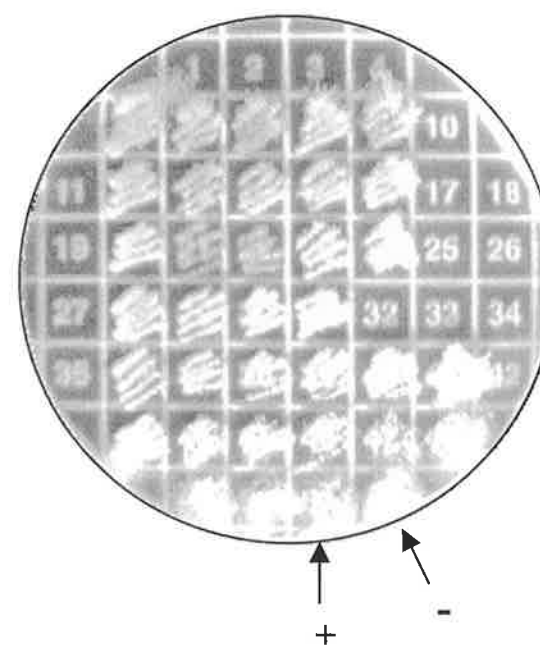
A

Screen plate



B

Stock plate



into a blue product. This product was not present in Patches 12-16, which each contained pB42AD-R817 plasmid and the negative control plasmid pLexA-LAM. Therefore, R817 did not bind LexA-LAM, indicating that the interaction with LexA-PHTH was dependent on the PHTH domain and not the LexA residues. Patches 20-22 and 24 were negative controls while 23 was the positive control containing pLexA-53 and pB42AD-T plasmids. Patches 28-31 contained the four clones from the first screen, while patches 36-41 and 43-50 contained fourteen clones from the second screen. Patches 51 and 52 were duplicates of the positive and negative controls from patches 23 and 20, respectively.

3.4.7 Yeast Colony PCR Obtained cDNA Library Inserts of Positive Clones

Library plasmids from the 10 bluest clones from plate R8 were sought for characterisation. These correspond to clones R816, R817, R818, R821, R835, R836, R839, R840, R841 and R842, all of which are represented in Figure 3.9. Plasmid rescue from yeast into *Escherichia coli* KC8 (Section 2.3.3.7) was attempted as a first step in verification of the two-hybrid result. This experiment was largely unsuccessful, due to the insufficient amount of plasmid harvested from the yeast, and was abandoned in favour of yeast colony PCR after several unsuccessful attempts.

A small scraping of yeast lysed in 1% NP40 was used as template in yeast colony PCR reactions (Section 2.3.3.8). In all the reactions, the pB42AD sequencing primers (5' and 3') were used to amplify the cDNA library insert in the pB42AD vector. These primers were chosen because they are specific for the vector backbone and should amplify different insert sequences indiscriminately. Three controls were used in the PCR reactions, including no DNA template, pB42AD with no insert and pB42AD-T, which were expected to yield no product, 156 bp and 2100 bp products, respectively. As shown in Figure 3.10A, these indicated no contamination of PCR reaction ingredients, as well as small and large size inserts. Two samples of pB42AD-T template were used; the first was purified plasmid DNA obtained by midiprep (Section 2.3.1.18) and the second was from a scraping of positive control yeast that was prepared at the same time as the potential positive clones. This showed that lysed yeast was an adequate template for yeast colony PCR.

Seven out of ten clones yielded approximately 800 bp PCR fragments (Figure 3.10A and B). Three of these also contained a second band of lower intensity that probably arose due to the yeast containing two different library plasmids; the less intense band most likely resulted from a library plasmid encoding a non-interacting protein that was being lost by

Figure 3.10 Analysis of Yeast Two-Hybrid Potential Positive Clones by Yeast Colony PCR

Library encoded inserts in pB42AD plasmid of potential positive clones were amplified by yeast colony PCR. A small scraping of yeast grown on screen medium was lysed in 1% NP40 and used as a template in yeast colony PCR reactions with pB42AD 5' and 3' sequencing primers (#136 and #81; Section 2.1.22). Three controls used in the PCR reactions included no DNA template, pB42AD with no insert and pB42AD-T, which were expected to yield no product, 156 bp and 2100 bp products, respectively. Samples were run on a 1.5% agarose-TAE gels at 75 V for 45 min, stained with ethidium bromide and photographed under UV light. SPP1 and PUC19 markers were used for size comparison of DNA bands; the positions of molecular weight standards are indicated.

A List of clones used as yeast colony PCR templates (or markers):

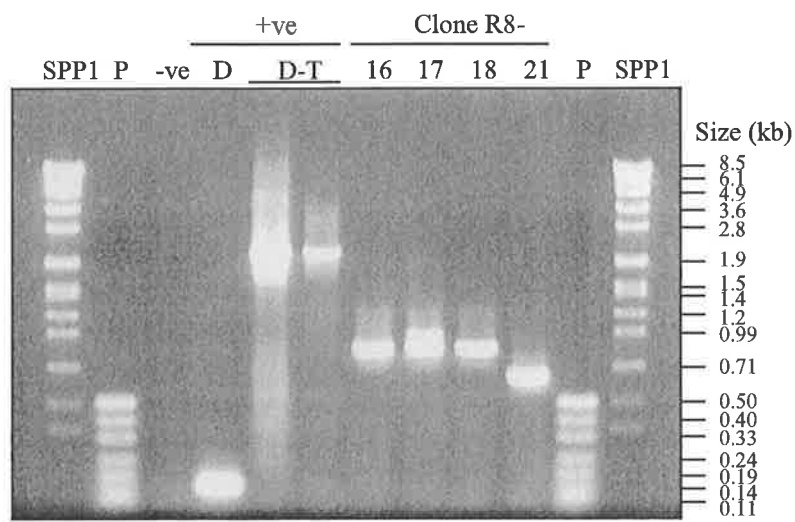
lane	1:	SPP1 markers	
	2:	pUC19 markers	
	3:	no DNA template	(negative control)
	4:	pB42AD	(positive control, purified plasmid DNA)
	5:	pB42AD-T	(positive control, purified plasmid DNA)
	6:	pB42AD-T	(positive control, lysed yeast)
	7:	R8-16	
	8:	R8-17	
	9:	R8-18	
	10:	R8-21	
	11:	pUC19 markers	
	12:	SPP1 markers	

B List of clones used as yeast colony PCR templates (or markers):

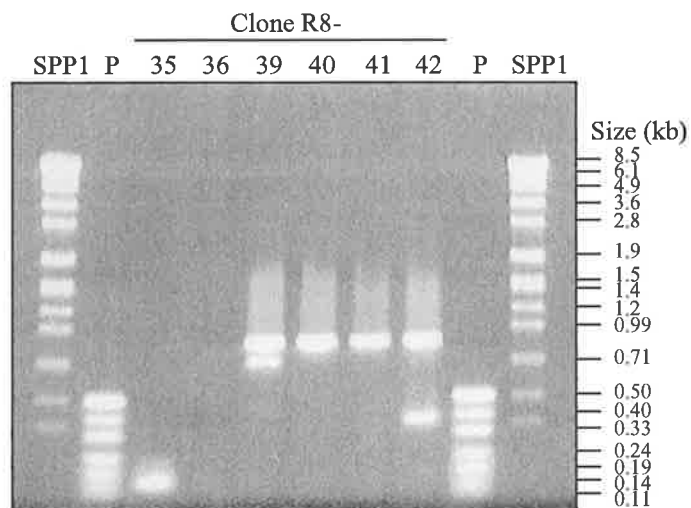
lane	1:	SPP1 markers
	2:	pUC19 markers
	3:	R8-35
	4:	R8-36
	5:	R8-39
	6:	R8-40
	7:	R8-41
	8:	R8-42
	9:	pUC19 markers
	10:	SPP1 markers

Screen medium: synthetic-dropout -His/-Trp/-Ura/-Leu medium containing galactose, raffinose, XGAL and BU salts and lack glucose.

A



B



segregation. The other three clones yielded 600 bp (R821), 180 bp (R835) and no detectable (R836) PCR products.

3.4.8 Actinin-4 Binds Tec PHTH Domain

The PCR products were purified and analysed by DNA sequencing. Sequence analysis results are tabulated below (Table 3.2) and show that a human α -actinin isoform, Actinin-4, accounts for at least five of the obtained PHTH domain interacting proteins. Those five sequences were identical and encoded a fusion of 220 in frame amino acids (listed in Figure 3.11) to the B42AD. The reading frame was determined from the pB42AD vector map and Multiple Cloning Site Sequence (CLONTECH Laboratories, 1996) and was the appropriate reading frame for Actinin-4 sequence, as determined by comparison with the published translation of Actinin-4 (Honda *et al.*, 1998a, Honda *et al.*, 1998b). In Figure 3.11, the different regions of sequence are coloured according to the domains to which they belong. Sequences that are attributed to cloning artefact from creation of the cDNA library are coloured grey. Importantly, it is expected that multiple copies of the Actinin-4 sequence obtained during the screening process arose during amplification of the cDNA library.

The sequence was not determined for four clones: one (R836) that gave no detectable PCR product, one (R835) that yielded a PCR product that was similar in size to that amplified from the empty pB42AD plasmid and two of which were the same size as the Actinin-4 clones (R818 and R842).

Table 3.2 Tec PHTH Domain Potential Protein Ligands

Clone Number	PCR Product Size (base pairs)	DNA Sequence (ND = not determined)
R816	800	Actinin-4
R817	800	Actinin-4
R818	800	ND
R821	600	Cytochrome C Oxidase
R835	180	ND
R836	-	ND
R839	800	Actinin-4
R840	800	Actinin-4

The PCR products were cloned into pB42AD and the library-encoded proteins were retested for their ability to bind the Tec PHTH domain. Actinin-4 continued to give a positive result (patches 5-9 in Figure 3.9A). Clone R821 encoded a fragment of Cytochrome C oxidase, a commonly recognised yeast two-hybrid false positive (Serebriiskii and Golemis,

Figure 3.11 Nucleotide and Amino Acid Sequence of Clone R817

The nucleotide sequence of clone R817 (top) coding strand was determined by automated DNA sequencing of R817 yeast colony PCR product and translated using DNASIS software to give the corresponding 220 amino acid sequence (single letter code, below). The stop codon is indicated by asterisk (*).

The *Eco*RI and *Xho*I restriction sites derived from the multiple cloning site of the pB42AD plasmid are boxed. The nucleotide sequence was used as a query in a search of the GenBank database using BLAST software (Section 2.2.2) where it was found to be identical to a section of Actinin-4 code.

The reading frame was determined from the pB42AD vector map (CLONTECH Laboratories, 1996) and is the appropriate reading frame for the Actinin-4 sequence, as determined by comparison with the published translation of Actinin-4 (Honda *et al.*, 1998a, Honda *et al.*, 1998b). The numbering of the amino acids is consistent with the published translation of the full-length protein (Honda *et al.*, 1998b). Residues that contribute to the core structures of Spectrin Repeats 2 (blue), 3 (green) and 4 (yellow) are indicated in colour, while the loop regions between the helices are shown in black. Sequences that are attributed to cloning artefact from creation of the cDNA library are coloured grey.

Amino- and carboxyl- terminal residues of R817 deletion series fragments (see Figure 3.14) are numbered and indicated with (>) and (<), respectively. Residues of the HIKE-like motif in the BC-loop of the third spectrin repeat (H602, I603 and K604) that were substituted with alanines in experiments described in Section 3.4.9 are numbered.

EcoRI

GAA	TTC	GCG	GCC	GCG	TCG	ACC	CAC	AAT	GTC	AAC	ACC	CGG	TGC	CAG	45
E	F	A	A	A	S	T	H	N	V	N	T	R	C	Q	496
489															
AAG	ATC	TGT	GAC	CAG	TGG	GAC	GCC	CTC	GGC	TCT	CTG	ACA	CAT	AGT	90
K	I	C	D	Q	W	D	A	L	G	S	L	T	H	S	511
498>															
CGC	AGG	GAA	GCC	CTG	GAG	AAA	ACA	GAG	AAG	CAG	CTG	GAG	GCC	ATC	135
R	R	E	A	L	E	K	T	E	K	Q	L	E	A	I	526
518>															
GAC	CAG	CTG	CAC	CTG	GAA	TAC	GCC	AAG	CGC	GCG	GCC	CCC	TTC	AAC	180
D	Q	L	H	L	E	Y	A	K	R	A	A	P	F	N	541
538>															
AAC	TGG	ATG	GAG	AGC	GCC	ATG	GAG	GAC	CTC	CAG	GAC	ATG	TTC	ATC	225
N	W	M	E	S	A	M	E	D	L	Q	D	M	F	I	556
GTC	CAT	ACC	ATC	GAG	GAG	ATT	GAG	GGC	CTG	ATC	TCA	GCC	CAT	GAC	270
V	H	T	I	E	E	I	E	G	L	I	S	A	H	D	571
558>															
CAG	TTC	AAG	TCC	ACC	CTG	CCG	GAC	GCC	GAT	AGG	GAG	CGC	GAG	GCC	315
Q	F	K	S	T	L	P	D	A	D	R	E	R	E	A	586
578>															
ATC	CTG	GCC	ATC	CAC	AAG	GAG	GCC	CAG	AGG	ATC	GCT	GAG	AGC	AAC	360
I	L	A	I	H	K	E	A	Q	R	I	A	E	S	N	601
CAC	ATC	AAG	CTG	TCG	GGC	AGC	AAC	CCC	TAC	ACC	ACC	GTC	ACC	CCG	405
H	I	K	L	S	G	S	N	P	Y	T	T	V	T	P	616
602	603	604													
CAA	ATC	ATC	AAC	TCC	AAG	TGG	GAG	AAG	GTG	CAG	CAG	CTG	GTG	CCA	450
Q	I	I	N	S	K	W	E	K	V	Q	Q	L	V	P	631
AAA	CGG	GAC	CAT	GCC	CTC	CTG	GAG	GAG	CAG	AGC	AAG	CAG	CAG	TCC	495
K	R	D	H	A	L	L	E	E	Q	S	K	Q	Q	S	646
<638															
AAC	GAG	CAC	CTG	CGC	CGC	CAG	TTC	GCC	AGC	CAG	GCC	AAT	GTT	GTG	540
N	E	H	L	R	R	Q	F	A	S	Q	A	N	V	V	661
<658															
GGG	CCC	TGG	ATC	CAG	ACC	AAG	ATG	GAG	GAG	ATC	GGG	CGC	ATC	TCC	585
G	P	W	I	Q	T	K	M	E	E	I	G	R	I	S	676
ATT	GAG	ATG	AAC	GGG	ACC	CTG	GAG	GAC	CAG	CTG	AGC	CAC	CTG	AAG	630
I	E	M	N	G	T	L	E	D	Q	L	S	H	L	K	691
<678															
CAG	TAT	GAA	CAC	AGC	TTC	TGT	GGC	TGG	CTG	TAA	TAC	TGT	ACA	ACT	675
Q	Y	E	H	S	F	C	G	W	L	*					
<696															
GTT	TCT	GAC	CAT	TAA	ATG	CTG	TTG	TAC	TCT	GAA	AAA	AAA	AAA	AAA	720
<i>XhoI</i>															
AAA	AAC	TCG	AG												731

2001). This clone gave only pale blue colour colonies after retesting in yeast (patch 41 in Figure 3.9A).

Since the library-encoded Actinin-4 sequence was only a fragment of the Actinin-4 protein, which is depicted in Figure 3.12A, the full-length clone was obtained from Dr S. Hirohashi (National Cancer Center Research Institute, Tokyo). Full length Actinin-4 encodes a 911-residue protein that contains two calponin homology domains at the N-terminus, four spectrin repeats that make up the central rod domain, and two EF-hands at the C-terminus (Figure 3.12A). α -actinin molecules form head to tail dimers with intermolecular association between spectrin repeats 2 and 3, leaving spectrin repeat 1 to pair with spectrin repeat 4. Each spectrin repeat contains three α -helices; A, B and C, that form a triple helical bundle structure. The spectrin repeats are depicted in different colours in Figure 3.12B, which is a ribbon diagram of the homodimeric central rod domain of Actinin-2 (Djinovic-Carugo *et al.*, 1999). The library-encoded fragment spanned the third spectrin repeat and flanking sequences that include helix C of spectrin repeat two and half of spectrin repeat 4 (Figures 3.11 and 3.12D). The equivalent region of Actinin-2 is illustrated in Figure 3.12C to indicate the approximate topology of R817.

3.4.9 Site-Directed Mutagenesis of the HIKE-like Motif in Actinin-4 Does not Disrupt Binding of Tec PHTH Domain

Site-directed mutagenesis was used in a preliminary attempt to identify specific residues involved in Tec-Actinin binding since a recent report (Alberti, 1998) proposed that PH domain ligands include HIKE motifs; motifs with an amino acid consensus sequence $\text{HIK}(\text{X}_8)\text{E}$ where X is any amino acid. There is a HIKE-like motif in the BC loop of Actinin-4 spectrin repeat 3 at position 602-613 of the native protein: **HIKLSGSNPYTT** (Figures 3.11). Structure prediction by Dr Booker using the INSIGHT II computer program showed the HIKE-like sequence forming a protruding motif. It was suggested that this protrusion could occupy a cleft in the Tec PHTH domain, a model of which is depicted shown in Figure 3.13A. To test this hypothesis, the HIK residues in a subclone of R817 that spanned I498-S696 (see Figures 3.11 and 3.14A) were replaced with alanines using site-directed mutagenesis (Section 2.3.1.3). Electropherograms from DNA sequencing of the wildtype and mutant HIK-encoding sequence are illustrated in Figures 3.13B and C. The mutant peptide was assayed for PHTH domain-binding in the yeast two-hybrid assay. Like wildtype, the H602A/I603A/K604A triple

Figure 3.12 Actinin-4 Protein Domain Structure and PHTH domain Binding Region

- A Diagrammatic representation of the protein domain structure of full-length Actinin-4. α -actinins form head to tail homodimers through interaction of four spectrin repeat domains in the rod domain. The spectrin repeat domains are coloured 1: pink, 2: blue, 3: green and 4: yellow. At the extreme amino terminus there are two copies of the calponin homology (CH) domain shown in purple. These actin binding domains enable alpha actinins to cross-link actin filaments into bundles and networks. At the carboxyl terminus there are two copies of the EF hand motif that bind calcium, shown in orange.
- B Ribbons diagram showing the tertiary structure of the rod domain from Actinin-2 (Ylanne *et al.*, 2001). The rod is a homodimer of four spectrin repeats that each consist of three alpha helices and are coloured the same as in part (A). The helices fold back on one another in an up-down-up fashion and form triple helical bundle structures.
- C Ribbons diagram showing the tertiary structure of the region in Actinin-2 (residues V477 to N684) equivalent to the segment of Actinin-4 encoded by the R817 clone (residues H489 to S696). This diagram is used to illustrate the approximate topology of the Tec PHTH domain binding fragment of Actinin-4. Adjacent spectrin repeats are joined by continuous alpha helix that is unlike the traditional flexible loops seen in the linkers between the A and B helices (AB loop) and between the B and C helices (BC loop).
- D Linear representation of the protein domain structure of the Actinin-4 fragment encoded in clone R817. The spectrin repeats are coloured the same as in part (A). R817 encodes the three alpha helices of spectrin repeat three (A, B, C) and flanking sequences, which include helix C of spectrin repeat two and helix A and part of helix B of spectrin repeat four.

The positions of the amino-terminus (N) and carboxyl-terminus (C) of each molecule are indicated. Figures in parts (B) and (C) were generated using MOLMOL software (Koradi *et al.*, 1996) by Dr Kasper Kowalski. PDB code: 1hci (Ylanne *et al.*, 2001).

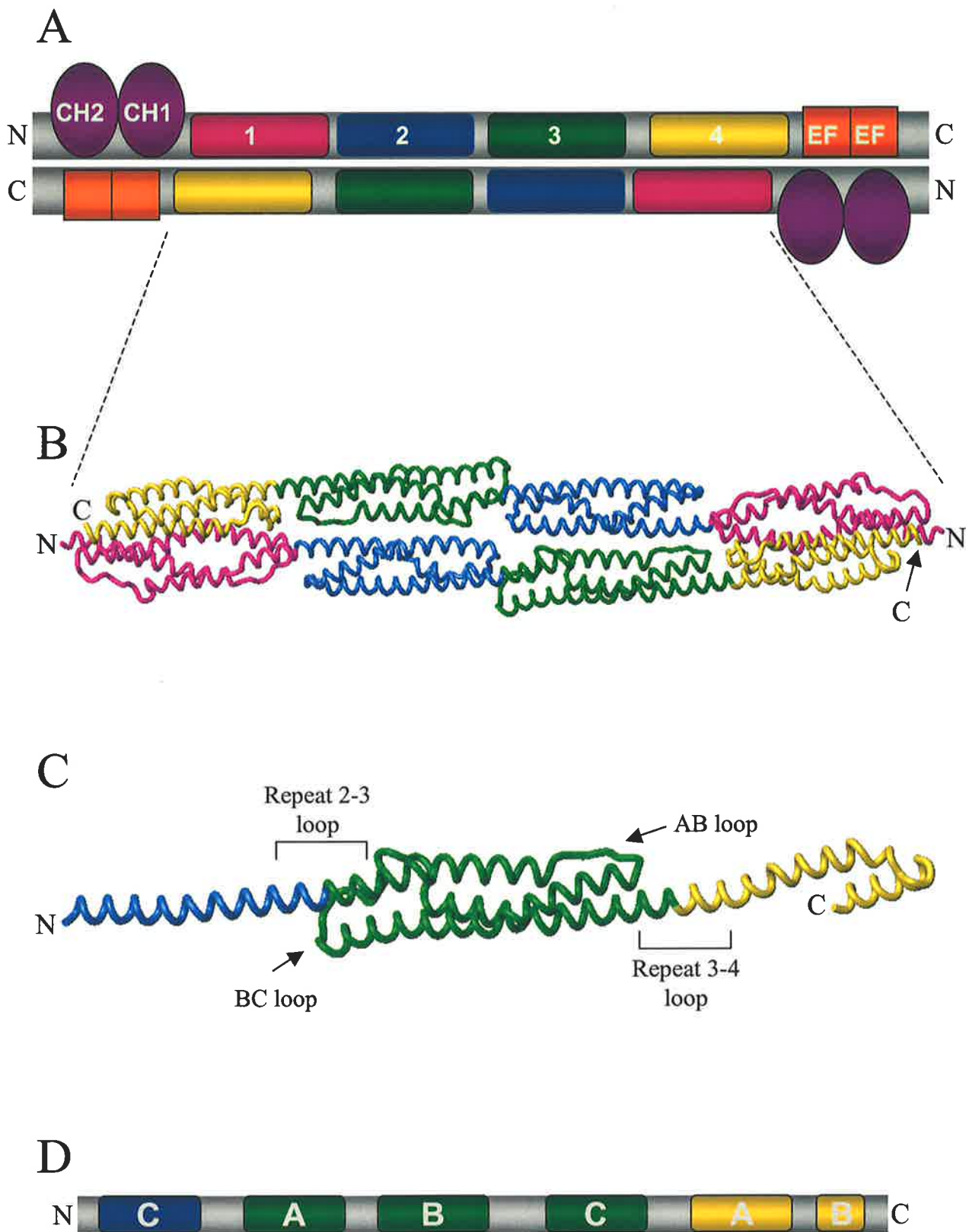


Figure 3.13 Mutation of the HIKE-like Motif in Clone R817

- A Model of the Actinin-4 central rod domain tertiary structure, shown on the diagonal. The structure contains an antiparallel homodimer of the second and third spectrin repeats and was modelled on that of Actinin-2 (PDB code: 1hci ; Ylanne *et al.*, 2001) using SwissModel (Section 2.2.2). The left molecule of the homodimer is shown as a ribbons diagram with Repeat-2 shown in blue and Repeat-3 shown in green. The right molecule of the homodimer is shown as an electrostatic polarisation surface diagram with basic residues shown in blue and acidic residues shown in red; the H602, I603 and K604 residues of the HIKE-like motif (Alberti, 1998) in the BC loop of repeat three are shown in ball and stick form. The protruding HIKE-like motif in Actinin-4 spectrin repeat 3 could occupy a cleft in the Tec PHTH domain. The structure of the Tec PHTH domain was modelled on that of Btk (PDB code: 1btk; Hyvonen and Saraste, 1997) using SwissModel (Section 2.2.2) and is shown in ribbons and electrostatic polarisation surface diagrams for size comparison to the Actinin-4 central rod domain. All images are drawn to the same scale to enable size comparison of the interacting proteins. The PH domain (green), Btk motif (red) and the Zn^{2+} ion (grey) are distinguished by colour in the ribbons diagram. The positions of the amino-terminus (N) and carboxyl-terminus (C) of each molecule are indicated. Figures were generated using MOLMOL software (Koradi *et al.*, 1996) by Dr Kasper Kowalski.
- B Electropherogram from DNA sequencing of the wild type subclone of R817 that encodes residues I493-S696 of Actinin-4 (see Figure 3.14A). Codons for the H602, I603 and K604 residues of the HIKE-like motif in the BC loop of repeat three are boxed.
- C Electropherogram from DNA sequencing of the HIK-mutant subclone of R817 that was derived from the clone in part (B). Site directed mutagenesis (Section 2.3.1.3) using primers #164 and #165 (Section 2.1.22) on pB42AD-I493-S696 template was used to exchange H602, I603, and K604 codons with A602, A603 and A604 codons. The alanine codons are boxed and contain a *NotI* restriction enzyme site for easy identification of mutant clones.
- D The wild type and HIK-mutant peptides described in parts (B) and (C) were assayed for PHTH domain binding in the yeast two-hybrid assay as described in Figure 3.9. EGY48-[p8opLacZ, pLexA-PHTH] yeast were transformed with pB42AD-I498-S696 or pB42AD-I498-S696(H602A/I603A/K604A) plasmid. Yeast clones and control yeast were replica plated onto a screen plate containing XGAL and a stock plate, grown at 30°C for 3 days and photographed.

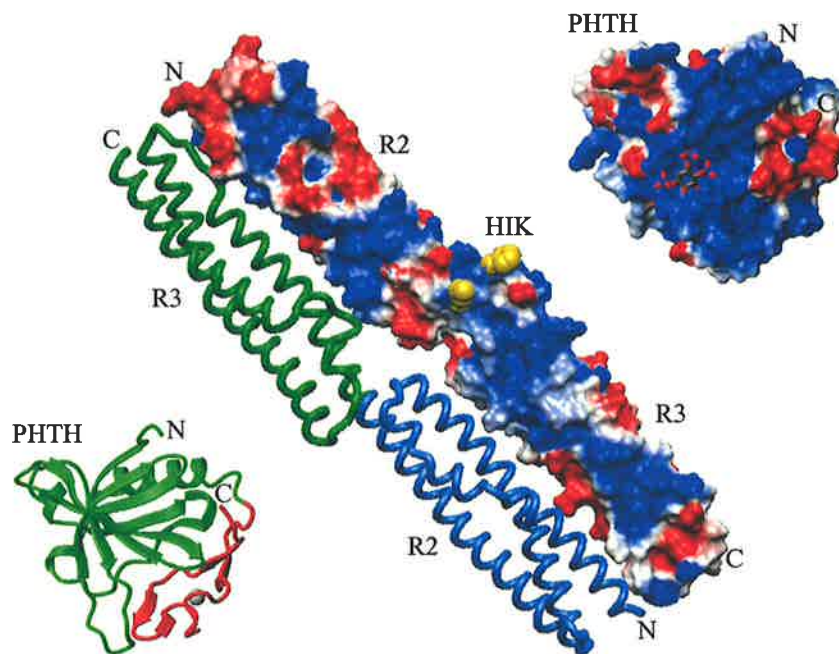
Screen plate: synthetic-dropout -His/-Trp/-Ura/-Leu medium containing galactose, raffinose, XGAL and BU salts and lack glucose.

Stock plate: synthetic-dropout -His/-Trp/-Ura medium containing glucose. Enables growth of the yeast without requirement for interaction of the LexA and B42AD fusion proteins.

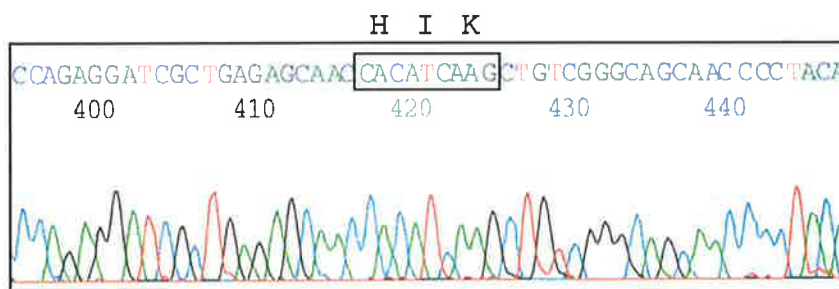
(+ve) control: EGY48[p8op-LacZ, pLexA-53, pB42AD-T]

(-ve) control: EGY48[p8op-LacZ, pLexA, pB42AD]

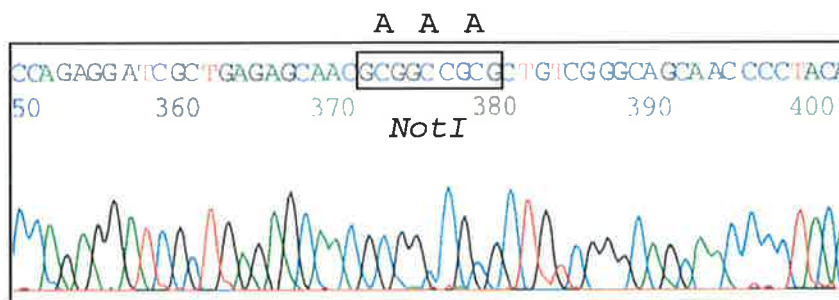
A



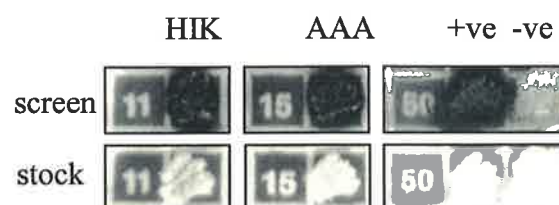
B



C



D



mutant bound to the Tec PHTH domain (Figure 3.13D) indicating this substitution did not affect the interaction between Actinin-4 and Tec.

3.4.10 Minimal PHTH-binding Region of Actinin-4 Contains Spectrin Repeat Three

Deletion analysis was performed to identify residues required for interaction with the Tec PHTH domain. At first, truncations of the library-encoded fragment R817 were created by PCR (Section 2.3.1.2) using primers #143-#163 (Section 2.1.22). Since R817 contained more than one spectrin repeat, primers for the deletion series were carefully designed with the aid of OligoTM software to avoid false priming or amplification of undesirable sequences by stipulating unique 3' ends. This software also predicted primer melting temperature, primer self annealing and formation of primer dimers and, therefore, enabled the design of primer pairs with compatible binding characteristics and minimal complications.

The deletion series PCR products encoded successive 20 amino acid truncations from the N- or C-terminus of R817 and are illustrated in Figure 3.14A. Each was cloned into the pB42AD plasmid, verified by DNA sequencing and transformed into yeast containing pLexA-PHTH. Six copies of each clone were assayed for PHTH-binding in direct yeast two-hybrid assays. Pictures of screen and stock replica plates that summarise the deletion series assay are shown in Figure 3.14B. When comparing yeast patches 28 and 36 (Figure 3.14B), the N-terminal truncation of residues spanning K518-A537, resulted in loss of PHTH-binding. Those residues were: KTEKQLEAI-DQLHLEYAKRA, where the hyphen indicates the start of Repeat-3. Similarly, when comparing patches 7 and 43 (Figure 3.14B), deletion of residues spanning E639-A658 also resulted in loss of binding. These residues were: EEQSKQQSN-EHLRRQFASQA, where the hyphen indicates the start of Repeat-4. The boundaries of Repeat-3 and Repeat-4 were identified by analogy to that illustrated for Repeat-19 of human erythrocyte α -spectrin (Pascual *et al.*, 1997). Actinin-4 Repeat-3 was predicted to begin at residue D527, while Repeat-4 was predicted to begin at residue E648 and in each case the preceding 10 residues were attributed to the linker regions between repeats. The K518-A537 and E639-A658 truncations removed linkers between repeats as well as the first eleven residues of the Repeats 3 and 4, respectively. The truncations may affect the ability of spectrin repeat three to form correct triple helical structure and/or prevent dimerisation.

The recent crystal structure of Repeat2-3 of Actinin-2 revealed α -helical continuity rather than traditional flexible loops in the linkers between individual spectrin repeats

Figure 3.14 Mapping the Interacting Residues of R817 and Tec

A Diagrammatic representation of the sub-domains of R817, with linear representation of the deletion series below. N- and C-terminal residues of R817 deletion series fragments are listed in single letter code and numbered consistent with the published translation of the full-length protein. The pink star denotes the alanine mutant of the HIKE-like motif in the BC loop of Repeat-3 described in the previous figure. Binding to LexA-PHTH in the yeast two-hybrid assay is indicated by a tick (yes) or cross (no).

B Yeast two hybrid assay of LexA-PHTH bait with the deletion series of R817 from part (A) and fragments encoding combinations of spectrin repeats. The deletion series of R817 was created by PCR, cloned into the pB42AD plasmid and tested for PHTH binding in direct yeast two-hybrid assays. The full length Actinin-4 clone was obtained from T. Yamada (Japan), and fragments encoding Repeats2-3 (N403-Q645), Repeats 3-4 (K518-D758) and Repeats 1-4 (A271-D758) were cloned by PCR and also tested in the yeast two-hybrid assay. A representative clone from each deletion or fragment was restreaked onto a summary stock plate, grown at 30°C for 3 days and used to inoculate a velvet stamp which was replica plated onto (left) a screen plate containing XGAL and (right) a stock plate. The two replica plates were grown at 30°C for 3 days and photographed. Each clone contains EGY48 yeast with the reporter plasmid p8opLacZ as well as pLexA-PHTH plasmid and the pB42AD- plasmid with insert listed below. Exceptions include Patch 5 that has no insert in the pB42AD plasmid and the controls in Patches 51-52. List of patch number and corresponding Actinin-4 residues encoded in the pB42AD insert:

Patch 5:	no insert	Patch 7:	I498-A658
Patch 12:	R817	Patch 14:	I498-E678
Patch 20:	I498-S696	Patch 22:	K518-Q645
Patch 28:	K518-S696	Patch 30:	N403-Q645
Patch 36:	A538-S696	Patch 38:	K518-D758
Patch 43:	I498-L638	Patch 45:	A271-D758
Patch 51:	pLexA-53 and pB42AD-T (positive control)		
Patch 52:	pLexA and pB42AD (negative control)		

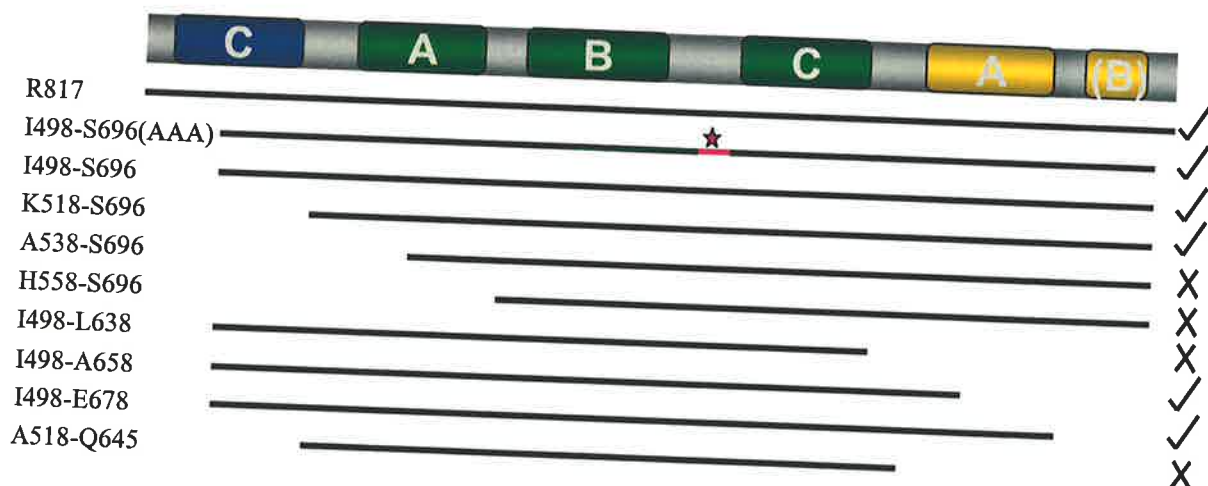
C Yeast two-hybrid assay of B42AD-R817 with fragments of Tec amino terminal domains expressed from pLexA. Triplicate colonies of each yeast clone were prepared, grown and replica plated as in part (B). List of patch number and corresponding Tec residues encoded in the pLexA insert:

Patches 7-9:	PH domain (M1-N113)
Patches 14-16:	Btk motif (N114-S152)
Patches 22-24:	PH-TH-SH3-SH2 domains (M1-S345)
Patches 30-32:	TH-SH3 domains (N114-E245)
Patch 51:	pLexA-53 and pB42AD-T (Positive control)
Patch 52:	pLexA and pB42AD (Negative control)

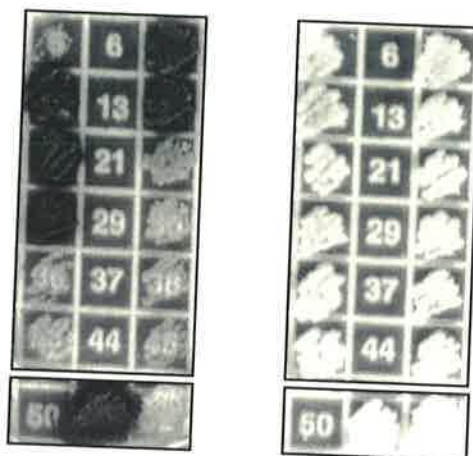
Screen plates: synthetic-dropout -His/-Trp/-Ura/-Leu medium containing galactose, raffinose, XGAL and BU salts and lack glucose. Interaction of the LexA and B42AD fusion proteins is required for activation of the LEU2 and LacZ reporter genes.

Stock plates: synthetic-dropout -His/-Trp/-Ura medium containing glucose. Enables growth of the yeast without requirement for interaction of the LexA and B42AD fusion proteins.

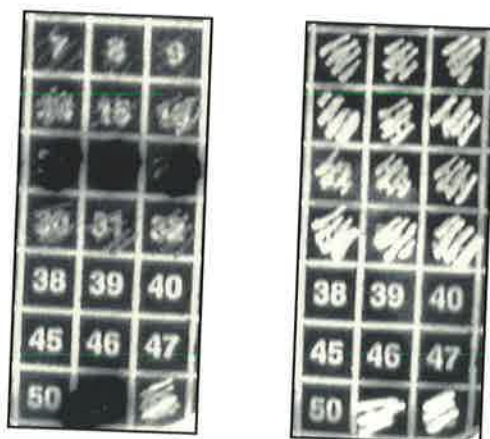
A



B



C



(Djinovic-Carugo *et al.*, 1999). Inspection of these regions in Figure 3.12C shows that these rod sections may stabilise the structure of the more flexible AB and BC loops of Repeat-3. Alternatively, the deleted peptides could have contained residues that directly contact the PHTH domain. To assess this, the twenty-residue peptides could be tested in the yeast two-hybrid assay for PHTH domain binding. Currently, it is not known whether these specific residues are involved in direct binding of PHTH.

To create a subclone of R817 that contained Repeat-3 and flanking residues, but not Repeat-2 and Repeat-4 residues, the sequence encoding Actinin-4 Repeat 3 with extension -9 and +8 was cloned. This was denoted Rpt3 and encodes K518-Q645. Rpt3 has the same N-terminal residues as the PHTH-binding clone in Patch 28 but has a 13 residue C-terminal truncation compared with the PHTH-binding clone in Patch 7. Yeast containing this clone were pale blue in colour after 5 days growth at 30°C but not at 3 days (Figure 3.14B), and this may be due to the C-terminal truncation. Unlike R817 protein, Rpt3 protein does not homodimerise (see Section 5.4.8), indicating that Tec may prefer to bind Actinin-4 dimers.

Combinations of Actinin-4 spectrin repeats that were expected to dimerise were cloned by PCR and tested in the yeast two-hybrid assay. These are included in Figure 3.14B. The clone containing repeats 2-3 has the same C-terminus as Rpt3. None of repeats 2-3 (Patch 30), repeats 3-4 (Patch 38) and repeats 1-4 (Patch 45), bound to PHTH in the yeast two-hybrid assay (Figure 3.14B). This was unexpected, but may be due to steric hindrance from the extra residues. Either the rigid rod structures constructed by the extra spectrin repeat sequences prevent the proper activation of the reporter genes, or they require cooperative interactions with other molecules or other Tec domains to induce a conformational change that permits PHTH domain-binding.

3.4.11 The PHTH Domain Functional Unit Is Required for Actinin-4-binding

The individual PH and TH regions of the PHTH domain and other fragments of Tec N-terminal domains were tested for binding to R817 in the yeast two-hybrid assay. As shown in Figure 3.14C, neither the PH domain nor Btk motif alone bound to R817. It is not known whether either of these regions alone forms their correct tertiary structures, as extensive contacts exist between the two regions in Btk (Hyvonen and Saraste, 1997) and are probably necessary for proper folding of the TH domain. The combined TH domain and SH3 domain did not bind R817 (Figure 3.14C). However, the region spanning all of the N-terminal domains of Tec, PH-TH-SH3-SH2, bound to R817 (Figure 3.14C) suggesting that the extra

sequences do not conceal the binding site. It is anticipated that the conformation of the N-terminal domains in the absence of the kinase domain matches their conformation when Tec is in the open, active state (see Figure 1.3).

3.5 Discussion

The activity of intracellular signalling molecules is regulated by interactions mediated with cellular proteins and factors. Many signalling molecules contain a number of discrete domains each capable of mediating protein-protein interactions. The specific ligands of each domain determine signalling pathways with which the molecule can participate and this influences the overall outcome from the initial stimulus.

PH domains are involved in targeting of molecules to specific cellular sites. Some participate in reversible interactions with lipid factors, a process that is regulated by the abundance of ligand, which is controlled by specific kinases, phosphatases and lipases. PH domains of Tec-family kinases bind 3'-phosphorylated phosphatidylinositol lipids and contribute to the regulation of protein tyrosine kinase activity through modulation of intramolecular interactions. Although PI 3,4,5-P₃-binding interactions are not detected in the yeast two-hybrid assay, it was expected that novel and previously identified Tec-family PH domain protein ligands would be identified as binding partners of the Tec PHTH domain. While a number of protein ligands for PH domains have been identified, their interactions have not generally been well characterised.

3.5.1 Identification of Actinin-4 as a Ligand of Tec PHTH Domain.

The studies described in Section 3.4 aimed to isolate protein ligands of the Tec PHTH domain as a preliminary step to better understand how Tec is involved in intracellular signalling. Using the yeast two-hybrid assay, Actinin-4 was identified as a putative ligand of the Tec PHTH domain. Five independent yeast clones containing Actinin-4 sequence were isolated in the screen. Actinin-4 is a newly identified isoform of non-muscle α -actinin (Honda *et al.*, 1998a). The minimum binding region of Actinin-4 was found to include the third spectrin repeat. The equivalent region in the related non-muscle α -actinin isoform, Actinin-1, has been shown to bind intracellular signalling molecules, such as protein kinase N (Mukai *et al.*, 1997) and CLP-36 (Bauer *et al.*, 2000).

The crystal structure of the dimer formed by the spectrin repeats of Actinin-2 was recently elucidated. The solvent exposed surface of the Actinin-2 rod domain is

predominantly acidic (Djinovic-Carugo *et al.*, 1999, Ylanne *et al.*, 2001). These residues are well conserved amongst the different α -actinin isoforms and are suggested to form a common interaction surface for ligands (Ylanne *et al.*, 2001). Positively charged and hydrophobic residues are common elements in α -actinin-binding proteins (Galliano *et al.*, 2000). In particular, lysine residues have recently been identified as critical binding determinants in two α -actinin ligands: cysteine rich protein and α -1 integrin (Harper *et al.*, 2000, Loster *et al.*, 2001). It is likely that charge based interactions are important for the binding of Tec and Actinin-4, as structural studies have identified charge polarisation in the PH domain of Tec-family kinases (Hyvonen and Saraste, 1997, Baraldi *et al.*, 1999, Okoh and Vihinen, 1999). Furthermore, there are 21 lysine residues (14%) in the PHTH domain bait used in the yeast two-hybrid screen providing plenty of scope for involvement of lysine residues in binding to Actinin-4.

Some flexibility may exist in the intersubunit interactions of Actinin-4 dimers. Both staggered and aligned pairings of spectrin repeats were identified during investigations of their packing in the central rod domain of α -actinin dimers (Taylor and Taylor, 1993, Flood *et al.*, 1997, Winkler *et al.*, 1997, Djinovic-Carugo *et al.*, 1999). The flexibility may affect the residues and surfaces available for Tec PHTH domain binding. Phosphoinositides have been shown to affect the conformation and site-specific interactions of α -actinin. Intramolecular interactions in the C-terminal region of α -actinin inhibit titin-binding and this was relieved by phosphoinositide-binding (Young and Gautel, 2000). Therefore, the binding of Tec to full-length Actinin-4 may depend on a conformational change induced by phosphoinositides that remove steric inhibition or may induce a conformational change in the intersubunit interactions and thereby exert its effect on Actinin-4 function.

The repeat three domain of Actinin-4 contains a HIKE-like motif, which is a candidate PH domain binding site (Alberti, 1998). However, removal of this motif by exchanging the HIK residues for alanines using site-directed mutagenesis did not abrogate PHTH domain binding in the yeast two-hybrid assay. Therefore, this region is not expected to contribute significantly to PHTH domain binding.

3.5.2 Actinin-4 is a Cytoskeleton Structural Protein

α -actinins belong to the spectrin superfamily of actin cross-linking proteins, which includes spectrin, α -actinin, dystrophin and utrophin (Pascual *et al.*, 1997). It is thought that α - and β -spectrin evolved from α -actinin by gene duplication events. Interestingly, β -spectrin

contains a C-terminal PH domain, but it is not known whether this domain interacts with any of the spectrin repeats in α - or β -spectrin tetrameric complexes. Sequence analysis shows that, of all the spectrin repeats in β - and α -spectrin, β -spectrin repeats b1 and b2 are most highly conserved with repeats 1 and 2 of α -actinin, respectively, whereas α -spectrin repeats a20 and a21 are most similar to repeats 3 and 4 of α -actinin, respectively (Pascual *et al.*, 1997). The spectrin repeats are involved in antiparallel dimerisation.

α -actinins bind actin through the calponin homology domain and cross-link F-actin into bundles or networks and, thus, contribute to the structural framework of cells (Djinovic-Carugo *et al.*, 1999). Because they form head to tail homodimers, they have an actin-binding site and two EF hands at each end of the dimer. Calcium regulation has been implicated in F-actin-binding of different α -actinin isoforms (Trave *et al.*, 1995). Binding of non-muscle α -actinin to F-actin is inhibited by calcium concentrations higher than 10^{-7} M, whereas binding of the muscle form is Ca^{2+} insensitive (Burridge and Feramisco, 1981, Landon *et al.*, 1985).

Four α -actinin isoforms are described in the literature. Two of these, Actinin-2 and Actinin-3, are muscle-specific proteins that form part of the contractile machinery in skeletal muscle cells (Beggs *et al.*, 1992). The non-muscle isoforms include Actinin-1 and Actinin-4 and these localise to different subcellular compartments (Honda *et al.*, 1998a). Actinin-1 is localised at stable structures such as focal adhesions, cell contacts and at the end of stress fibres, while Actinin-4 is localised to actively moving regions of cells (Araki *et al.*, 2000).

3.5.3 Actinin-4 Is A Newly Identified Isoform of α -Actinin

A report by Honda *et al.*, 1998a, identified human Actinin-4 as an 884-residue protein. This was revised to include an extra 27 in frame amino acids at the extreme N-terminus that result from use of an earlier translation initiation site (Honda *et al.*, 1998b). The new protein is 911 amino acids in length and has a predicted molecular mass of 105 kDa. This is similar to the report by Nikolopoulos *et al.*, 2000, who identified Actinin-4 as a 913 amino acid protein. Mouse Actinin-4 shares more than 98% sequence identity with human Actinin-4. Of the fourteen differences, there are eight conservative substitutions, four semi-conservative substitutions, one non-conservative difference and one gap. The region encoded by the Tec-binding R817 clone includes the non-conserved residue and two of the conservative differences (A499N, A525T, V660M; residue of human protein listed first). Therefore, mouse and human Actinin-4 proteins, like mouse and human Tec proteins, are highly conserved.

3.5.4 Possible Functions Of Actinin-4

To date, there have been only several reports addressing possible Actinin-4 function. Actinin-4 was found to be concentrated in sharply stretched SW480 cells (colon cancer cell line) and at the leading edge of A-431 cells (human vulvar epidermoid cancer cell line) migrating into an artificial wound (Honda *et al.*, 1998a). Therefore, the subcellular localisation of Actinin-4 in cancer cells was correlated with cell motility. Combined with histological subtyping of breast cancer cells, this was extrapolated to predict the metastatic potential of breast cancer cells. Nuclear translocation of Actinin-4 was identified in several cell lines where PI3K signalling was inhibited by treatment with Wortmannin or when actin polymerisation was inhibited by treatment with Cytochalasin D. Consequently, cytoplasmic Actinin-4 was placed in the PI3K signalling pathway and suggested to regulate the actin cytoskeleton (Honda *et al.*, 1998a). Cytoskeletal organisation was previously shown to be altered by interaction of α -actinin with the phosphatidylinositol signal transduction pathway (Burn *et al.*, 1985, Fukami *et al.*, 1994).

In macrophages, Actinin-4 was identified at surface regions of cells in actively moving structures. F-actin bundling by Actinin-4 was greatest in circular or curved ruffles during M-CSF stimulation and in phagocytic cups during phagocytosis of latex beads (Araki *et al.*, 2000). When these structures closed on top to form macropinosomes and phagosomes, respectively, F-actin levels diminished and, consequently, the ratio of Actinin-4/F-actin was increased as determined by fluorescence ratio imaging analysis (Araki *et al.*, 2000). Actinin-4, unlike Actinin-1, did not localise to podosomes, which are cell-substratum contact sites equivalent to focal adhesion plaque-like structures in non-macrophage cells (Araki *et al.*, 2000). Localisation at the cell membrane in ruffles and phagocytic cups might result from specific intermolecular interactions with proteins that undergo membrane translocation. As described in this thesis, Tec provides one such link to the cell membrane as membrane targeting of Tec-family kinases was previously shown to result from binding of PI 3,4,5-P₃ to the PH domain (Varnai *et al.*, 1999).

Other researchers identified an inverse correlation between growth in soft agar (tumorigenicity) and amount of Actinin-4 protein ectopically expressed in highly tumorigenic neuroblastoma stem cells (Nikolopoulos *et al.*, 2000). Expression of Actinin-4 was also correlated with substrate adhesiveness, which plays a pivotal role in malignant transformation of cells (Nikolopoulos *et al.*, 2000). Therefore, Actinin-4 was described to exhibit tumour suppressor activity.

Heritable genetic mutations in the gene encoding Actinin-4 have been implicated in the kidney disease focal and segmental glomerulosclerosis (Kaplan *et al.*, 2000). The mutations led to increased binding of Actinin-4 to actin filaments. It was postulated that the disease results from altered mechanical characteristics of the glomerular podocyte, which was shown to express high levels of Actinin-4 protein.

3.5.5 Ligands of Non-Muscle α -Actinins

Several ligands of α -actinins have been identified. Actinin-1 associates with several cytoskeletal and membrane associated proteins. The globular N-terminal head of α -actinin contains the actin-binding domain. PI 4,5- P_2 was shown to dramatically increase actin polymerisation by α -actinin (Fukami *et al.*, 1992) and enhance binding of α -actinin with PI3K (Shibasaki *et al.*, 1994) and PKN, a Rho activated serine/threonine protein kinase (Mukai *et al.*, 1997). Spectrin repeat 3 of both skeletal and non-muscle α -actinin were also shown to bind PKN (Mukai *et al.*, 1997). Furthermore, PKN phosphorylated a bacterially expressed N-terminal region of skeletal muscle α -actinin (Mukai *et al.*, 1997).

Focal adhesion kinase (FAK) was shown to bind Actinin-1 and phosphorylate Y12 in the actin-binding domain (Izaguirre *et al.*, 2001). Tyrosine phosphorylation decreased F-actin-binding of Actinin-1. In platelets, this coincided with FAK activation and platelet spreading. Therefore, tyrosine phosphorylation of Actinin-1 by FAK was suggested to affect cytoskeletal organisation (Izaguirre *et al.*, 2001). The Tec-family member Bmx is also a ligand of FAK (Chen *et al.*, 2001). Other signalling molecules that bind to α -actinins include extracellular signal-regulated kinase (Leinweber *et al.*, 1999) and MAPK (Christerson *et al.*, 1999).

Spectrin repeats 2 and 3 in the central rod domain of Actinin-1 were shown to interact with CLP-36, a PDZ-LIM domain protein (Bauer *et al.*, 2000). Actinin-4 was subsequently shown to bind CLP-36 (Vallénus *et al.*, 2000). PDZ-LIM domain proteins are thought to act as adapters between kinases and the cytoskeleton (Guy *et al.*, 1999, Zhou *et al.*, 1999). The rod region also binds the cytoplasmic tail of the NMDA receptor (Wyszynski *et al.*, 1997). The cytoskeletal proteins vinculin (Wachsstock *et al.*, 1987, McGregor *et al.*, 1994), zyxin (Crawford *et al.*, 1992) and palladin (Parast and Otey, 2000) also bind to α -actinins.

The C-terminal tail of non-muscle α -actinin binds to β -subunits of integrins (Otey *et al.*, 1990, Sampath *et al.*, 1998). PI 3,4,5- P_3 , and not PI 4,5- P_2 , disrupts the interaction of Actinin-1 with β -3 integrin (Greenwood *et al.*, 2000). In studies of adhesion formation and

disassembly in migrating cells, adhesive components including α -5 integrin, paxillin and Actinin-1 were shown to be serially recruited into adhesion complexes but not serially removed; instead, the complexes were seen to disperse (Laukaitis *et al.*, 2001). Other adhesion receptors that bind to α -actinins include cadherins (Knudsen *et al.*, 1995, Nieset *et al.*, 1997), ICAMS (Carpen *et al.*, 1992, Heiska *et al.*, 1996) and Tir, a bacterial secreted protein used in infection of enteropathogenic *E. coli* (Goosney *et al.*, 2000).

3.5.6 Cytoskeletal Reorganisation Downstream of Tec Signalling

The actin cytoskeleton is a complex protein network that provides cellular structure. It is fundamental for cellular dynamics such as shape change and migration. Rapid cytoskeletal reorganisation is seen upon activation of numerous cell surface receptors. A prime example involves particle engulfment by macrophage cells during Fc- γ R-mediated phagocytosis. Atmosukarto identified a novel role for Tec during Fc- γ R-mediated phagocytosis by macrophage cells (Atmosukarto, 2001). Tec was shown to relocate to the phagosome like Syk kinase, a known effector of phagocytosis. Furthermore, Tec was postulated to play a role in the sustained phosphorylation and signalling of PLC- γ , akin to the function of Btk in B cell receptor signalling that involves extracellular calcium influx (Fluckiger *et al.*, 1998, Perez-Villar and Kanner, 1999). Studies with inhibitors led to the suggestion that Tec acts as a link between PI3K activation and actin cytoskeleton rearrangement (Atmosukarto, 2001). These studies are consistent with a cytoskeletal assembly role previously demonstrated for the Tec-family member Btk (Yao *et al.*, 1999).

Laffargue *et al.*, 1997, identified translocation of Tec to the cytoskeleton of activated platelets and suggested that Tec participates in platelet signalling downstream of integrin activation. The interaction of the PH domain of Bmx with the FERM domain of FAK was also associated with integrin signalling as well as migration of metastatic carcinoma cells (Chen *et al.*, 2001). α -actinin has also been implicated in cytoskeletal reorganisation in activated platelets during platelet function (Takubo *et al.*, 1998).

An emerging theme of intracellular signalling involves protein targeting, rather than diffusion, in the assembly of multimolecular protein complexes in which signalling domains are nucleated by scaffolding proteins. Non-muscle α -actinin has been postulated to act as one such scaffold protein (Izaguirre *et al.*, 2001). Signalling molecules may be recruited upon ligand-dependent conformational change that exposes new ligand-binding sites. For example, PI 3,4,5-P₃ ligand interaction with the Btk PH domain is thought to induce a conformational

change that endorses further interactions with the other domains after promoting the release of (multiple low affinity) inhibitory intramolecular interactions (Saito *et al.*, 2001).

Common elements exist in signalling pathways that involve Tec-family kinases and α -actinins. The function of these two classes of proteins is closely linked with phosphoinositide and calcium signalling. In particular, PI3K subunits and products have been identified as ligands of non-muscle α -actinins. Furthermore, both classes of protein directly bind F-actin and have been implicated in cytoskeletal remodelling. Actinin-4 is, therefore, suitably identified as a novel ligand of the Tec PTH domain. This consolidates the link between signalling of Tec-family kinases and intracellular actin dynamics. Further characterisation of the interaction between these proteins is necessary to confirm the interaction in a cellular context and identify its functional significance and this is discussed in the following chapter.

CHAPTER

4

Tec And Actinin-4 In Mammalian Cells

4.1 Introduction

Interaction of signalling molecules with their upstream activators and downstream effectors is dependent on interactions mediated by their constituent protein domains. To avoid aberrant signalling, enzyme activation is a tightly regulated stepwise process, involving intermolecular interactions with other proteins and factors as well as intramolecular interactions. Cell activation by growth factors and antigens frequently results in rapid rearrangement of cytoplasmic networks. Importantly, the cytoskeleton, which endows cells with structure, order and shape, has dynamic properties (Machesky and Schliwa, 2000). The signalling pathways involved in actin cytoskeleton restructuring were recently shown to include PI3Ks and small molecular weight GTPases of the Rho-family - many of the known regulatory proteins for the latter contain one or two PH domains (Machesky and Hall, 1997, Jimenez *et al.*, 2000). These and other common elements exist in the signalling pathways independently identified for non-muscle α -actinins and Tec-family kinases.

Tec-family proteins have recently been implicated in direct association with cytoskeletal elements through PH domains. Btk was shown to bind F-actin through a stretch of amino acids, rich in basic groups, near the N-terminus of the PH domain (Yao *et al.*, 1999); these residues are conserved in Tec. The PH domain of Bmx was shown to bind FAK and this interaction has an essential role in integrin signalling and cell migration (Chen *et al.*, 2001).

α -actinins bind F-actin through calponin homology domains and cross-link actin filaments to provide rigidity to localised regions of cells. The different isoforms are associated with different subcellular structures and therefore could be regulated by different signals. Actinin-1, but not Actinin-4, is concentrated in focal adhesion structures (Honda *et al.*, 1998a, Araki *et al.*, 2000). Actinin-2 and Actinin-3 are muscle α -actinins and localise to the sarcomere of the Z-disk of skeletal muscle cells (Beggs *et al.*, 1992). Actinin-4 preferentially localises to actively moving structures such as dorsal ruffles of macrophages (Araki *et al.*, 2000). Actinin-4 has been proposed to provide a constructive brace to maintain recently formed structures such as early macropinosomes, which are created by tip closure of circular cell surface ruffles, and phagocytic cups during phagocytosis of latex beads (Araki *et al.*, 2000). Although direct involvement in contraction of these structures and, therefore, force generating machinery has not been demonstrated, the possibility has not been ruled out.

Actinin-4 is a 105 kDa protein that exists as a dimer through the pairing of spectrin repeats that make up the central rod domain. This domain binds the PDZ domain of CLP-36, a PDZ-LIM domain family protein (Vallénus *et al.*, 2000). Actinin-1 also binds CLP-36

despite Actinin-1 and Actinin-4 having different subcellular localisations. Actinin-1 is concentrated in stress fibres and focal contacts whereas Actinin-4 is localised to actively moving regions of cells and has been linked to the metastatic potential of cancer cells (Honda *et al.*, 1998a, Araki *et al.*, 2000, Nikolopoulos *et al.*, 2000).

The presence of EF hands, which bind calcium, and calponin homology domains, which bind actin and phospholipids, suggests that the activity of α -actinins could be commonly regulated by calcium and phosphoinositides (Honda *et al.*, 1998a, Djinovic-Carugo *et al.*, 1999, Matsudaira, 1991). Tec-family proteins have similarly been implicated in calcium and phosphoinositide signalling. The PH domain provides a reversible membrane anchor through binding to phosphoinositide products of PI3K. Furthermore, Btk has been implicated in the sustained calcium response during BCR signalling through phosphorylation and activation of PLC- γ (Fluckiger *et al.*, 1998). Tec has recently been implicated in PLC- γ activation in cardiac cells (Bony *et al.*, 2001). Inositol 1,4,5-P₃ is produced from PIP₃ by activated PLC- γ and triggers the release of calcium from endoplasmic reticulum stores. The amplitude and duration of calcium signalling is dependent on the intensity of the BCR stimulus (Healy *et al.*, 1997). In Tec transfected T cells, Tec appears to regulate nuclear import of the transcription factor nuclear factor of activated T cells (NF-AT) and influences cytoplasmic free calcium increase and, therefore, Tec is also critically involved in transcriptional gene regulation (Yang *et al.*, 2000).

Both Tec and Actinin-4 are expressed in many cell types. Although transcripts encoding five Tec isoforms have been described, only two of these were identified in a range of mouse tissues (Merkel *et al.*, 1999). Other researchers have not distinguished expression of the different isoforms. Tec3 and Tec4 transcripts arise by alternative splicing of the 66 bp exon 8. The 72 kDa Tec4 protein has similar primary sequence to other members of the Tec-family. In contrast, Tec3 protein lacks the C-terminal 22 amino acids of the SH3 domain. This deletion is expected to disrupt proper folding and function of the SH3 domain and, therefore, regulation of the kinase activity. Specifically, Tec3 is thought to be intrinsically more active than Tec4 due to lack of intramolecular contacts that contribute to a closed inactive conformation of the kinase domain. It is not clear whether Tec3 exists in cells as Tec3-specific antibodies are not available for Western Blot analysis. Tec3 is expected to migrate further than Tec4 during sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as it has a lower predicted molecular weight. However, increased

phosphorylation may exist for Tec3 compared with Tec4, as a result of higher intrinsic activity, and thus make it difficult to resolve the two proteins by SDS-PAGE.

To date, phosphorylation of Actinin-4 has not been tested, however, tyrosine phosphorylation of Actinin-1 has been detected in activated platelets (Izaguirre *et al.*, 1999), specifically, in the soluble fraction of PMA-stimulated-platelet lysates. The N-terminal region of skeletal muscle α -actinin has been identified as a substrate of protein kinase N, a serine/threonine kinase that also binds the central rod domain of Actinin-1 (Mukai *et al.*, 1997). Furthermore, Izaguirre *et al.*, 2001, recently reported phosphorylation of Y12 in the actin-binding domain of Actinin-1 by FAK. They found that tyrosine phosphorylation reduced the amount of α -actinin that co-sedimented with actin filaments (Izaguirre *et al.*, 2001). Therefore, the association of α -actinins with the actin cytoskeleton may be regulated by tyrosine and/or serine/threonine kinases. There is a possibility that Tec phosphorylates Actinin-4 and contributes to the regulation of its function. But first it needs to be established that Tec and Actinin-4 interact in living cells.

4.2 Aims

The aim of the work described in this chapter was to verify the Tec:Actinin-4 interaction in mammalian cells and to demonstrate a functional significance for this interaction.

4.3 Approaches

Several approaches were used to investigate the interaction of Tec and Actinin-4 in mammalian cells. Indirect immunofluorescence microscopy was used to identify the subcellular localisation of endogenous Tec and Actinin-4 proteins and sites where the two proteins colocalise. The interaction of endogenous Tec and Actinin-4 in mammalian cells was assessed by co-immunoprecipitation (co-IP) and Western blot analysis using Tec- and Actinin-4-specific antibodies.

These techniques are dependent on highly specific antibodies. Santa Cruz provides a commercially available Tec antibody raised in goat, sc-1109, that is suitable for immunofluorescence studies and Western blot analysis. Actinin-4 antibodies have been produced and extensively characterised by Honda and co-workers (Honda *et al.*, 1998a). The polyclonal antibody raised in rabbit is suitable for Western blot and IP. The monoclonal antibody NCC-Lu632, which has IgM subtype, is suitable for indirect immunofluorescence

but not for IP or Western blot (Dr T Yamada, personal communication). Actinin-4 antibodies are not commercially available but samples can be obtained from the lab of Dr S Hirohashi (Honda *et al.*, 1998a).

In a second approach, a binding assay was established to analyse the effect of specific mutations of Tec on Actinin-4-binding. Epitope-tagged Tec and Actinin-4 proteins were expressed in mammalian cells and subjected to co-IP and Western blot analysis using available epitope-tag-specific antibodies. Specific amino acid substitutions were introduced into Tec proteins by site-directed mutagenesis (Stratagene, see Section 3.3.7) of the corresponding cDNAs. This second approach used Myc epitope-tag fused to Actinin-4 and enhanced green fluorescent protein (EGFP) fused to Tec. The latter enabled direct visualisation of the different Tec proteins and mutants in living cells. EGFP has proven to be a powerful new tool in investigating biomolecular signals contained with protein domains that influence subcellular distribution.

The design of the Tec mutants was based on Btk and kinase activity mutants described in the literature. In Btk, K17, K18 and K19 are critical F-actin-binding determinants and substitution for alanines severely reduces actin binding (Yao *et al.*, 1999). By analogy, it is expected that K18, K19 and K20 in Tec mediate F-actin-binding and that this would be prevented by substitution to alanines: K18A/K19A/K20A. A second mutant, R29C, was designed to mimic the R28C Btk mutant, which affects phospholipid head group binding to the Btk PH domain and causes XLA in humans and Xid in mice (Rawlings *et al.*, 1993, Thomas *et al.*, 1993, Baraldi *et al.*, 1999). The third mutant, Y187E, was designed to cause constitutive activation of Tec kinase activity and therefore this mutant was expected to have an open conformation. The equivalent residue in Btk, Y223, is autophosphorylated upon activation of Btk, in an event that completes the activation of Btk kinase activity (Park *et al.*, 1996, Rawlings *et al.*, 1996). In the kinase domain, mutation of the predicted ATP-binding residue K397 to glutamate (K397E) was expected to block autophosphorylation and produce a dominant negative form of the kinase, as does Y551F in Btk (Saito *et al.*, 2001), and have closed conformation. The open and closed conformations of Tec kinase are expected to each have a different repertoire of ligands, depending on which residues are exposed and available for interaction.

More recently, phagocytosis experiments were commenced to demonstrate a functional significance for the Tec:Actinin-4 interaction, however, time constraints did not permit further investigation into the role of the interaction during phagocytosis.

4.4 Results

4.4.1 Endogenous Tec And Actinin-4 Colocalise In Mammalian Cells

Several mammalian cell lines were analysed for the presence of Tec and Actinin-4 proteins in order to demonstrate sites of potential interaction in mammalian cells. Initially, the HepG2 (Aden *et al.*, 1979), MCF-7 (Soule *et al.*, 1973) and COS-1 (Gluzman, 1981) adherent cell lines were analysed. The human liver cancer cell line HepG2 was chosen because Tec was first identified in liver and, as described in Chapter 3, a liver cDNA library was used in the yeast two-hybrid screen. The human breast cancer cell line MCF-7 was chosen because it was previously shown to contain Actinin-4. The monkey kidney fibroblast cell line COS-1 was chosen because it was planned for use as a host in later transient transfection experiments and it was therefore necessary to establish whether it contained endogenous Tec and Actinin-4. In later experiments, the haematopoietic cell lines Dami and U937 were analysed for Tec and Actinin-4 expression; Dami is a human megakaryocytic cell line, U937 is a promonocytic cell line. These cells were planned for use in phagocytosis experiments.

Immunofluorescent staining analysis of mammalian cell lines was used to investigate the intracellular localisation of endogenous Tec and Actinin-4 proteins. The MCF-7, HepG2 and COS-1 cell lines were analysed since it was established by Western Blot in parallel experiments that they contained the desired proteins (see Section 4.4.3). Cells were grown adherently on glass coverslips before methanol fixing, permeabilising and immunostaining. Twenty-four hours after plating, adherent cells had attained a flattened morphology. In general, all three cell types had similar morphology with the nucleus comprising less than half of the cell. The extensive cytoplasm made these cells ideal for studies of Tec and Actinin-4 distribution. In contrast, the Dami and U937 cells grow in suspension and, like other haematopoietic cells, are typically round with very little cytoplasm. Although they can be induced to adhere to tissue culture plastic with poly-L-lysine or cellular fibronectin, these cells are quite small (approximately the same size as a COS-1 cell nucleus) and were not used in the early colocalisation experiments due to their size and limited amount of cytoplasm.

Using the sc-1109 and NCC-Lu632 antibodies, immunofluorescent staining studies were carried out in COS-1, MCF-7 and HepG2 cells. At first, FITC conjugated secondary antibodies were used to visualise Tec or Actinin-4 in different samples. Remarkably, the staining pattern of the two antibodies was indistinguishable. Negative control samples stained without primary antibody gave no detectable signal when the same image exposure times were used, indicating that the staining was dependent on the primary antibodies. As shown in

Figure 4.1, Tec and Actinin-4 localised throughout the cytoplasm of flattened cells in fine filamentous, web-like structures. Points where filaments overlap appear as localised regions of increased staining and in captured images often look like spots. The ultra fine structures were most obvious when looking down the microscope with the naked eye, but were difficult to capture on film or digitally where the brighter regions often masked them. Small amounts of Tec and Actinin-4 were detected in spots in the nucleus, however, staining was most intense in the perinuclear region where the cells were most voluminous. Tec staining in this region in J774 macrophage cells was concomitantly shown to be dispersed by addition of BrefeldinA (a golgi-disrupting compound) to the culture medium (Atmosukarto, 2001).

In order to show colocalisation, it was necessary to co-stain cells for Tec and Actinin-4 using different secondary antibodies. For these experiments, Cy3 conjugated secondary antibody was used to visualise Tec (Figure 4.1, red panels). When comparing the staining pattern of Tec visualised with the two different secondary antibodies, the Cy3 conjugated antibody gave a more spotty appearance than the FITC conjugated antibody (data not shown). This was most likely due to precipitation of the secondary antibody and was reduced somewhat by centrifugation of the antibody before use in staining. As shown in Figure 4.1A, B and C, fine cytoplasmic structures and intense perinuclear staining were detected when co-staining COS-1, MCF-7 and HepG2 cells with Tec (shown in red) and Actinin-4 (shown in green) antibodies, respectively. Overlaying of the Tec and Actinin-4 images demonstrated substantial colocalisation (shown in yellow) in adherent cells, ignoring spots in the Tec staining attributed to the precipitated antibody (Figure 4.1A, B and C merge panels). Corresponding cell nuclei were identified by staining cells with Hoechst 33258 (shown in blue) (Figure 4.1). Negative control experiments, in which cells were stained with either one or the other primary antibody and then both secondary antibodies, were performed in parallel. Results from the controls indicated no cross-reaction between antibodies.

In these cells, Tec and Actinin-4 were extensively colocalised throughout the cytoplasm and their subcellular distribution pattern suggests a role for them associated with common cell architecture elements.

4.4.2 Endogenous and Epitope-Labelled Tec and Actinin-4 Have Varying Degrees of Solubility

IP experiments were used to demonstrate the interaction of Tec and Actinin-4 in mammalian cells. For IP experiments, pools of soluble Tec and Actinin-4 were required.

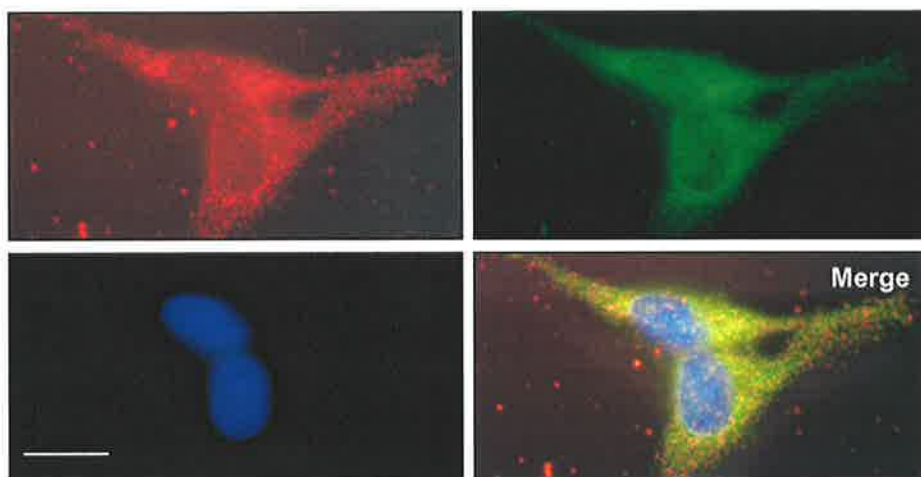
Figure 4.1 Colocalisation of Tec and Actinin-4 in Adherent Cells

Mammalian cells were grown adherently on 22mm² coverslips before fixation with methanol and immunofluorescent staining. Samples were co-stained for Tec and Actinin-4 proteins, which were detected with Cy3- and FITC-conjugated secondary antibodies, respectively. Cell nuclei were stained with Hoechst 33258. Cy3 images are shown in red in the upper left panels, FITC images are shown in green in the upper right panels and Hoechst 33258 images are shown in blue in the lower left panels. Composite images constructed by overlaying the Cy3, FITC and Hoechst 33258 images are shown in the bottom right panels (merge), where colocalisation of Tec and Actinin-4 is shown in yellow. The following mammalian cells were analysed:

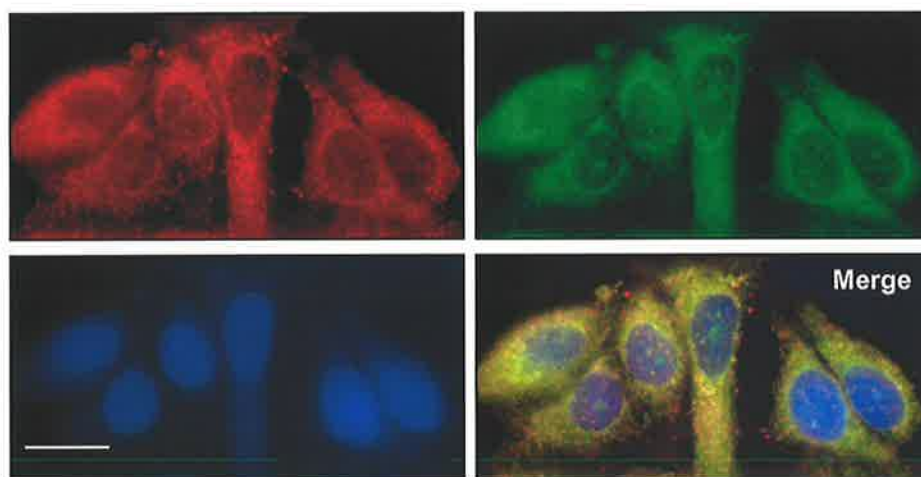
- A COS-1 monkey kidney fibroblast cells (Gluzman, 1981).
- B MCF-7 human breast adenocarcinoma cells (Soule *et al.*, 1973).
- C HepG2 human liver hepatocellular carcinoma cells (Aden *et al.*, 1979).

Primary antibodies were used at 1/1000 and 1/2 dilutions for anti-Tec and anti-Actinin-4, respectively, while corresponding secondary antibodies were used at 1/1000 dilution for anti-goat-Cy3 and 1/200 dilution for anti-mouse-IgM-FITC and anti-goat-FITC. Fluorescent images were captured using an Olympus Provus AX70 microscope (with filter sets: U-MNIBA for FITC; U-MWIG for TRITC/Cy3; and U-MWU for Hoechst 33258) attached to a Photometrix Cool Snap FX camera was used with V++ software.

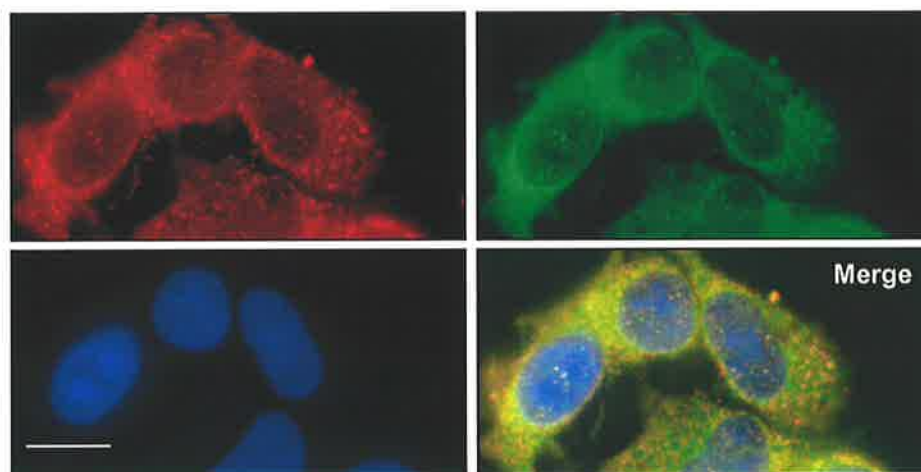
A



B



C



Therefore, the presence of soluble Tec and Actinin-4 proteins in mammalian cells was investigated.

4.4.3 Endogenous Tec and Actinin-4 Both Exist in Soluble and Insoluble Fractions of Mammalian Cell Lysates

MCF-7, HepG2 and COS-1 cells were grown on tissue culture plastic and lysed with cytoskeletal lysis buffer (Section 2.1.4) as described in Section 2.3.4.4. The Triton-X100 soluble cytoplasmic fraction was separated from the Triton-X100 insoluble cytoskeletal rich pellet by centrifugation. Samples were resolved by polyacrylamide gel electrophoresis and probed for Tec (72 kDa) using the sc-1109 polyclonal antibody. As seen in Figure 4.2A, the majority of Tec in these cells is present in the supernatant. The same membrane was then probed for Actinin-4 (105 kDa) using the anti-Actinin-4 polyclonal antibody. As seen in Figure 4.2B, Actinin-4 is present in both the supernatant and pellet fractions. It is clear from other Western blots that a fraction of the cellular pool of Tec partitions in the pellet (Figure 4.2C). Interestingly, both Tec and Actinin-4 in the pellet fraction have slightly larger size that may be due to post-translational modification.

Western blot of U937 and Dami whole cell lysates and supernatants showed these cells also contain Tec and Actinin-4 proteins (Figure 4.1C and data not shown). Therefore, all five cell lines tested contain a pool of endogenous Tec and Actinin-4 proteins that are solubilized by cytoskeletal lysis buffer and suitable for IP experiments.

4.4.4 Solubility of Epitope-Tagged Tec and Actinin-4 in COS-1 Cells

In order to test the involvement of specific residues of Actinin-4 and Tec in binding, as well as the activation state of Tec on Actinin-4-binding, a system for expression of wildtype and variants of Tec and Actinin-4 was prepared. The solubility of these proteins in mammalian cells was investigated prior to use in IP reactions.

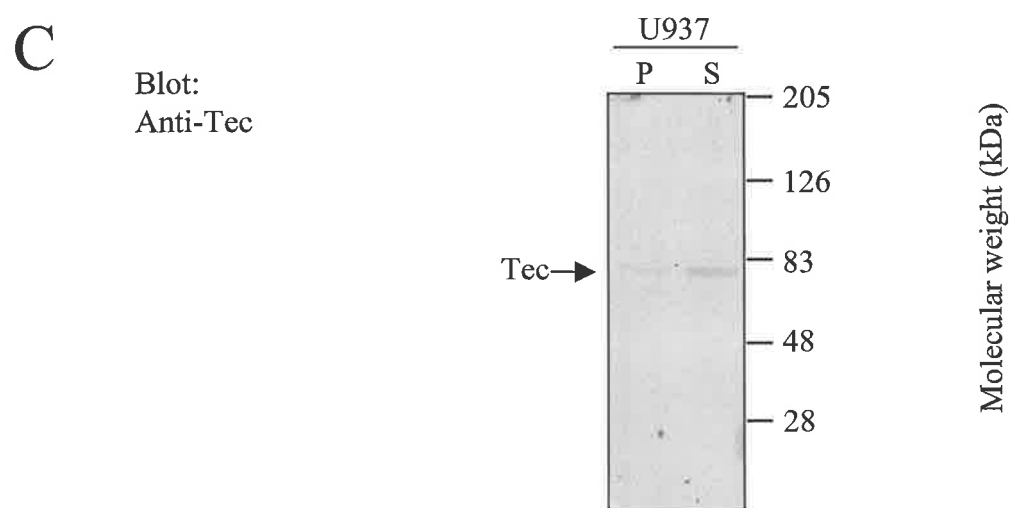
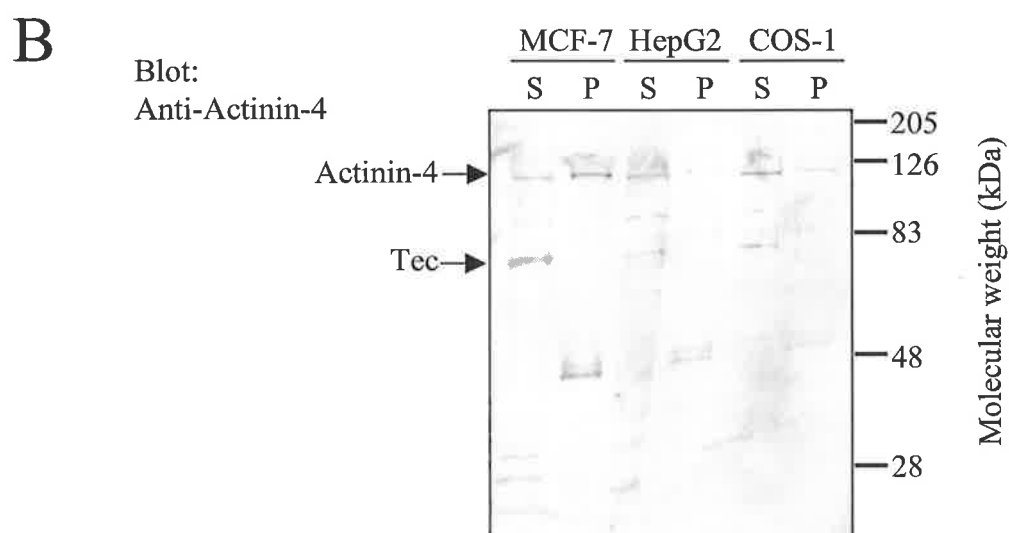
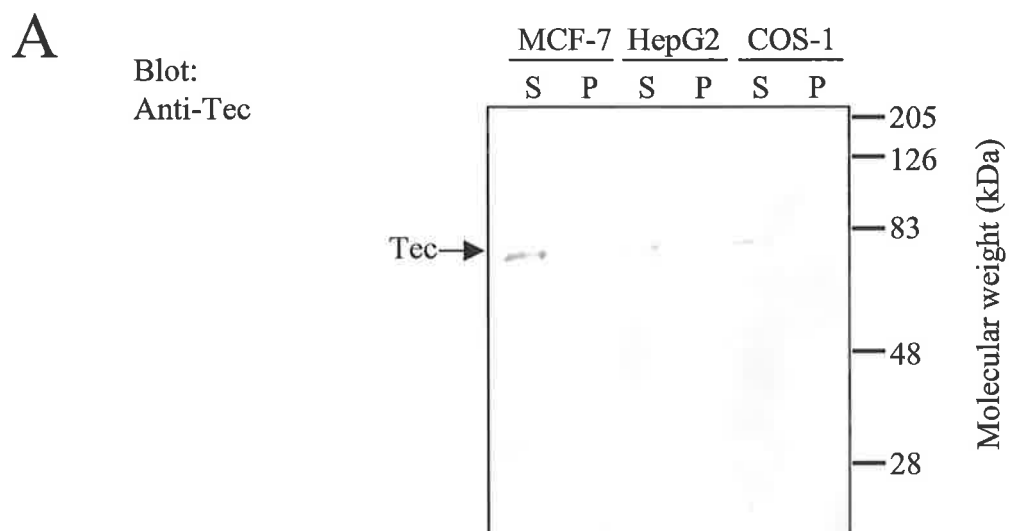
Transient transfection of vectors encoding epitope-tagged proteins into mammalian cells is part of a well-established system for showing interaction of proteins (Zhang *et al.*, 2001). Antibodies that bind to the epitope-tag can be used to identify and immunoprecipitate the epitope-tagged protein of interest, without requirement for antibodies specific for the protein of interest. This system also enables expression of epitope-tagged protein with altered amino acid sequence, which can be useful for studying specific residues involved in interaction with other factors without using homologous recombination or gene replacement.

Figure 4.2 Western Blot of Endogenous Tec and Actinin-4 Separated into Triton-X100 Soluble and Insoluble Fractions

MCF-7, HepG2 and COS-1 cells were grown adherently on 10cm tissue culture dishes before lysis with cytoskeletal lysis buffer. U937 cells were grown in suspension in a T75cm² flask before lysis with cytoskeletal lysis buffer. Supernatant (S) and pellet (P) fractions obtained by centrifugation were run on (A) 12.5% or (C) 8% Tris-tricine gels which were transferred to nitrocellulose and probed by Western blot.

- A Samples probed with goat-anti-Tec primary antibody and sheep-anti-goat-AP secondary antibody.
- B The same blot as in (A) was blocked and probed a second time using rabbit-anti-Actinin-4 primary antibody and goat-anti-rabbit-AP secondary antibody. Since the blot in (A) was developed using the alkaline phosphatase method, which produces a coloured precipitate, it was not stripped before re-probing.
- C Samples probed with goat-anti-Tec primary antibody and rabbit-anti-goat-HRP secondary antibody.

Primary antibodies were used at 1/5,000 and 1/9,000 dilution for anti-Tec and anti-Actinin-4, respectively, while AP-conjugated secondary antibodies were used at 1/10,000 dilution and HRP-conjugated secondary antibodies were used at 1/5,000 dilution. Alkaline phosphatase (AP) conjugated secondary antibodies were developed with NBT/BCIP. Horseradish peroxidase (HRP) secondary antibody was developed with enhanced chemiluminescence and exposed to X-ray film. Sigma Colour Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.



4.4.5 Differing Solubilities Of Various Tec Proteins Expressed As C-Terminal Fusions Of EGFP

During these studies, fusion proteins consisting of Tec and EGFP (EGFP Excitation maximum = 488 nm; emission maximum = 507 nm; CLONTECH) were created. EGFP fusion proteins have fluorescent properties similar to the native *Aequorea victoria* jellyfish GFP protein from which they were derived that allows visualisation of fusion protein localisation. The EGFP tag was used for two reasons. Firstly, it enabled examination of the intracellular distribution of Tec isoforms Tec3 and Tec4, the Actinin-4-binding PHTH domain (pleckstrin homology domain and Btk motif of the Tec homology domain) as well as variants of these proteins that contained engineered amino acid substitutions. Secondly, the rabbit anti-GFP antibody efficiently immunoprecipitates soluble EGFP-tagged proteins.

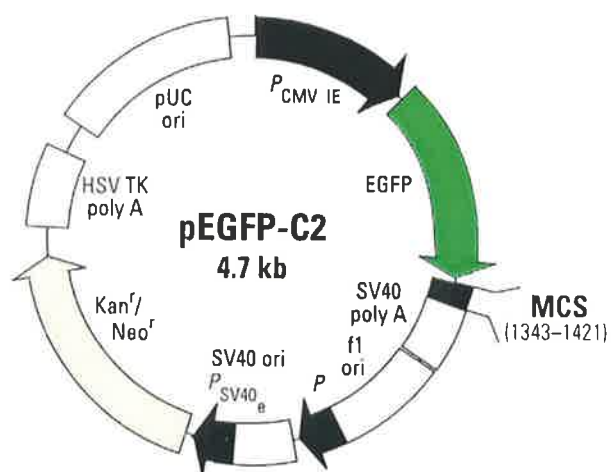
Epitope-tagged clones were constructed as described in Figure 4.3 and transiently transfected into COS-1, MCF-7 and HepG2 cells. These fusion proteins contain the EGFP moiety at the N-terminus and are expressed from the pEGFP-C2 vector. Expression is driven from the strong constitutive cytomegalovirus immediate early (CMV IE) promoter (Figure 4.3A). All transfections were transient to avoid creation and analysis of non-representative stably transfected clones. The transfection efficiency was very low in MCF-7 and HepG2 cells and therefore those cells were not used in further transfection experiments. The COS-1 cell line is widely used as a model system for transient transfection experiments. The transfected COS-1 cells were harvested 24 h after transfection, and lysed by a variety of lysis buffers or sonication in a number of different experiments. Western blot analysis of cells lysed in cytoskeletal lysis buffer shown in Figure 4.4A shows that EGFP and EGFP-PHTH were expressed at higher levels than EGFP-Tec3 and EGFP-Tec4. Transfection or expression of the larger fusion proteins (approximately 100 kDa) was less efficient than that of the smaller proteins (27 kDa and 46 kDa) and in Tec3 samples this was exacerbated by poor growth of transfected cells, many of which rounded up and lifted off the tissue culture plastic.

When whole cell lysates were separated into supernatant and pellet fractions, the majority of EGFP-Tec3 and EGFP-Tec4 partitioned in the insoluble pellet fraction (Figure 4.4B and C). After stripping and re-probing both of these immunoblots in parallel, the insoluble EGFP-Tec4 and EGFP-Tec3 proteins were found to be phosphorylated (Figure 4.4D) whereas the soluble fractions contained negligible phosphorylated EGFP-Tec (data not shown). Both Western blots were treated in an identical manner and, in particular were concurrently exposed side by side to the same piece of XRAY film. The lack of other

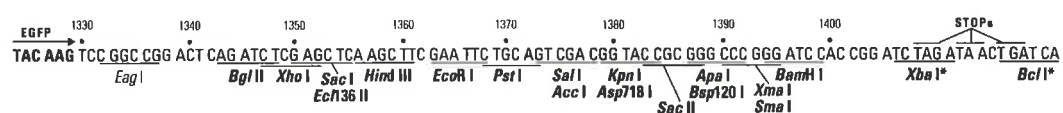
Figure 4.3 Cloning of EGFP-Tec

- A Vector map of the plasmid pEGFP-C2 that expresses the enhanced green fluorescent protein (EGFP) from the strong, constitutive cytomegalovirus immediate early promoter (pCMV IE) and has the C2 variant of the multiple cloning site (Clontech). pEGFP-C2 was kindly provided by Michael Lees (Dr Murray Whitelaw laboratory).
- B Sequence and restriction map of the multiple cloning site of the C2 variant of pEGFP plasmid (Clontech).
- C Diagrammatic representation of the PHTH domain encoding sequence that was cloned in frame into the *EcoRI* restriction site of pEGFP-C2 and the Tec4 encoding sequence that was cloned in frame into the *EcoRI* and *SmaI* (blunt) restriction sites of pEGFP-C2. A partial restriction map is indicated. PHTH cDNA was excised from pLexA-PHTH using *EcoRI*. Tec4 was excised from pBluescriptSK+TecIV using *EcoRI* and *Ecl136II* (blunt) restriction enzymes. *Ecl136II* is an isoschizomer of *SacI* and cuts the cDNA in the 3' untranslated region 134 bp downstream from the stop codon. Tec3 differs from Tec4 due to lack of the 66 bp exon8 that is contained within the *AccI/PstI* restriction fragment. pEGFP-C2-Tec3 was cloned by replacing the 343 bp *AccI/PstI* fragment in pEGFP-C2-Tec4 with the 277 bp *AccI/PstI* fragment from pGEX2T-TecIII. The orientation and sequence of the insert in each clone was confirmed by automated DNA sequencing.
- D Linear representation of the EGFP protein and the EGFP-Tec fusion proteins used in this study. In each case, the EGFP tag is at the N terminus of the protein. Predicted molecular weight was calculated using pI/MW software and is indicated for each fusion protein.

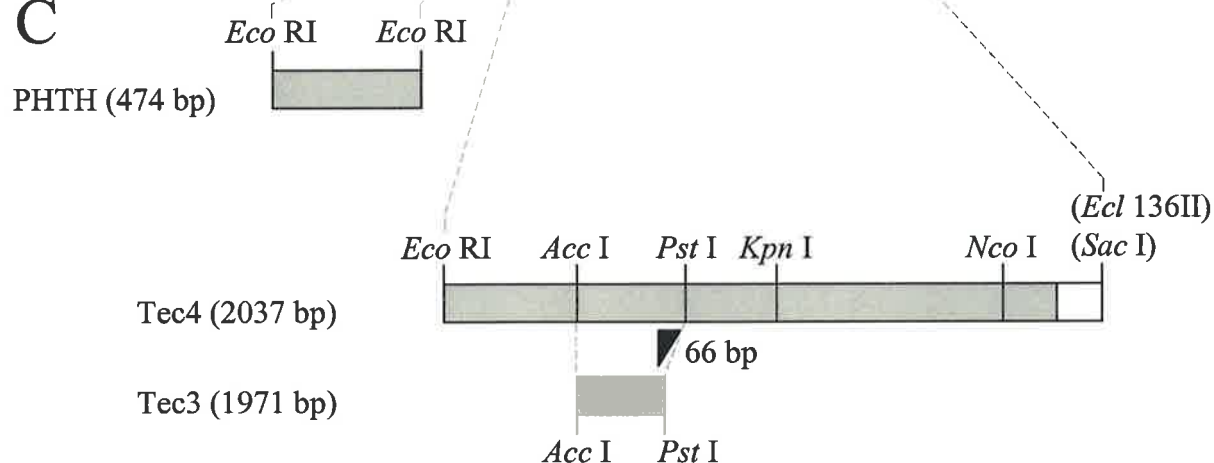
A



B



C



D

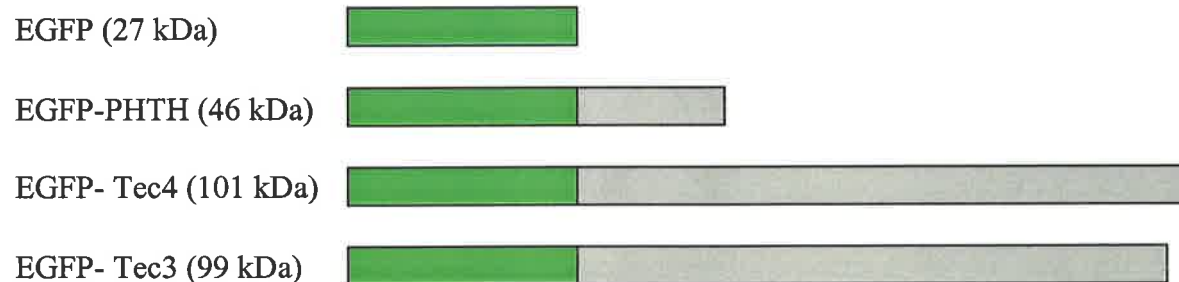


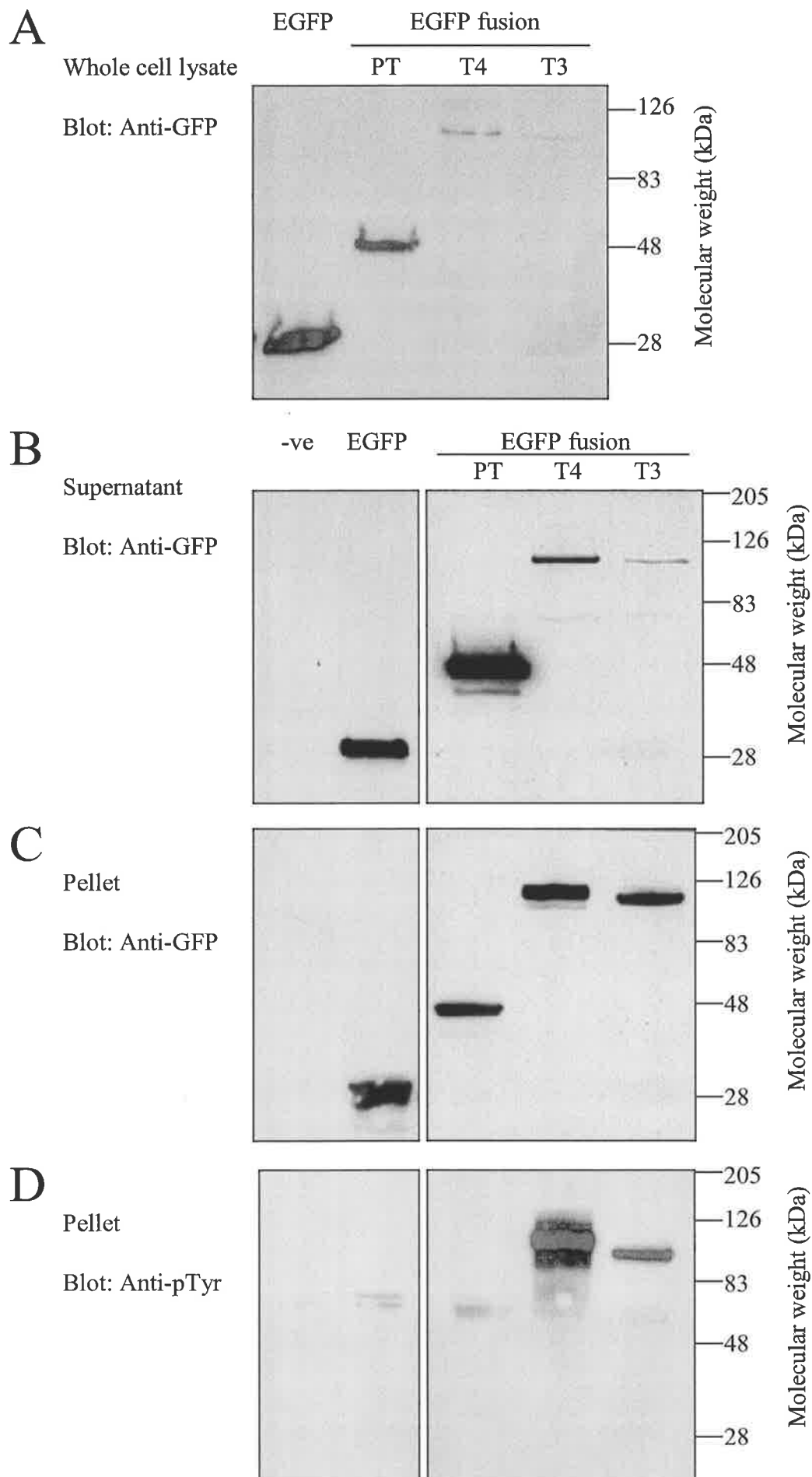
Figure 4.4 Western Blot of COS-1 Cells Transiently Transfected With EGFP-Tec Plasmids

COS-1 cells were grown adherently on 10 cm tissue culture dishes and transfected with pEGFP-C2 control or pEGFP-C2-Tec plasmid using the LF2000 reagent. Transfected and untransfected control (-ve) cells were lysed with cytoskeletal lysis buffer twenty-four hours post transfection. Aliquots of whole cell lysate were set aside. From the remainder, supernatant (S) and pellet (P) fractions were obtained by centrifugation. Samples were run on 10% Tris-tricine gels which were transferred to Hybond-C and probed by Western blot with rabbit-anti-GFP and goat-anti-rabbit-HRP antibodies and visualised using ECL.

- A Whole cell lysate samples.
- B Supernatant fractions.
- C Pellet fractions.
- D The same blot as in part (C) was stripped and probed a second time using mouse-anti-phosphotyrosine primary antibody and sheep-anti-mouse-HRP secondary antibody.

Primary antibodies were used at 1/6,000 and 1/4,000 dilution for anti-GFP and anti-phosphotyrosine, respectively, while secondary antibodies were used at 1/3,000. Sigma Colour Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

EGFP:	enhanced green fluorescent protein.
PT:	PHTH domain
T3:	Tec3
T4:	Tec4



detectable tyrosine phosphorylated proteins in these Western blots was most likely due to the short exposure time that was required to visualise the overexpressed and highly phosphorylated Tec4- and Tec3-containing bands.

The insoluble Tec4 fusion protein was remarkably hyperphosphorylated (Figure 4.4D). This observation is in contrast to a previous report that EGFP-Tec3 is more highly phosphorylated than EGFP-Tec4 (Atmosukarto, 2001). EGFP alone and EGFP-PHTH present in the pellet fractions were not phosphorylated (Figure 4.4D). The detection of these proteins in the pellet fraction could result from the presence of a small amount of residual supernatant upon centrifugation and separation of the fractions, combined with the relatively high expression level of these fusion proteins compared with the EGFP-Tec3 and EGFP-Tec4 fusion proteins.

4.4.6 Mouse and Human Tec PHTH Domain and Mouse Tec4 and Tec3 Were Expressed in COS-1 Cells as N-Terminal Fusions of EGFP

When creating EGFP fusion proteins there is a possibility that the EGFP moiety affects the function and subcellular localisation of the attached protein. Therefore, it is commonplace to produce separate fusion proteins where the protein of interest is attached to the N-terminus or C-terminus of EGFP. Since the PH domain resides at the extreme N-terminus of the native Tec protein, it was important to create and test Actinin-4-binding of Tec-EGFP having N-terminal PHTH domain.

To investigate the positional effect of the EGFP fusion partner relative to the Tec PHTH domain or Tec4 and Tec3 proteins, in binding to Actinin-4, Tec-EGFP fusions were created that contained the EGFP moiety at the C-terminus. The subcloning of these constructs is described in Figure 4.5. The Tec-EGFP fusion proteins were expressed at similar levels as their EGFP-Tec counterparts, respectively, and had similar solubility when whole cell extracts and supernatant and pellet fractions were analysed by Western blot (data not shown).

The Actinin-4 binding ability of human PHTH (hPHTH) domain compared to that of mouse PHTH (mPHTH) domain was also investigated. As described in Section 3.4.3, these proteins have 95.4% identity (see Figure 3.5). Construction of hPHTH-EGFP and mPHTH-EGFP is described in Figure 4.5. Oligonucleotide primers that bind mouse and human Tec cDNAs were designed and used in PCR reactions during cloning into the pEGFP-N2 vector. Human Tec cDNA obtained from Dr H Mano (Japan) was used as a template in PCR reactions to clone hPHTH domain. The lower primer used in the PCR

Figure 4.5 Cloning of Tec-EGFP

- A Features engineered into Tec cDNAs using PCR primer pairs #178 and #179 (for human PHTH), #253 and #179 (for mouse PHTH) or #253 and #254 (for Tec4) are indicated (see Section 2.1.22). The sequences of the upper primers are listed showing the *Eco*RI restriction site used for cloning into pEGFP-N2, the Kozak consensus sequence for translation initiation and the reading frame. The expected translation start methionine residue and following amino acids are written in single letter code below the appropriate codon. The complement of the lower primer sequences, showing the *Bam*HI restriction site used for cloning into pEGFP-N2 and the reading frame are listed above the amino acid sequence. The last six residues of PHTH domain are shown followed by a linker between PHTH and EGFP that contains a consensus thrombin cleavage site. This site will be useful in purification of recombinant PHTH domain protein.
- B Vector map of the plasmid pEGFP-N2 that expresses the enhanced green fluorescent protein (EGFP) from the strong, constitutive cytomegalovirus immediate early promoter (pCMV IE) and has the N2 variant of the multiple cloning site (Clontech). pEGFP-N2 was kindly provided by Tricia Pelton (Prof Peter Rathjen laboratory).
- C Sequence and restriction map of the multiple cloning site of the N2 variant of EGFP plasmid (Clontech).
- D Diagrammatic representation of the Tec sequences that were cloned in frame into the *Eco*RI and *Bam*HI restriction sites of pEGFP-N2. Human and mouse PHTH cDNAs were amplified by PCR using primers #178 and #179, or #253 and #179, on pBluescriptIIS+hTec4 and pEGFP-C2-mTec4 templates, respectively, and cloned into the *Eco*RI and *Bam*HI restriction sites of pEGFP-N2. Tec3 and Tec4 cDNAs were constructed in several steps. The kinase domain encoding sequence was amplified by PCR using primers #8 and #254 (Section 2.1.22) on pKS+mTecKinKB1.0 to remove the stop codon and cloned *Kpn*I/*Bam*HI into pBluescriptIIS+. The sequence was verified by automated DNA sequencing. A trimolecular ligation reaction was used to insert the *Acc*I/*Kpn*I fragment from pEGFP-C2-Tec4 and the new *Kpn*I/*Bam*HI fragment into the *Acc*I/*Bam*HI sites of pEGFP-N2-mPHTH. Subsequently, pEGFP-N2-Tec3 was created by replacing the 343 bp *Acc*I/*Pst*I fragment in pEGFP-N2-Tec4 with the 277 bp *Acc*I/*Pst*I fragment from pEGFP-C2-Tec3. The sequence and reading frame of the insert in each clone was confirmed by automated DNA sequencing.
- E Linear representation of the EGFP protein and the EGFP-Tec fusion proteins used in this study. In each case, the EGFP tag is at the C-terminus of the protein. Fusions of EGFP with human and mouse Tec PHTH domain were created and are denoted hPHTH-EGFP and mPHTH-EGFP, respectively. Predicted molecular weight was calculated using pI/MW software and is indicated for each fusion protein.

B



amplification also encoded a thrombin cleavage site for use in later purification of PHTH domain from PHTH-EGFP expressed in mammalian cells (Figure 4.5A) and this is discussed further in Chapter 5.

Expression of hPHTH-EGFP was not as high as expression of mPHTH-EGFP. This was also observed by direct immunofluorescence of transfected cells (data not shown) and may be due to translation initiating from different Kozak sequences that were engineered in the respective primers (see Figure 4.5A). The PHTH domain containing EGFP fusion proteins had similar solubility (see Figure 4.7C and data not shown).

4.4.7 Site-Directed Mutagenesis was Used to Alter Specific Residues of Tec Regulatory Domains

To investigate the importance of specific residues of Tec in binding to Actinin-4, amino acid substitution mutants of Tec were created. Production of mutants that affected the activation state, and therefore the open/closed conformation of Tec were also attempted. The design of the mutants was based on analogous Tec-family mutants reported in the literature. Site-directed mutagenesis was attempted to alter specific residues in the PH, SH3 and kinase domains. Four separate pairs of mutagenesis primers were designed to create amino acid substitutions at four different sites using PCR. These encoded the changes R29C, Y187E, K397E and the triple mutant K18A/K19A/K20A.

Automated DNA sequencing was used to analyse the mutant clones, which were tentatively identified by different restriction enzyme pattern (encoded in the mutagenesis primers where appropriate), and confirmed that only the desired mutations were introduced. As shown in Figure 4.6, K18A/K19A/K20A, R29C and Y187E clones were successfully created. The triple lysine to alanine mutant was created for mouse PHTH domain, Tec3 and Tec4. The Y187E mutant was created for Tec3. The R29C mutant was created for mPHTH but time did not permit cloning it into pEGFP vector or cloning of the other mutants.

4.4.8 Substitution Mutants Have Different Solubility to Wildtype Tec

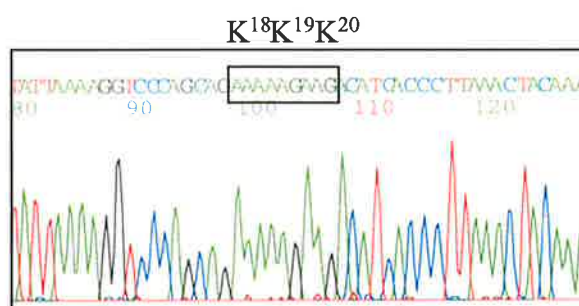
The mutants were cloned into the pEGFP-C2 vector, as described for wildtype Tec sequences in Figure 4.3, and expressed as fusions with EGFP in COS-1 cells in preparation for co-IP experiments with Myc epitope-tagged Actinin-4. As shown in Figure 4.7A, the Tec3 mutants were expressed poorly. When the cell lysates were separated by centrifugation into supernatant and pellet fractions, the mutant and wildtype Tec proteins were found to have

Figure 4.6 Electropherograms of Tec Nucleotide Sequence Before and After Site Directed Mutagenesis

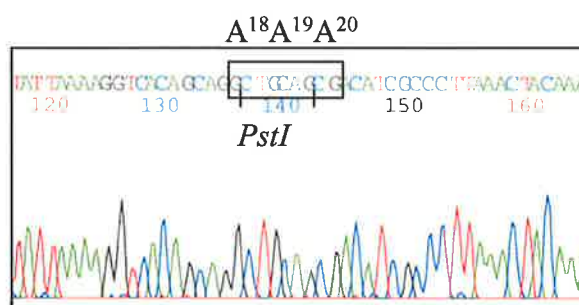
Site directed mutagenesis was performed to alter specific residues in the PHTH, SH3 and kinase domains of mouse and human Tec. Mutagenesis reactions were performed with templates cloned into the pBluescript plasmid. Restriction fragments containing desired mutations were then excised and cloned into pEGFP-C2 or pEGFP-N2 plasmid for expression in mammalian cells. The sequences of wild type and mutant Tec coding regions were analysed by automated DNA sequencing in which sequence electropherograms were generated. The sequence encompassing each mutagenesis site is shown for representative wild type Tec and corresponding mutant clones. Primer sequences are listed in Section 2.1.22.

- A The region encompassing codons for lysine residues 18-20 in wild type Tec. The sequence of the coding strand is shown and the three lysine codons of interest are boxed.
- B The region encompassing codons for the K18A/K19A/K20A triple substitution mutant. The sequence of the coding strand equivalent to that in part (A) is shown and the three alanine codons of interest are boxed. The *Pst*I restriction site used to discriminate mutant from wild type clones is indicated. Codons for lysine residues 18-20 were substituted with codons for alanine residues using primers #166 and #167 in mutagenesis reactions on pKS+(E-)mTec3 template. The mutant *Eco*RI/*Acc*I 412 bp fragment was then swapped with corresponding wild type sequence in pEGFP-C2-Tec3 and pEGFP-C2-Tec4 plasmids to produce plasmids that express EGFP-Tec3(K18A/K19A/K20A) and EGFP-Tec4(K18A/K19A/K20A) fusion proteins, respectively. Subsequently, PHTH(K18A/K19A/K20A) encoding sequence was amplified by PCR using primers #1 and EGFP-SP on pEGFP-C2-Tec3(K18A/K19A/K20A) template, and cloned into pEGFP-C2 to produce plasmid that expresses EGFP-PHTH(K18A/K19A/K20A) fusion protein. Similarly, primers #253 and #179 and pEGFP-C2-Tec3(K18A/K19A/K20A) template were used in a PCR reaction to amplify PHTH(K18A/K19A/K20A) encoding sequence for cloning into pEGFP-N2 to produce plasmid that expresses PHTH(K18A/K19A/K20A)-EGFP fusion protein. These primers include a 5' Kozak site and remove the 3' stop codon.
- C The region encompassing the codon for tyrosine residue 187 in wild type Tec. The sequence of the coding strand is shown and the tyrosine codon of interest is boxed.
- D The region encompassing the codon for the Y187E substitution mutant. The sequence of the coding strand equivalent to that in part (C) is shown and the glutamate codon of interest is boxed. The *Nco*I restriction site used to discriminate mutant from wild type clones is indicated. The codon for tyrosine residue 187 was substituted with a glutamate codon using primers #172 and #173 in mutagenesis reactions on pKS+(E-)mTec3 template. The mutant *Acc*I/*Pst*I 277 bp fragment was then swapped with corresponding wild type sequence in pEGFP-C2-Tec3 plasmid to produce a plasmid that expresses EGFP-Tec3(Y187E) fusion protein.
- E The region encompassing the codon for arginine residue 29 in wild type Tec. The sequence of the coding strand is shown and the arginine codon of interest is boxed.
- F The region encompassing the codon for the R29C substitution mutant. The sequence of the coding strand equivalent to that in part (E) is shown and the cysteine codon of interest is boxed. The codon for arginine residue 29 was substituted with a cysteine codon using primers #225 and #226 in mutagenesis reactions on pKS+mPHTH(943) template. This mutant is yet to be cloned into pEGFP-C2 plasmid to produce a plasmid that expresses EGFP-PHTH(R29C) fusion protein.

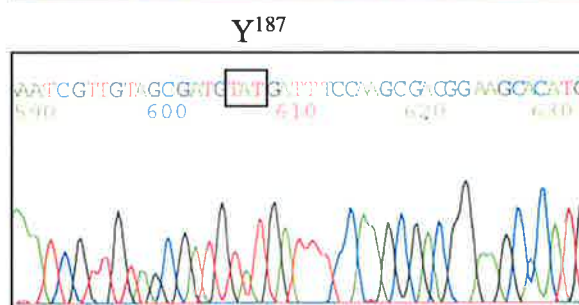
A



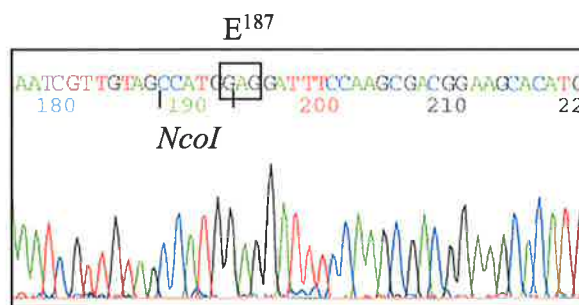
B



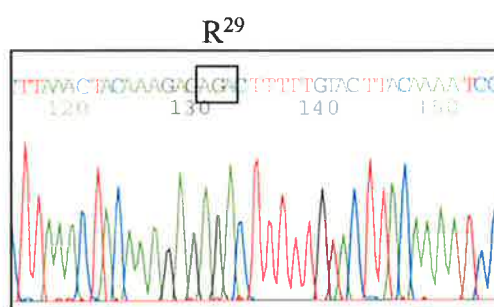
C



D



E



F

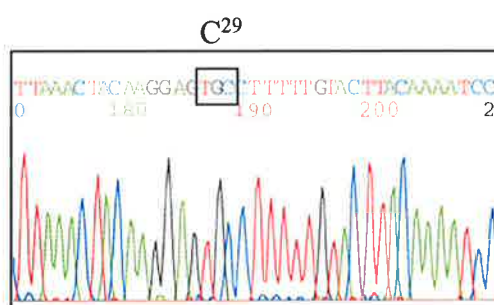


Figure 4.7 Analysis of Solubility of EGFP-Tec Variants

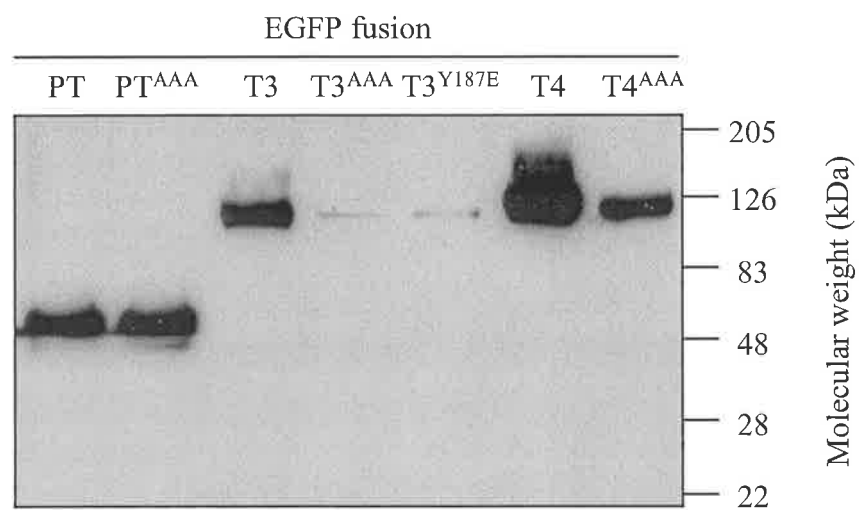
COS-1 cells were grown adherently on 10 cm tissue culture dishes and transfected with pEGFP-Tec plasmid using the LF2000 reagent. The different clones expressed fusions of amino terminal EGFP with wild type or mutant Tec sequences. Cells were lysed with cytoskeletal lysis buffer twenty-four hours post transfection. Aliquots of whole cell lysate were set aside. From the remainder, supernatant (S) and pellet (P) fractions were obtained by centrifugation. Samples were run on 10% Tris-tricine gels which were transferred to Hybond-C and probed by Western blot with rabbit-anti-GFP and goat-anti-rabbit-HRP antibodies and visualised using ECL.

- A Whole cell lysate samples.
- B Supernatant and pellet fractions of samples containing wild type and K18A/K19A/K20A triple mutant EGFP-Tec3 and EGFP-Tec4 fusion proteins.
- C Supernatant and pellet fractions of samples containing wild type and K18A/K19A/K20A triple mutant EGFP-PHTH fusion proteins and Y187E mutant EGFP-Tec3 fusion protein.

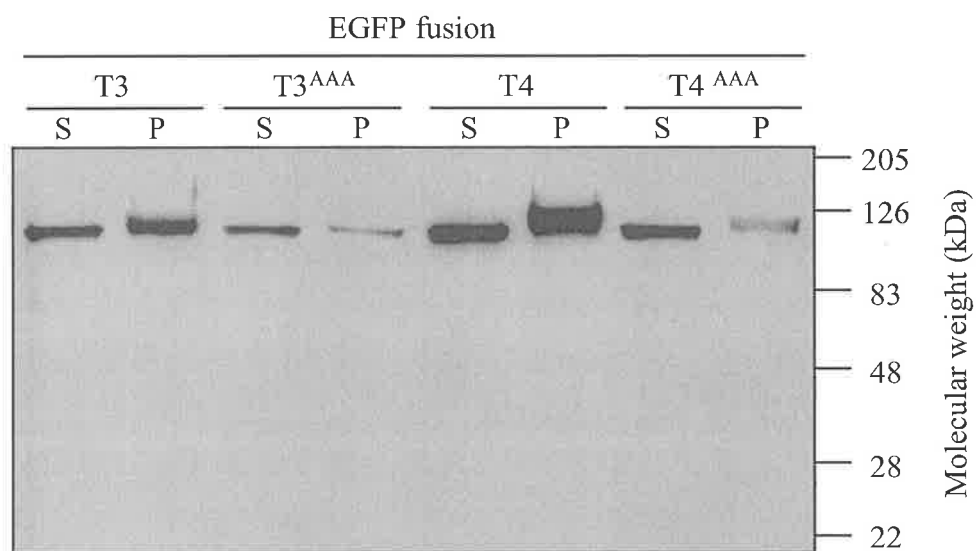
Primary antibody was used at 1/2500, while secondary antibody was used at 1/1500 dilution. Sigma Colour Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

EGFP:	enhanced green fluorescent protein.
PT:	PHTH domain
PT ^{AAA} :	PHTH domain K18A/K19A/K20A triple mutant
T3:	Tec3
T3 ^{AAA} :	Tec3 K18A/K19A/K20A triple mutant
T3 ^{Y187E} :	Tec3 Y187E substitution mutant
T4:	Tec4
T4 ^{AAA} :	Tec4 K18A/K19A/K20A triple mutant

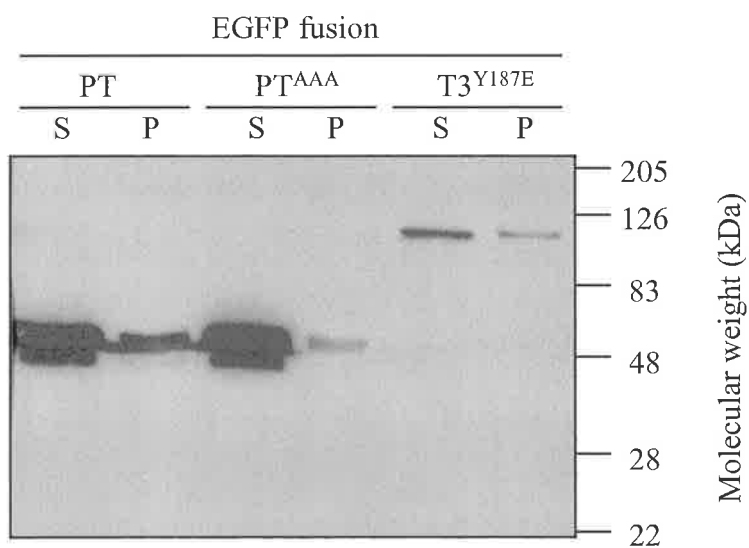
A



B



C



different solubility. Western blot of wildtype Tec3- and Tec4-containing samples indicated a stronger band in the pellet than supernatant (Figure 4.7B). Conversely, the corresponding samples from the mutants had a fainter band in the pellet than supernatant (Figure 4.7B). The K18A/K19A/K20A triple mutation increased the solubility of EGFP-Tec3 and EGFP-Tec4. Similarly, the Y187E substitution increased the solubility of EGFP-Tec3. The majority of wildtype EGFP-PHTH protein partitioned in the soluble fraction and this was not affected by the K18A/K19A/K20A triple mutation, which is within the PH domain (Figure 4.7C).

4.4.9 The Majority of Myc Tagged Truncated Actinin-4 Partitions in The Soluble Fraction

The full-length Actinin-4 cDNA was obtained from the lab of Dr S Hirohashi (Japan) and epitope-tagged Actinin-4 was cloned. Since Btk (a Tec-family member) and α -actinin both bind F-actin, there is a possibility that co-IP of Tec and Actinin-4 from cultured cells is mediated indirectly through actin binding. Therefore, the actin-binding domain was excluded when Myc epitope-tagged Actinin-4 (Myc-A4) was cloned. The Myc-A4 clone was constructed as described in Figure 4.8 and transiently transfected into COS-1 cells. Expression from the pXMT2 vector is driven by the strong constitutive Adenovirus major late promoter (Figure 4.8C) and translation is initiated at the Kozak consensus sequence that was engineered into the coding region by PCR (Figure 4.8A). This fusion protein contains the Myc epitope-tag near the N-terminus (Figure 4.8D). The transfected COS-1 cells were harvested 24 h after transfection, and lysed by cytoskeletal lysis buffer. Western blot analysis, shown in Figure 4.9 shows that two bands of Myc-A4, that differ by approximately 10 kDa, are detected in transfected COS-1 cells when using the anti-Myc monoclonal antibody but not in untransfected control cells. The same two bands are identified in anti-Myc immunoprecipitates from supernatant fractions of Myc-A4 transiently transfected cells but not from untransfected cells (Figure 4.9B). The anti-Myc antibody was produced and harvested from the Myc 1-9E10.2 hybridoma cell line culture supernatant as described in Section 2.3.4.2. The same two bands are identified when anti-Actinin-4 antibody is used in the Western blot instead of anti-Myc antibody, indicating that the correct protein is made (data not shown).

As seen in Figure 4.9, Myc-A4 is present in both the supernatant and pellet fractions with the majority partitioning in the soluble fraction. Once again, Myc-A4 in the pellet fraction migrates slower than Myc-A4 in the soluble fraction and this may be due to

Figure 4.8 Cloning of Epitope Tagged Actinin-4 Lacking the Actin Binding Domain, Myc-A4

- A Features engineered into Actinin-4 cDNA lacking the actin binding domain encoding region (A4) using PCR primers #176 and #177 (Section 2.1.22) are indicated. The sequence of the upper primer is listed showing the *Eco*RI restriction site used for cloning into pXMT2, the Kozak consensus sequence for translation initiation, the reading frame and the *Xba*I restriction site for insertion of in frame Myc epitope tag encoding sequences. The expected translation start methionine residue and following amino acids are written in single letter code below the appropriate codon. The first alanine shown corresponds to residue 271 of the native Actinin-4 protein. The complement of the lower primer sequence, showing the *Eco*RI restriction site used for cloning into pXMT2 and the reading frame, is listed above the amino acid sequence of the last seven residues and the natural stop codon.
- B Diagrammatic representation of the Myc epitope tagged Actinin-4 encoding sequence that was cloned into the *Eco*RI restriction site of pXMT2 plasmid. The 1958 bp A4 fragment was PCR amplified from pBKCMV-Actinin-4 plasmid (kindly supplied by Dr Tesshi Yamada, Japan) with primers #176 and #177 and cloned into the intermediate pGEM-T plasmid. After cloning A4 into the *Eco*RI restriction site of modified pBluescriptIIKS+, in which the *Xba*I restriction site was removed, Myc-A4 was cloned by ligation of three copies of the Myc epitope tag encoding sequence into the *Xba*I site engineered at the beginning of the A4 sequence. The 3x Myc epitope tag encoding sequence (126 bp) was excised from pGEMT-6Myc that was kindly provided by Dr Dan Peet (Dr Murray Whitelaw laboratory). A partial restriction map showing *Xba*I (X), *Bam*HI (B), *Pst*I (P) and *Sac*I (S) sites is indicated. The orientation of 3x Myc and sequence of the Myc-A4 insert in pKS+(X) was confirmed by automated DNA sequencing.
- C Vector map of the plasmid pXMT2 that expresses insert sequences from the strong, constitutive adenovirus major late promoter (MLP). pXMT2 was kindly provided by Tricia Pelton (Prof Peter Rathjen laboratory). The *Eco*RI site within the multiple cloning site (MCS) where Myc-A4-encoding sequence was inserted is indicated. The orientation of Myc-A4 was confirmed by *Pst*I restriction digestion that excised a 420 bp fragment and not a 1600 bp fragment. One *Pst*I site is in the MCS and the other is within Actinin-4 coding sequence.
- D Linear representation of the Myc-A4 fusion protein used in this study. The Myc tag is near the amino terminus of the protein. Predicted molecular weight, shown in brackets, was calculated using pI/MW software.

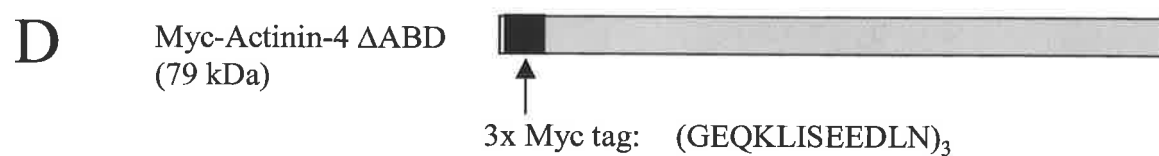
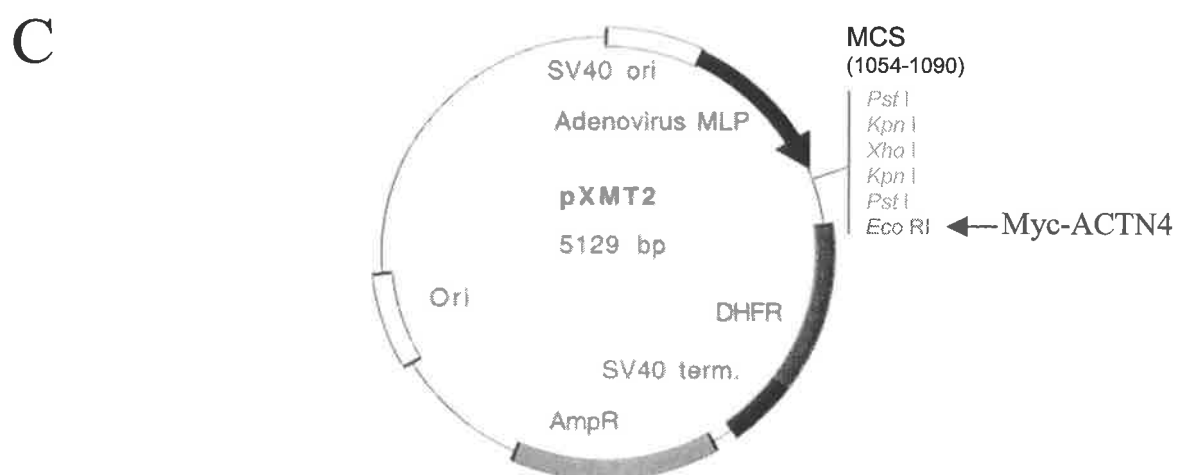
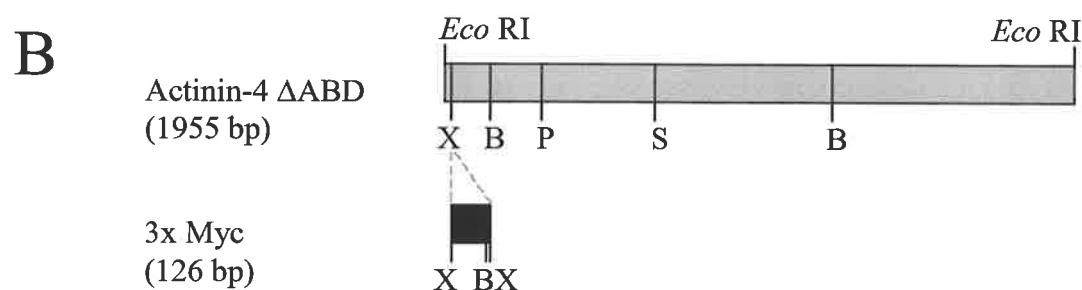
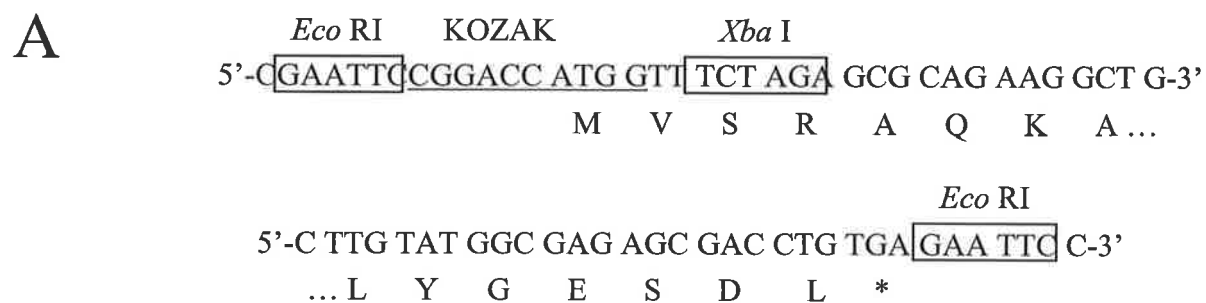


Figure 4.9 Western Blot of COS-1 Cells Transiently Transfected With pXMT2-Myc-A4

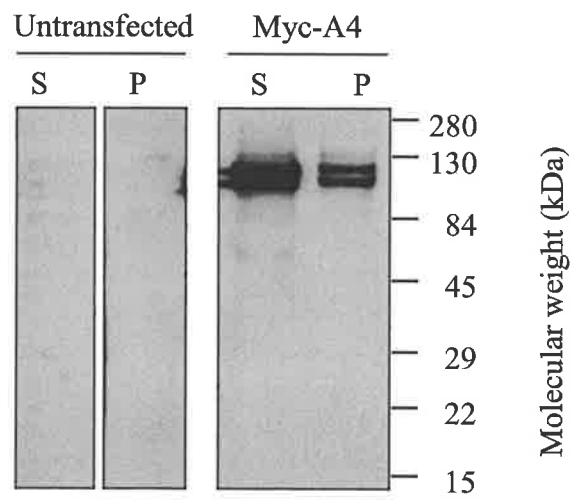
COS-1 cells were grown adherently on 10 cm tissue culture dishes and transfected with pXMT2-Myc-A4 (Myc-A4) plasmid using the LF2000 reagent. Transfected and untransfected control cells were lysed with cytoskeletal lysis buffer twenty-four hours post transfection.

- A Supernatant (S) and pellet (P) fractions were obtained by centrifugation and samples were run on a 10% Tris-tricine gel which was transferred to Hybond-C and probed by Western blot with mouse-anti-Myc and sheep-anti-mouse-HRP antibodies and visualised using ECL.
- B Supernatant fractions from the transfected (Myc-A4) and untransfected (U) samples were subjected to immunoprecipitation (IP) analysis with mouse-anti-Myc antibody and protein-G-agarose. IP pellets were washed four times and samples were analysed by Western blot as in part (A).

Primary antibody was used at 1/2 dilution, while secondary antibody was used at 1/3000. Sigma Colour Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated. The predicted molecular weight of Myc-A4 is 79 kDa and was calculated using pI/MW software.

A

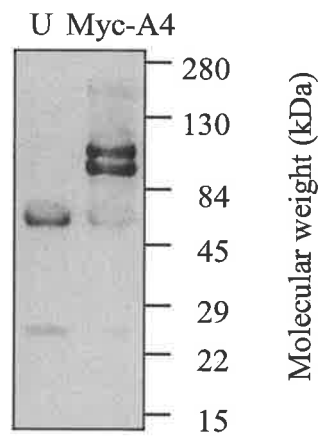
Blot:
Anti-Myc



B

IP: Anti-Myc

Blot: Anti-Myc



post-translational modification. The predicted molecular weight of Myc-A4 is 79 kDa, however, the observed bands appear to have slightly larger molecular weight. Correspondingly, the endogenous Actinin-4 protein migrates slower than its predicted molecular weight 105 kDa (Figure 4.2B).

4.4.10 Expression of the EGFP-Tec Proteins was Verified by Fluorescence Microscopy

Tec proteins were fused in frame to either the C- or N-termini of the fluorescent EGFP moiety. EGFP-fusion proteins can be directly visualised in living cells by fluorescence microscopy without the need for fixation and immunostaining. There is, however, a possibility that overexpression of the EGFP-Tec fusion proteins in this system affects their subcellular distribution pattern.

To estimate the transfection efficiency of the various pEGFP-Tec plasmids and to ensure EGFP-Tec fusion protein expression in cells prior to lysis in preparation for the co-IP experiments, transfected COS-1 cells were investigated by light and fluorescence microscopy. As shown in Figures 4.10-4.12, differences in subcellular localisation pattern were identified for EGFP-PHTH, EGFP-Tec3 and EGFP-Tec4 proteins and the intracellular distribution patterns were altered by specific amino acid substitutions. The subcellular localisation patterns of the various EGFP-Tec fusion proteins were not affected by the relative position of the EGFP moiety (data not shown). The images shown are representative of the major fluorescence patterns observed for each clone.

Both EGFP alone and EGFP-PHTH were present diffusely throughout the cell and nucleus and in fine filamentous, web-like structures in the cytoplasm (Figure 4.10A and B). When PHTH was fused to the N-terminus of EGFP, only faintly fluorescing cells were detected due to lower expression. Images of these cells were not recorded although they had similar fluorescence pattern to pEGFP-PHTH transfected cells. Remarkably different subcellular distribution patterns were identified for EGFP-Tec4 (Figure 4.11A) and EGFP-Tec (Figure 4.12A) compared with EGFP-PHTH (Figure 4.10B). The subcellular distribution of EGFP-Tec3 and EGFP-Tec4 were extensively analysed and documented by Atmosukarto; 2001, in experiments performed in parallel with those described here.

In the majority of EGFP-Tec4 expressing cells, fluorescence was most intense in a large juxta nuclear globule (Figure 4.11A) thought to contain endoplasmic reticulum (ER) or Golgi network components. However, this was not directly tested using ER- or Golgi-specific markers. These cells often contained punctate fluorescence in the cytoplasm, most likely associated with vesicles that originated from the Golgi (Atmosukarto, 2001). In some

Figure 4.10 Subcellular Localisation of EGFP and EGFP-PHTH Wild Type and Actin Binding Mutant

COS-1 cells were grown adherently on sterile glass coverslips in 6-well trays and transfected with plasmids that express EGFP, EGFP-PHTH or EGFP-PHTH(K18A/K19A/K20A) fusion proteins using the LF2000 reagent. Live, transfected cells were visualised by direct fluorescence microscopy twenty-four hours post transfection. The coverslip was inverted onto a microscope slide and the cells were analysed at 100× magnification under oil immersion with a BIORAD MRC-600 Laser Scanning Confocal Imaging System coupled to an Olympus IMT-2 Inverted Research Microscope. Duplicate samples were analysed at 60× magnification using a Nikon inverted (Eclipse TE300) microscope and images were captured on 100ASA photographic film. Images were captured digitally using the confocal laser scanning microscope, except for images in the right panels of parts (A) and (B), which were captured on photographic film.

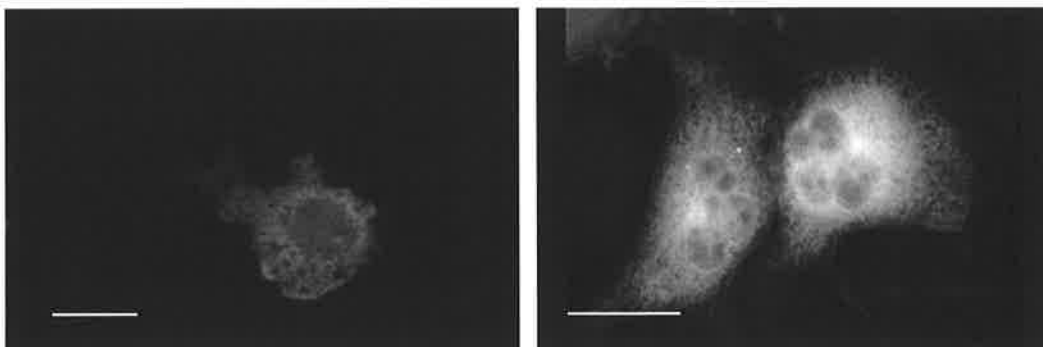
- A Cells transfected with empty pEGFP-C2 plasmid showing the subcellular distribution of EGFP protein.
- B Cells transfected with pEGFP-C2-PHTH plasmid showing the subcellular distribution of EGFP-PHTH fusion protein.
- C Cells transfected with pEGFP-C2-PHTH(K18A/K19A/K20A) plasmid showing the subcellular distribution of EGFP-PHTH(K18A/K19A/K20A) fusion protein.

EGFP: enhanced green fluorescent protein.

Scale bar 20 μ M

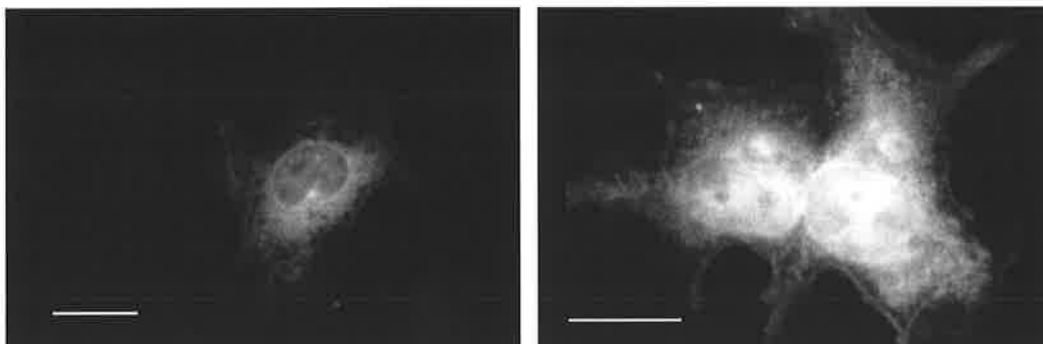
A

EGFP



B

EGFP-PHTH



C

EGFP-PHTH(K18A/K19A/K20A)

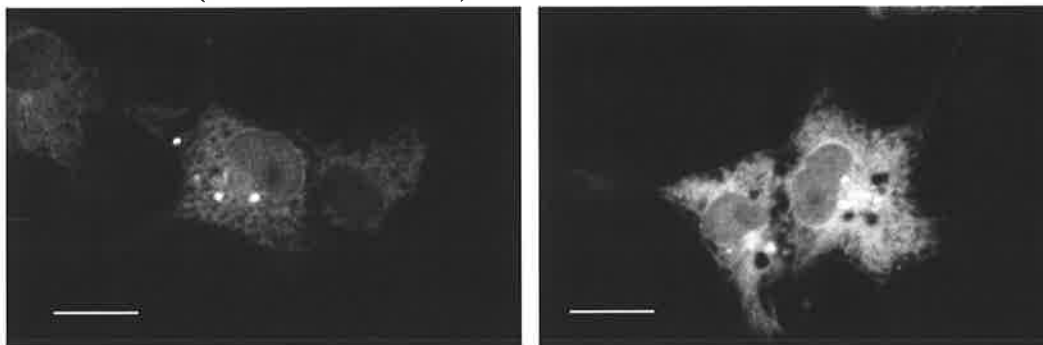


Figure 4.11 Subcellular Localisation of EGFP-Tec4 and Actin Binding Mutant

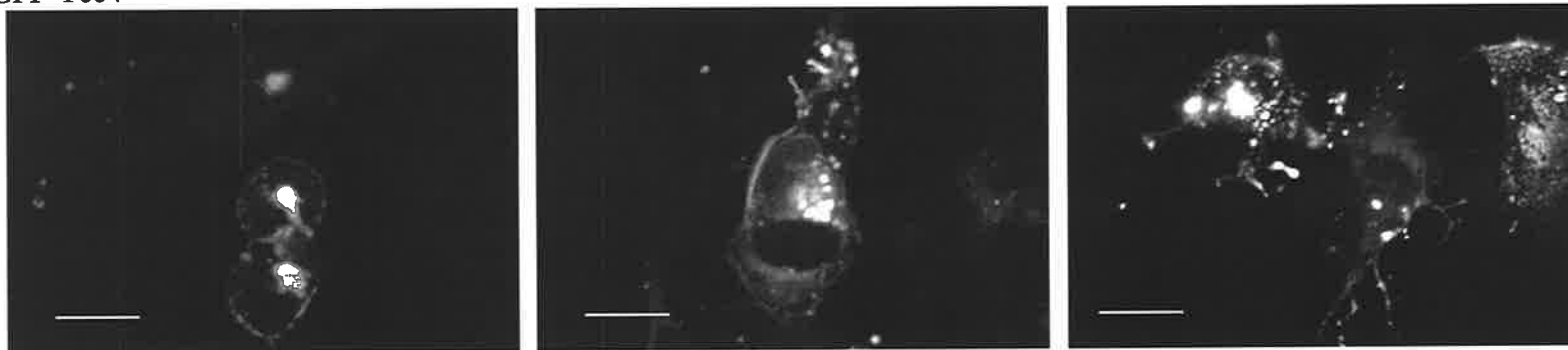
COS-1 cells were grown adherently on sterile glass coverslips in 6-well trays and transfected with plasmids that express EGFP-Tec4 or EGFP-Tec4(K18A/K19A/K20A) fusion proteins using the LF2000 reagent. Live, transfected cells were visualised by direct fluorescence microscopy twenty-four hours post transfection and images were captured digitally. The coverslip was inverted onto a microscope slide and the cells were analysed at 100× magnification under oil immersion with a BIORAD MRC-600 Laser Scanning Confocal Imaging System coupled to an Olympus IMT-2 Inverted Research Microscope.

- A Cells transfected with pEGFP-C2-Tec4 plasmid showing the subcellular distribution of EGFP-Tec4 fusion protein.
- B Cells transfected with pEGFP-C2-Tec4(K18A/K19A/K20A) plasmid showing the subcellular distribution of EGFP-Tec4(K18A/K19A/K20A) fusion protein.

EGFP: enhanced green fluorescent protein.
Scale bar 20 μ M

A

EGFP-Tec4

**B**

EGFP-Tec4(K18A/K19A/K20A)

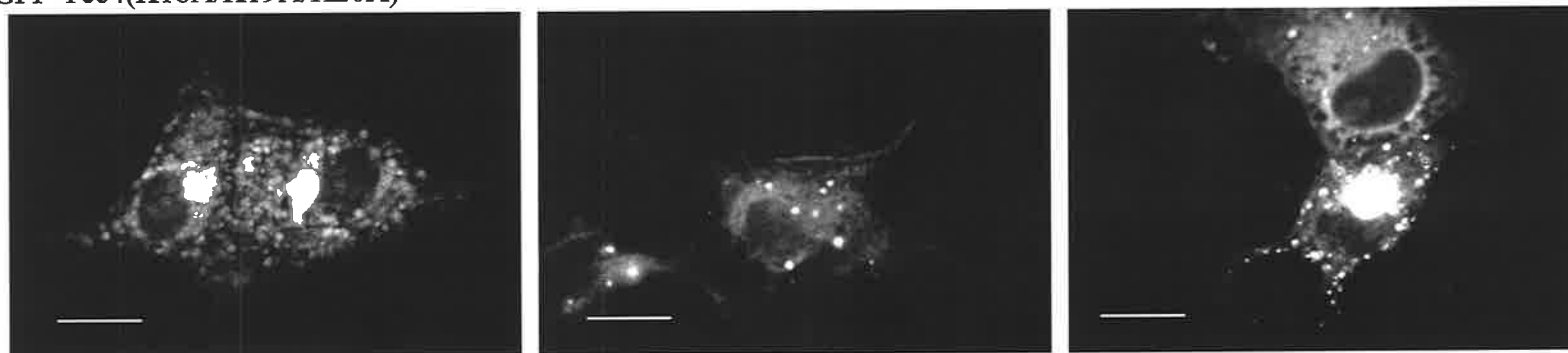


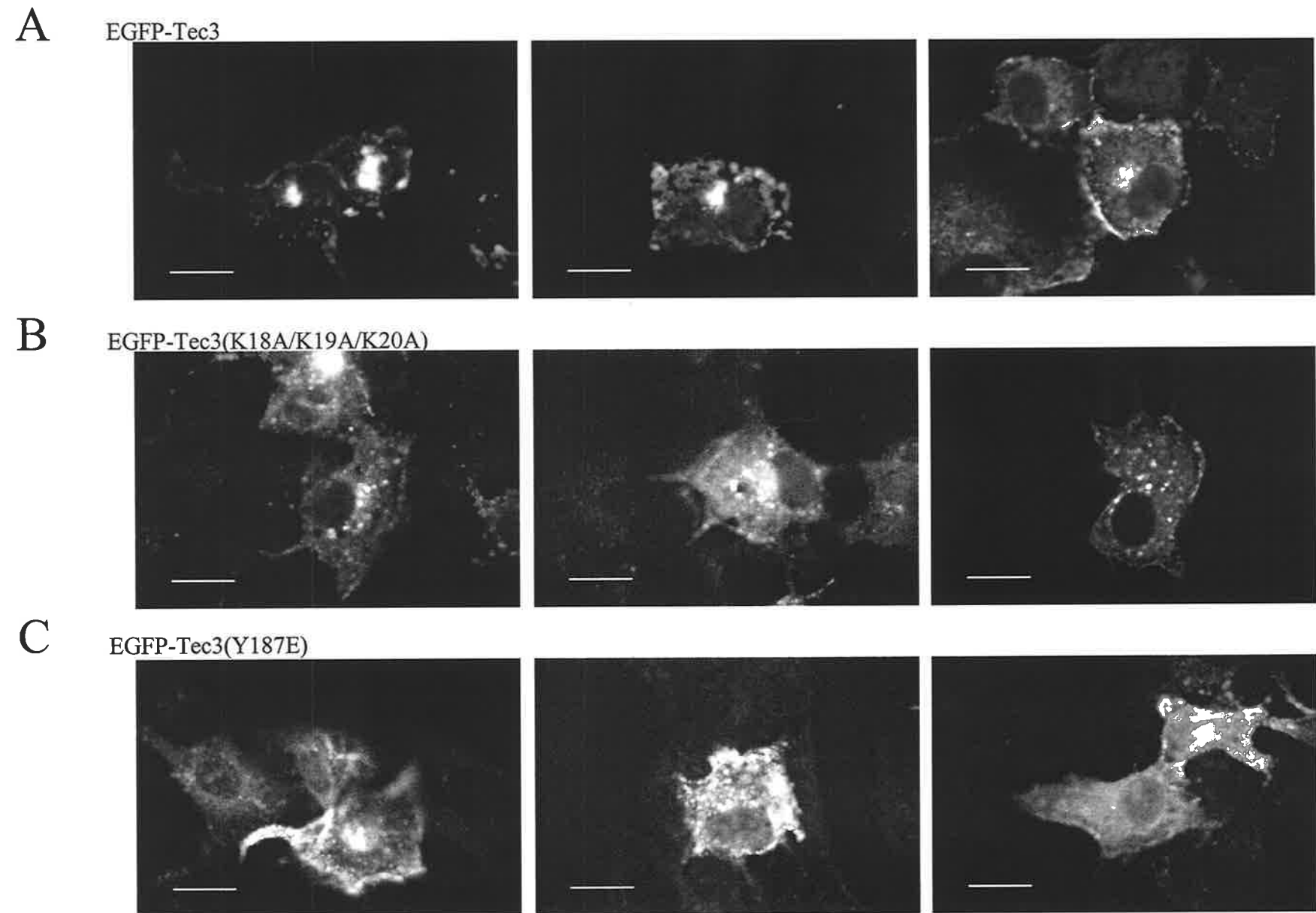
Figure 4.12 Subcellular Localisation of EGFP-Tec3 Wild Type, Actin Binding Mutant and Y187E Mutant

COS-1 cells were grown adherently on sterile glass coverslips in 6-well trays and transfected with plasmids that express EGFP-Tec3, EGFP-Tec3(K18A/K19A/K20A) or EGFP-Tec3(Y187E) fusion proteins using the LF2000 reagent. Live, transfected cells were visualised by direct fluorescence microscopy twenty-four hours post transfection and images were captured digitally. The coverslips were inverted onto microscope slides and the cells were analysed at 100× magnification under oil immersion with a BIORAD MRC-600 Laser Scanning Confocal Imaging System coupled to an Olympus IMT-2 Inverted Research Microscope.

- A Cells transfected with pEGFP-C2-Tec3 plasmid showing the subcellular distribution of EGFP-Tec3 fusion protein.
- B Cells transfected with pEGFP-C2-Tec3(K18A/K19A/K20A) plasmid showing the subcellular distribution of EGFP-Tec3(K18A/K19A/K20A) fusion protein.
- C Cells transfected with pEGFP-C2-Tec3(Y187E) plasmid showing the subcellular distribution of EGFP-Tec3(Y187E) fusion protein.

EGFP: enhanced green fluorescent protein.

Scale bar 20 μ M



cells this was accompanied by plasma membrane fluorescence. The region surrounding pEGFP-Tec4 transfected COS-1 cells sometimes contained punctate fluorescence suggesting blebbing or exocytosis of the fluorescent vesicles.

EGFP-Tec3 expressing cells displayed obvious plasma membrane fluorescence in addition to intense fluorescence adjacent the nucleus (Figure 4.12A). Depending on the slice of the cell that was imaged, the plasma membrane fluorescence was visualised as a rim with surface ruffles and filopodial extensions or in surface patches indicative of membrane rafts (Figure 4.12A). Membrane localisation (a requirement for full activation of the kinase) of Tec3 would place it in close proximity to upstream activators upon cell stimulation.

Increased punctate fluorescence in the cytoplasm was observed in cells expressing the PH domain K18A/K19A/K20A triple mutation in EGFP-PHTH (Figure 4.10C), EGFP-Tec4 (Figure 4.12B) and EGFP-Tec3 (Figure 4.11B). At the same time, the juxta nuclear fluorescence was reduced in intensity. The spots were of varying sizes with larger ones closer to the nucleus. Plasma membrane fluorescence was also diminished in cells expressing the K18A/K19A/K20A triple mutation in EGFP-Tec3.

Extensive cytoplasmic projections and increased plasma membrane fluorescence were observed in cells expressing the EGFP-Tec3 Y187E substitution mutant (Figure 4.12C). These cells generally displayed fluorescence adjacent the nucleus. The membrane localisation and projections are consistent with Y187E being an activating mutation.

4.4.11 Endogenous Tec And Actinin-4 Interact In Mammalian Cells

Since the two proteins exist in the same subcellular localisation in adherent cells, IP experiments were performed to investigate the association of endogenous Tec and Actinin-4. As shown in Figure 4.13, when Actinin-4 antibody was used in IP reactions from MCF-7, HepG2 or COS-1 cell supernatant fractions, both Tec and Actinin-4 were identified in the immunoprecipitate. However, Tec was not identified in several co-IP experiments when immunoprecipitates were washed five times indicating the interaction of the two proteins in the Triton-X100 soluble fraction is weak. The anti-Tec antibody raised in goat was not suitable for use in IP experiments. Therefore, reciprocal IP experiments were not performed. To further characterise the interaction in mammalian cells, an expression system was prepared in which epitope-tagged proteins were created and subjected to IP analysis.

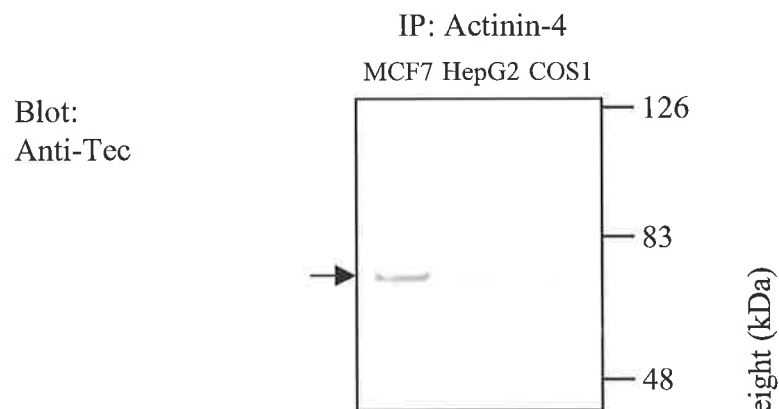
Figure 4.13 Co-immunoprecipitation of Endogenous Tec with Actinin-4

MCF-7, HepG2 and COS-1 cells were grown adherently on 10 cm tissue culture dishes before lysis with cytoskeletal lysis buffer. Anti-Actinin-4 immunoprecipitates (IP) from supernatant fractions were run on duplicate 10% Tris-tricine gels that were transferred to nitrocellulose and probed by Western blot.

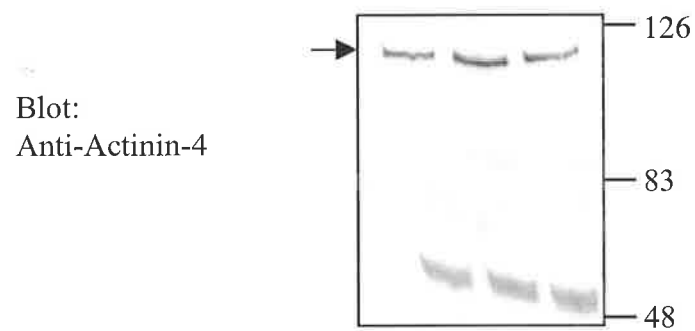
- A Anti-Actinin-4 IP probed with goat-anti-Tec primary antibody and sheep-anti-goat-AP secondary antibody.
- B Anti-Actinin-4 IP control probed with rabbit-anti-Actinin-4 primary antibody and goat-anti-rabbit-AP secondary antibody.

Primary antibodies were used at 1/5,000 dilution while secondary antibodies were used at 1/10,000 dilution. Alkaline phosphatase conjugated secondary antibodies were developed with NBT/BCIP. Sigma Colour Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

A



B



4.4.12 Epitope-Labelled Tec and Actinin-4 Interact In Mammalian Cells

Supernatants from COS-1 cells cotransfected with MycA4 and various EGFP-Tec expressing plasmids were subjected to IP analysis with anti-Myc and anti-GFP antibodies. As seen in Figure 4.14A, a fraction of the EGFP-Tec but not EGFP alone is present in anti-Myc immunoprecipitates. Similarly, a fraction of Myc-A4 is present in anti-GFP immunoprecipitates that contain EGFP-Tec fusion protein (Figure 4.14C). IP controls are shown (Figure 4.14B and D). Therefore, a portion of the epitope-tagged Tec and Actinin-4 proteins interacts in cultured cells.

Since the majority of EGFP-Tec3 and EGFP-Tec4 proteins were insoluble (see Section 4.4.5), difficulties in obtaining soluble EGFP-Tec3 and EGFP-Tec4 proteins for use in IP experiments were encountered. This was evident in the loading control shown in Figure 4.14D. While the bands were weak, the interaction was confirmed by experiments described in the following section.

4.4.13 EGFP-Tec Variants Have Similar Myc-Actinin-4-binding Characteristics

In further co-IP experiments, various pEGFP-Tec constructs were transiently co-transfected into COS-1 cells with pXMT2-Myc-A4 and immunoprecipitated with anti-GFP antibodies. The binding of Myc-A4 to the EGFP-Tec variants was evaluated by Western blot of the anti-GFP immune complexes with anti-Myc antibody (Figure 4.15 A and C). IP controls are shown (Figure 4.15B and D). Since the EGFP-Tec4 and Myc-A4 proteins migrate at similar positions, duplicate gels were used in the controls, rather than stripping and reprobing the original Western blot, to improve the clarity of the results.

Similar amounts of Myc-A4 were co-immunoprecipitated with the hPHTH-EGFP (Figure 4.15A) and EGFP-mPHTH fusion proteins (Figure 4.15C) but not with EGFP alone. It therefore appears that human and mouse Tec PHTH domains have similar Actinin-4-binding characteristics while having 7 amino acid differences in their sequence. However, most of those differences are conservative substitutions: V37M, S39T, V53F, I56V, F78Y, R101L and A126T, with the mouse residue and position listed before the human residue. It would be interesting to next assess binding of mouse Tec to mouse Actinin-4, which was recently cloned (Dear *et al.*, 2000).

The K18A/K19A/K20A triple substitution mutation in the PH domain did not appear to reduce the binding of Myc-A4 to Tec proteins (Figure 4.15A and C). This was irrespective of whether the EGFP moiety was fused to the C- or N-terminus of Tec, and whether PHTH,

Figure 4.14 Co-immunoprecipitation of Epitope Tagged Tec and Actinin-4 From Transiently Co-transfected COS-1 Cells

COS-1 cells were grown adherently on 10 cm tissue culture dishes and co-transfected with pXMT2-Myc-A4 plasmid and pEGFP control or pEGFP-Tec plasmid using the LF2000 reagent. Cells were lysed with cytoskeletal lysis buffer twenty-four hours post transfection. Immunoprecipitates (IP) from supernatant fractions were washed in IP wash buffer three times and samples were run on 10% Tris-tricine gels that were transferred to Hybond-C and probed by Western blot. Co-immunoprecipitated proteins were visualised using ECL.

- A Anti-Myc IP probed with rabbit-anti-GFP primary antibody and goat-anti-rabbit-HRP secondary antibody.
- B Anti-Myc IP control. The same blot as in part (A) was stripped, blocked and probed a second time using mouse-anti-Myc primary antibody and sheep-anti-mouse-HRP secondary antibody.
- C GFP IP probed with mouse-anti-Myc primary antibody and sheep-anti-mouse-HRP secondary antibody.
- D Anti-GFP IP control. The same blot as in part (C) was stripped, blocked and probed a second time using rabbit-anti-GFP primary antibody and goat-anti-rabbit-HRP secondary antibody.

Primary antibodies were used at 1/2 and 1/3000 dilutions for anti-Myc and anti-GFP, respectively, while secondary antibodies were used at 1/4000. Sigma Colour Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

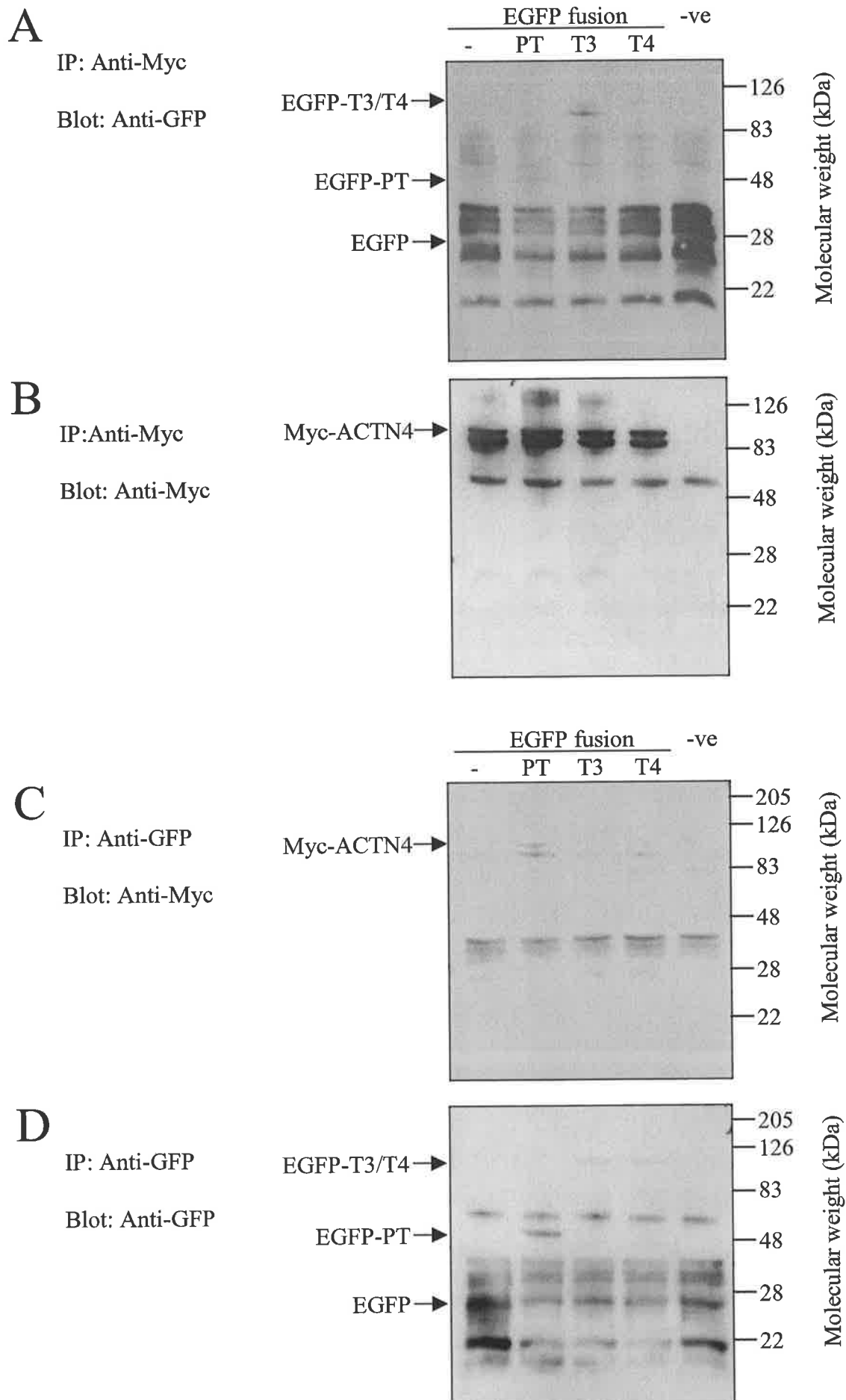


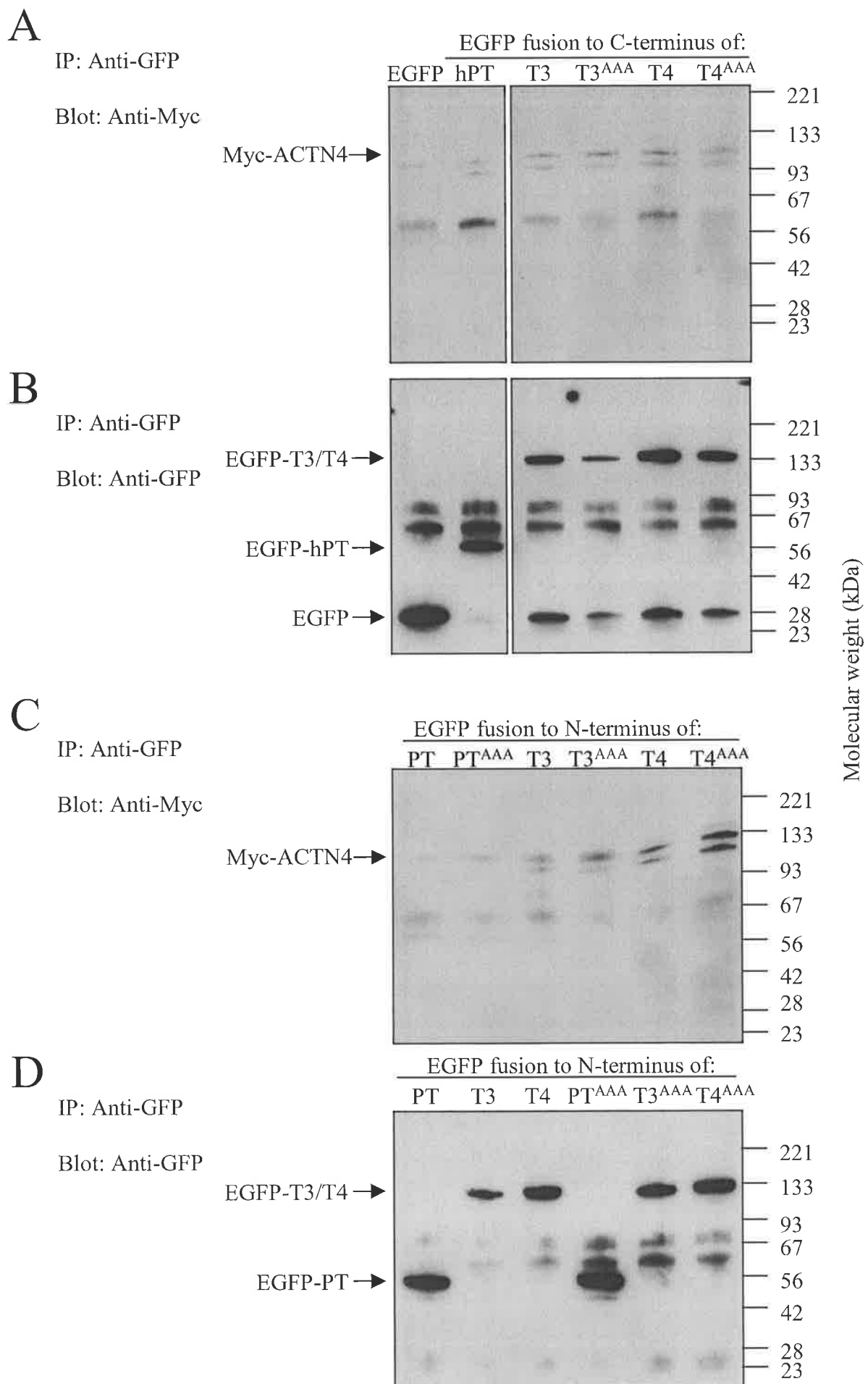
Figure 4.15 Co-immunoprecipitation of Epitope Tagged Tec Variants and Actinin-4 From Transiently Co-transfected COS-1 Cells

COS-1 cells were grown adherently on 10cm tissue culture dishes and co-transfected with pXMT2-Myc-A4 plasmid and pEGFP control or pEGFP-Tec plasmid using the LF2000 reagent. The Tec variants that were analysed for binding to Myc-A4 include human Tec PHTH domain (hPT), and the K18A/K19A/K20A triple mutants. The EGFP moiety was fused to either the C-terminus of the Tec protein in (in parts A and B) or to the N-terminus of the Tec protein (in parts C and D). Cells were lysed with cytoskeletal lysis buffer twenty-four hours post transfection. Anti-GFP immunoprecipitates (IP) from supernatant fractions were washed in IP wash buffer four times. Samples were run on duplicate 8% Tris-tricine gels, transferred to Hybond-C and probed by Western blot. Co-immunoprecipitated proteins were visualised using ECL.

- A GFP IP probed with mouse-anti-Myc primary antibody and sheep-anti-mouse-HRP secondary antibody.
- B GFP IP control. A duplicate of the blot in part (A) was probed with goat-anti-GFP primary antibody and donkey-anti-goat-HRP secondary antibody.
- C GFP IP probed with mouse-anti-Myc primary antibody and sheep-anti-mouse-HRP secondary antibody.
- D GFP IP control. A duplicate of the blot in part (A) was probed with goat-anti-GFP primary antibody and donkey-anti-goat-HRP secondary antibody.

Primary antibodies were used at 1/200 and 1/2 dilutions for goat-anti-GFP and mouse-anti-Myc, respectively, while secondary antibodies were used at 1/3000 dilution. Benchmark Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

EGFP:	enhanced green fluorescent protein.
PT:	PHTH domain
PT ^{AAA} :	PHTH domain K18A/K19A/K20A triple mutant
T3:	Tec3
T3 ^{AAA} :	Tec3 K18A/K19A/K20A triple mutant
T4:	Tec4
T4 ^{AAA} :	Tec4 K18A/K19A/K20A triple mutant



Tec3 or Tec4 sequences were used. Therefore, the presence of these three actin-binding lysine residues is not essential for the Actinin-4 binding capability of Tec.

4.4.14 Purified Actinin-4 Repeat-3 Binds to EGFP-Tec

To further confirm the interaction of Actinin-4 and Tec proteins, co-IP of purified Rpt3 protein with EGFP-Tec fusion proteins was investigated using the anti-GFP antibody. A fusion protein of glutathione-S-transferase and spectrin repeat 3 of Actinin-4 (GST-Rpt3), purified for use in experiments described in Chapter 5, was available for use in IP studies. Exogenous GST-Rpt3, or GST alone in control samples, was mixed with lysate supernatants from transfected COS-1 cells. GST-containing proteins that co-immunoprecipitated with EGFP-containing proteins were identified by Western blot. The GST-Rpt3 protein co-immunoprecipitated with rabbit anti-GFP antibody when Tec was fused to EGFP (Figure 4.16A). In control experiments, GST alone did not co-IP with any EGFP-containing proteins, as expected (Figure 4.16A). As a further control, the membrane was stripped and probed for GFP to show the amount of immunoprecipitated EGFP-containing protein. A background band observed in each sample, at approximately 55 kDa, is most likely antibody heavy chain. The difference in intensity of this band in lanes 5 and 6 was due to technical error where 10 times the amount of protein-A-Sepharose was added to two IP samples. Reciprocal studies in which glutathione agarose was used to pull down GST-containing proteins and their associated proteins were attempted several times. However, cross-reaction between EGFP and glutathione agarose in negative control samples meant that no conclusions could be drawn from those experiments.

4.4.15 Involvement of Tec and Actinin-4 in Phagocytosis by Differentiated U937 Cells

To demonstrate a functional significance for the Tec:Actinin-4 interaction in mammalian cells, an assay system was sought in which the interaction could be perturbed and the effect of this could be measured. Phagocytosis was identified as one such assay system. Phagocytosis involves massive architectural restructuring of cells through regulated cytoskeletal rearrangement when cells engulf particulate matter. Tec is hypothesized to transmit signals downstream of Fc- γ R-mediated phagocytosis (Atmosukarto, 2001). The proposed signalling pathway is analogous to the signalling pathway described for Btk downstream of the B cell receptor in B-lymphocytes and Itk downstream of the T cell receptor

**Figure 4.16 Co-immunoprecipitation of Exogenous GST-Rpt3 With EGFP-Tec
From Transiently Transfected COS-1 Cells**

COS-1 cells were grown adherently on 10 cm tissue culture dishes and transfected with pEGFP-C2 control or pEGFP-C2-Tec plasmid using the LF2000 reagent. Cells were lysed with cytoskeletal lysis buffer twenty-four hours post transfection. Supernatant fractions obtained by centrifugation were mixed with exogenous purified GST-Rpt3 protein and immunoprecipitated with rabbit-anti-GFP antibody. Immunoprecipitates were washed five times with lysis buffer and samples were run on a 10% Tris-tricine gel that was transferred to Hybond-C and probed by Western blot.

- A Anti-GFP IP samples probed with mouse-anti-GST primary antibody and sheep-anti-mouse-HRP secondary antibody.
- B Anti-GFP IP control. The same blot as in part (A) was stripped, blocked and probed a second time using rabbit-anti-GFP primary antibody and goat-anti-rabbit-HRP secondary antibody.

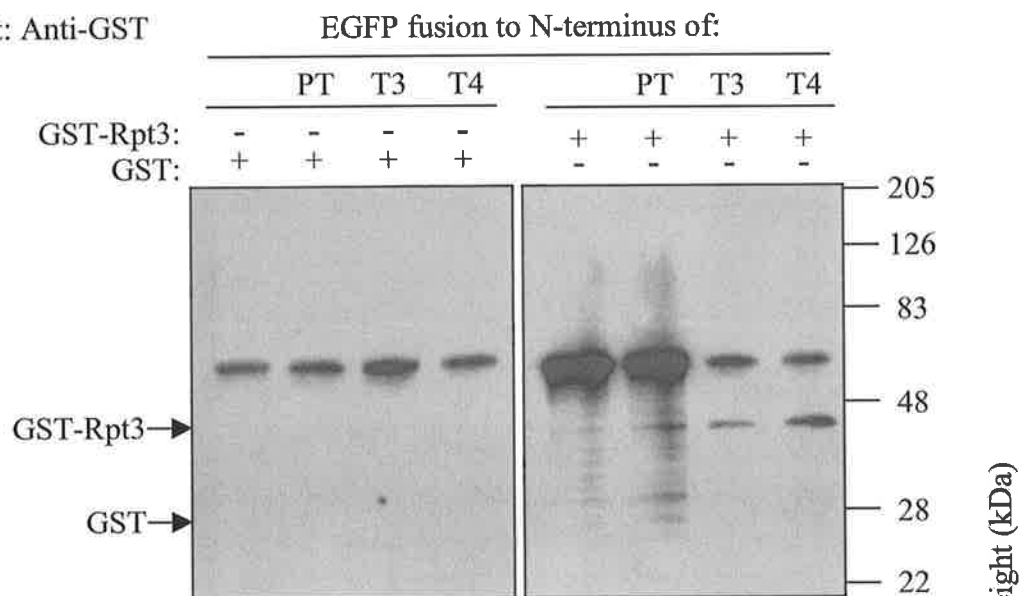
Primary antibodies were used at 1/4000 dilution, while secondary antibodies were used at 1/3,000. Sigma Colour Markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

EGFP:	enhanced green fluorescent protein
PT:	PHTH domain
T3:	Tec3
T4:	Tec4

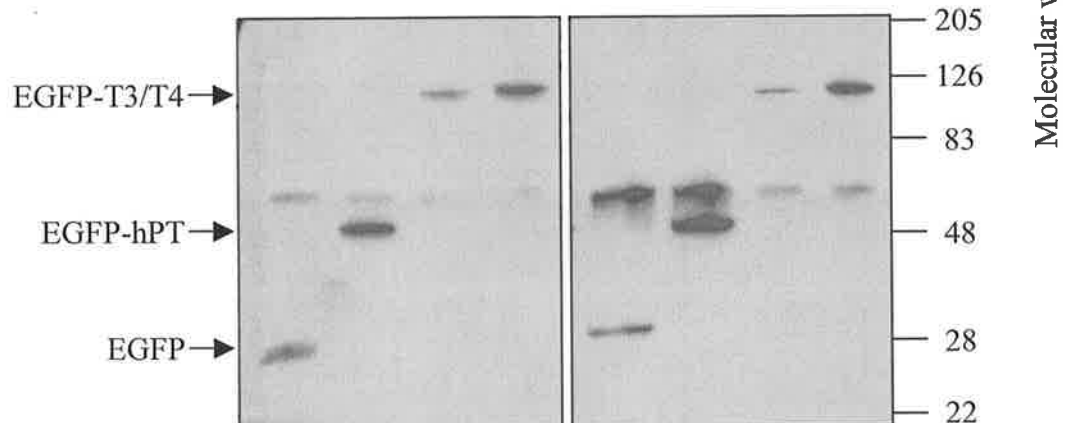
A

IP: Anti-GFP

Blot: Anti-GST



B



in T lymphocytes (see Figure 1.7). Therefore, a phagocytosis assay was set up to investigate the relationship of Tec and Actinin-4 during Fc- γ R-mediated phagocytosis.

The U937 human cell line (Sundstrom and Nilsson, 1976) was chosen for analysis in the phagocytosis assay since Tec and Actinin-4 proteins were identified in these cells by Western blot (Figure 4.1C and data not shown) and these cells are routinely used in phagocytosis experiments. U937 cells were derived from a histiocytic lymphoma and express many of the monocyte-like characteristics exhibited by cells of histiocytic origin. They grow non-adherently and bear receptors for Fc and C3, and phagocytose antibody-coated particles. Phorbol ester can be used to terminally differentiate U937 cells (Gidlund *et al.*, 1981). Upon differentiation with 10 ng.mL^{-1} PMA for 48 h, the PMA-U937 cells adhered to tissue culture plastic. Early attempts at staining phorbol myristate acetate differentiated U937 (PMA-U937) cells kept in suspension during phagocytosis experiments were unsuccessful due to loss of cells in wash steps during the immunofluorescence staining procedure. However, samples analysed by light microscopy at different time points of the phagocytosis experiments performed in suspension, showed particle uptake into these spherical cells. This confirmed that these cells were capable of phagocytosis.

To facilitate immunofluorescent staining of PMA-U937 cells in phagocytosis experiments, they were adhered to glass coverslips that were coated with cellular fibronectin (cFn) or poly-L-lysine (PLL), as they did not adhere to untreated coverslips. The two reagents attach cells in different ways. Adhesion to fibronectin-coated substratum is mediated by the integrin-family of transmembrane receptors, whereas adhesion to PLL, a non-specific attachment factor, is mediated through negatively charged ions of the cell membrane.

In preliminary phagocytosis experiments, PMA-U937 cells were exposed to Zymosan A at 37°C for up to 10 min to allow engulfment of the particles before fixing and staining for immunofluorescence analysis. Specifically, the PMA-U937 cells were grown on tissue culture plastic for 48 h, harvested and plated onto cFn ($1 \text{ }\mu\text{g.cm}^{-2}$) - or PLL ($10 \text{ }\mu\text{g.cm}^{-2}$) -treated coverslips for 10 min before exposure to IgG-opsonised Zymosan A or unopsonised Zymosan A particles on ice for twenty min. Coverslips were then transferred into preconditioned media ($37^\circ\text{C}/5\% \text{ CO}_2$) for the appropriate time (0, 2, 5, 10 min) before methanol fixation.

Prior to immunostaining, cells were visualised using light microscopy. Despite input of equivalent numbers of IgG-opsonised Zymosan A or unopsonised Zymosan A particles into experiment or control samples, respectively, less unopsonised Zymosan A than IgG-opsonised Zymosan A particles were engulfed or bound per cell at the culmination of the

experiments. Therefore, binding of unopsonised Zymosan A to PMA-U937 was less efficient than binding of IgG-opsonised Zymosan A. This result suggests that particle-binding is enhanced by IgG-opsonisation. In other controls, cells were not incubated at 37°C, but, instead, kept on ice. These cells retained spherical morphology whereas cells treated at 37°C tended to spread out.

When immunofluorescent staining analysis of mammalian cell lines was used to investigate the intracellular localisation of endogenous Tec and Actinin-4 proteins, no signal was detected. This was despite a number of different attempts, in which the concentration of antibodies was adjusted and different secondary antibodies were employed. In particular, secondary antibodies consisting of Fab₂ fragments conjugated to FITC or TRITC were used. These antibodies lack the Fc portion and, therefore, cannot cross react with Fc receptors involved in the phagocytosis process. Due to time constraints, the technical problems encountered in this section of work were not addressed further. However, independent literature implicates Tec and Actinin-4 in actin cytoskeleton rearrangement and localisation to the phagosome during phagocytosis (Araki *et al.*, 2000, Atmosukarto, 2001).

4.5 Discussion

During signal transduction, signals are propagated via the recruitment and activation of a plethora of intracellular molecules. Protein-protein interaction domains play a fundamental role in this process. Tec-family kinases have PHTH, SH3 and SH2 domains, each of which are capable of mediating specific intermolecular interactions. Furthermore, the PHTH and SH3 domains have been found to function as critical regulators of protein tyrosine kinase activity (Park *et al.*, 1996, Yamashita *et al.*, 1996, Scharenberg *et al.*, 1998, Nore *et al.*, 2000). Protein ligands of PH domains were not well characterised at the beginning of this PhD project and no link between Tec-family kinases and the cytoskeleton had been established.

As described in Chapter 3, an interaction between the PHTH domain of Tec and the third spectrin repeat of Actinin-4 was identified by yeast two-hybrid assay. As a precursor to demonstrating a biological significance for this interaction, it was necessary to establish that Tec and Actinin-4 interact in mammalian cells, which was confirmed using co-IP experiments. Since *in vitro* IP techniques are routinely used to dissect signalling pathways and identify physical links between proteins, this method was incorporated to test the interaction of various Tec and Actinin-4 proteins.

4.5.1 The Interaction of Tec and Actinin-4 in Mammalian Cells

In adherent MCF-7, COS-1 and HepG2 cells, the subcellular distribution patterns of Tec and Actinin-4 show evidence of colocalisation in an ultra fine filamentous network. During processing of the cells for immunofluorescence analysis, the cells were treated with phosphate-buffered saline containing 0.1% Triton-X100 prior to staining in order to permeabilise them. Although this is a common technique that is used to improve the access of antibodies into the cells, according to recent literature this may remove any soluble proteins and affect the staining pattern (Greenwood *et al.*, 2000). Therefore, only the Triton-X100 insoluble fraction may have been visualised by the staining method used.

Lysis and fractionation of the MCF-7, COS-1 and HepG2 cells by centrifugation into a Triton-X100 soluble cytoplasmic fraction and Triton-X100 insoluble cytoskeletal-rich pellet identified two cellular pools of Tec and Actinin-4. Both proteins were identified in both fractions although Tec was more prevalent in the soluble fraction. Therefore, it is foreseeable that only a portion of the two proteins interacts under the conditions in which the cells were grown.

Potentially, the cytoskeletal pool of Tec identified in experiments described in this thesis could be attached to the sub membranous cortical actin cytoskeleton framework through interaction with Actinin-4. It remains to be tested whether or not Actinin-4 is a substrate of the Tec kinase domain and, if so, whether tyrosine phosphorylation affects the F-actin-binding of Actinin-4. Phosphorylation has been detected in the actin-binding domain of Actinin-1 and this was shown to negatively affect F-actin-binding (Izaguirre *et al.*, 2001). Upon stimulation, Tec would be in a position to promote the release of Actinin-4 from the cytoskeleton, to break its rigidity and thus allow for cytoskeletal rearrangement. In the absence of Tec stimulation, Actinin-4 could remain in the cytoskeleton and contribute to cell structural integrity.

Co-IP experiments with epitope-labelled exogenous proteins indicate that a portion of the soluble Tec and Actinin-4 proteins interact in cultured cells. When anti-GFP antibody was used to immunoprecipitate EGFP-Tec proteins, human and mouse Tec PHTH domain were found to co-IP similar amounts of Myc epitope tagged Actinin-4 protein. This indicates that the sequence differences in the PHTH domains of human and mouse Tec do not affect Actinin-4 binding. It would be important to verify this result by testing the binding of Actinin-4 to full-length human Tec protein. Furthermore, the mouse Actinin-4 cDNA was

recently isolated by Dear and co-workers (Dear *et al.*, 2000). It would be useful to obtain this clone and test the interaction of mouse Tec and Actinin-4.

An unexpected and as yet unexplained result, was the identification by Western blot of two bands of Myc-epitope-tagged Actinin-4. These proteins were found to exist in similar amounts when analysed by Western blot of Triton-X-100 soluble and insoluble cell fractions as well as by IP with anti-Myc antibody. Chemical modification such as phosphorylation on tyrosine, serine or threonine residues may be the reason for the difference in size. However, preliminary experiments suggest that they are not phosphotyrosine variants. This is consistent with recent literature that identified the N-terminal region and not any other region of Actinin-1 as a site of tyrosine phosphorylation (Izaguirre *et al.*, 2001) and this region was removed during cloning of Myc-A4. Another possibility is that the smaller species is a proteolysis product of the other. A report by Selliah *et al.*, 1996, showed that α -actinin exists as a 105 kDa protein in resting T cells, but that an 80 kDa lower molecular form of α -actinin was produced by proteolytic cleavage by calpain after TCR stimulation of T cells. Interestingly, Btk was recently identified as an endogenous substrate of calpain proteolytic cleavage in thrombin stimulated platelets (Mukhopadhyay *et al.*, 2001b). Alternatively, the production of the doublet could be an artefact of overexpression in this transient transfection system.

4.5.2 The Effect of Specific Mutations on Tec Subcellular Localisation and Solubility

Obtaining sufficient soluble EGFP-Tec3 and EGFP-Tec4 fusion protein for co-IP experiments was difficult since the majority of the fusion protein partitioned in the Triton-X100 insoluble fraction (see Figure 4.4). A number of different lysis buffers as well as sonication were used to lyse the transfected cells in an attempt to increase yields of soluble fusion protein. These solutions contained different buffering agents (Tris or HEPES) as well as different detergents (Triton-X100 or NP40) combined in different ratios, and other chemicals such as glycerol and salts. However, none of the solutions significantly improved the solubility of EGFP-Tec.

A possible link between solubility and subcellular localisation was identified for the various EGFP-Tec proteins. Direct fluorescence microscopy of EGFP-Tec transiently transfected COS-1 cells, identified a large pool of fluorescence in a juxta nuclear region in the majority of transfected cells. This region of intense fluorescence was not obvious in COS-1 cells expressing EGFP alone but was detectable when they expressed EGFP-PHTH (see

Figure 4.10). The juxta nuclear fluorescence was diminished in size and brightness in COS-1 cells transfected with plasmids expressing EGFP-Tec fusion proteins containing K18A/K19A/K20A or Y187E amino acid substitution mutations in Tec PH or SH3 domains, respectively (see Figures 4.10 to 4.12). The fluorescence was dispersed into numerous smaller vesicles or localised to the cell membrane, respectively, and the majority of these mutant fusion proteins partitioned in the Triton-X100 soluble fraction (see Figure 4.7). In addition, a portion of endogenous Tec protein was observed in the juxta nuclear region by indirect immunofluorescence microscopy and a portion was detected in the insoluble pellet fraction by Western blot. It is therefore possible that association with the juxta nuclear structures correlates with Tec solubility. These structures are likely to include a pool of proteins trapped in or associated with the endoplasmic reticulum and golgi in the process of vesicular transport to or from the plasma membrane.

Decreased actin or phosphoinositide-binding for the triple mutant K18A/K19A/K20A could explain decreased distribution to the insoluble cytoskeletal fraction. However, the reason for increased solubility of the Y187E mutant is not immediately clear. There is a possibility that the loss of tyrosine phosphorylation due to substitution of tyrosine with glutamate prevents SH2-ligand-dependent translocation of Y187E to insoluble signal complexes.

Phosphorylation of EGFP-Tec3 and EGFP-Tec4 was only detected in the insoluble pellet fraction suggesting that phosphorylation is involved in determining Tec solubility. Indeed, Western blot analysis showed slower migration of insoluble Tec fusion protein compared with the soluble counterpart, consistent with increased molecular weight due to post-translational modification such as phosphorylation. Membrane localisation and phosphorylation are both requirements for full activation of Tec-family proteins (Park *et al.*, 1996, Rawlings *et al.*, 1996). A number of factors may contribute to determining the solubility of Tec. Besides phosphorylation, they include the abundance of phospho-binding ligand(s) as well as intramolecular and intermolecular interactions mediated between and with the different domains.

4.5.3 The Effect of Actin-binding Residues on Tec and Actinin-4 Binding

The PH and SH3 domain mutants were made in preparation for co-IP experiments that test the binding of Tec in different activation states and to assess the influence of particular key residues on binding. Many PH domains, including those of Tec-family kinases, have

strong electrostatic polarization (Blomberg and Nilges, 1997, Hyvonen and Saraste, 1997, Baraldi *et al.*, 1999, Okoh and Vihinen, 1999). Therefore, it is no surprise that specific charged residues have been implicated in the binding of PH domain ligands and subcellular targeting (Burks *et al.*, 1998, Yao *et al.*, 1999).

Tec binds Actinin-4 lacking the actin-binding domain, indicating that the interaction is not a consequence of both proteins binding to F-actin. The possibility of formation of endogenous Actinin-4 and Myc-A4 heterodimers cannot be excluded, although, Myc-A4 dimers are most likely the predominant species due to overexpression of Myc-A4. Alanine substitution of three lysine residues that are critical actin-binding determinants of the Tec PH domain did not appear to affect Actinin-4-binding. Although this substitution would decrease the net positive charge of the PHTH domain, the possibility still exists that the Tec:Actinin-4 interaction may depend on interaction of electrostatically polarized surfaces. In addition, F-actin may promote the interaction of Actinin-4 with Tec by bringing the proteins into close proximity.

Phosphoinositide-binding sites overlap with actin-binding regions in both Tec-family kinases and α -actinins. Both families of proteins have been demonstrated to bind PI 3,4,5-P₃, a product of PI3K activation. F-actin-binding competes with phosphoinositide-binding for the Btk PH domain (Yao *et al.*, 1999) and PI 3,4,5-P₃-binding has been proposed to alter the affinity of Actinin-1 for actin filaments (Greenwood *et al.*, 2000), although this has not been directly demonstrated. In rat embryonic fibroblasts, purified PI 3,4,5-P₃ was shown to mimic the effect of PDGF treatment, which induced redistribution of Actinin-1 from focal adhesion plaques and the insoluble actin cytoskeleton to the soluble cellular fraction (Greenwood *et al.*, 2000). The possibility exists that Tec-family kinases are involved in the influence of PI 3,4,5-P₃ on α -actinin function.

Residues in β 1- β 2 and β 3- β 4 loops of the Btk PH domain form the binding pocket of Inositol 1,3,4,5-P₄ (Baraldi *et al.*, 1999). K12 and R28 make specific contacts with the 3- and 4-phosphates while Q16, K17 and K18 stabilise the 5-phosphate. Interestingly, the basic residues K12, R13, K17, K18 and K19 have, in particular, been implicated in F-actin-binding (Yao *et al.*, 1999). K19E was identified as an XLA mutation and was suggested to operate by decreasing the affinity for negatively charged membrane surface (Baraldi *et al.*, 1999). Therefore, the K18A/K19A/K20A triple substitution in the Tec PH domain may have affected not only F-actin-binding but also PI 3,4,5-P₃-binding. It would be interesting to analyse the

binding of full length Actinin-4 (containing the PIP₃ binding site) to wildtype Tec and the triple lysine mutant of Tec.

PI 3,4,5-P₃-binding may modulate the Tec:Actinin-4 interaction by acting as a cooperative ligand or inducing their colocalisation. Therefore, the interaction of the two proteins may be measurably improved in IP experiments by stimulation of the PI3K pathway in cells prior to IP, providing that this does not concomitantly cause redistribution of Tec to the insoluble fraction. Furthermore, the timing of Tec and Actinin-4 movements during cell stimulation need to be evaluated.

4.5.4 Activation of Tec Kinases and Cytoskeletal Association

Activation of Tec-family kinases requires several steps. In the inactive state, intramolecular interactions between the proline-rich region of the TH domain and the SH3 domain are predicted to hold the molecule in a closed inactive conformation (Andreotti *et al.*, 1997, Pursglove *et al.*, 2002). Upon cellular activation, PI 3,4,5-P₃ binds to the PH domain of Tec-family kinases and induces translocation of the kinase to the plasma membrane (Li *et al.*, 1997, Fluckiger *et al.*, 1998, Scharenberg *et al.*, 1998). Apparent conformational change of Tec kinases, through disruption of TH-SH3 intramolecular interactions, combined with colocalisation with Src-family kinases leads to phosphorylation of a tyrosine residue (Site 1) in the activation loop of the kinase domain (Rawlings *et al.*, 1996, Andreotti *et al.*, 1997, Wahl *et al.*, 1997, Ma and Huang, 1998). Subsequent autophosphorylation of a tyrosine residue (Site 2) in the SH3 domain precedes full kinase activity (Y223 in Btk, Y187 in Tec) (Park *et al.*, 1996, Wahl *et al.*, 1997). Tec kinases are inactivated by successive dephosphorylation of Site 1 and Site 2 phospho-tyrosine residues (Wahl *et al.*, 1997). Furthermore, recent experiments suggest that PKC- β acts as a feedback loop inhibitor of Btk activation through a highly conserved PKC- β serine phosphorylation site in a short linker within the TH domain of Btk (Kang *et al.*, 2001). Therefore, phosphorylation is a major regulator of Tec-family kinase activation.

Activated Tec kinases colocalise with signalling complexes through adapter proteins of the SLP-family and lead to phosphorylation and activation of PLC- γ and downstream pathways (Fluckiger *et al.*, 1998, Su *et al.*, 1999, Tomlinson *et al.*, 1999, Bony *et al.*, 2001, Lewis *et al.*, 2001). Increased phospholipid turnover and inositol trisphosphate generation leads to increased calcium influx from intracellular stores and extracellular sources;

intracellular calcium concentrations provide cell-specific information that is important for cell homeostasis (Berridge, 1993).

Tec, Src and Syk/ZAP-70 kinases act in concert to phosphorylate multiple substrates and to activate a variety of signalling pathways, including Ras and phosphatidylinositol 3-kinase activation (Kurosaki, 1998). These pathways are involved in growth and differentiation processes and include changes in the cytoskeleton and transcriptional control (Kline *et al.*, 2001). In particular, activation of Rho/Ras/Rac/Cdc42 pathways has been demonstrated downstream of Tec kinases (Deng *et al.*, 1998, Mao *et al.*, 1998, van Leeuwen and Samelson, 1999, Nore *et al.*, 2000, Kline *et al.*, 2001). Rho-family GTPases are intimately linked with regulation of cytoskeletal architecture (Ridley and Hall, 1992, Guillemot *et al.*, 1997).

An emerging role for Tec kinases involves cytoskeletal reorganisation. Tec kinases are involved in signalling downstream of integrins in platelets (Laffargue *et al.*, 1997, Hamazaki *et al.*, 1998, Laffargue *et al.*, 1999, Oda *et al.*, 2000, Mukhopadhyay *et al.*, 2001a, Woods *et al.*, 2001). In activated platelets, phosphorylation of Tec correlates with translocation to the cytoskeleton (Laffargue *et al.*, 1997, Hamazaki *et al.*, 1998). Recently, this was also shown for Btk (Mukhopadhyay *et al.*, 2001a). Therefore, active Tec kinases may translocate to insoluble signal complexes. The possibility exists that improved Actinin-4-binding occurs upon redistribution of Tec to the insoluble fraction.

Time did not permit the production of Tec mutants that would be useful in evaluating the activation state dependence of Tec on Actinin-4-binding. The Tec4 mutants, Y187E and K397E, were designed to have altered kinase activity. It was anticipated that these mutants would have altered conformation and phosphorylation levels compared with wildtype Tec and that this could affect ligand binding. This issue needs to be addressed in future experiments that assess the conditions that promote Tec and Actinin-4 binding. It would be interesting to create double mutants in different Tec domains and evaluate their Actinin-4-binding characteristics.

4.5.5 Biological Consequences of Tec and Actinin-4 Interaction

The translocation of Tec kinases to the cytoskeleton upon stimulation of cellular receptors suggests that insoluble cytoskeletal proteins are targets of tyrosine kinase regulation. Since tyrosine kinase signalling pathways are activated immediately downstream of cellular receptors, this helps to explain the concomitant swiftness of cytoskeletal reorganisation. It is

expected that Tec kinases translocate to the sub-membranous cortical actin cytoskeleton and are, therefore, in close proximity to PI3-kinases and their products.

4.5.6 A Potential Role for Tec and Actinin-4 in Phagocytosis

Imaging techniques are used to define the intracellular localisation of specific proteins and are especially important in analysing protein movements upon cell stimulation. By identifying parallels between BCR, TCR and Fc- γ R signalling, and combining this knowledge with recently defined cytoskeletal links of Btk (Yao *et al.*, 1999) and Tec (described in this thesis), Atmosukarto (2001) predicted and demonstrated the involvement of Tec in Fc- γ R-dependent phagocytosis of IgG-opsonised particles. Importantly, that work complements the studies described in this thesis and helps to consolidate the functional significance of the Tec:Actinin-4 interaction. There is a rapid rise in cellular PI 3,4,5-P₃ levels upon Fc- γ R stimulation (Ninomiya *et al.*, 1994). This is expected to drive the PH domain-dependent recruitment of Tec to the phagosome in regions that accumulate F-actin, since studies with the PI3K inhibitor LY294002 abrogated recruitment of Tec to the phagosome (Atmosukarto, 2001). Further studies with the potent actin-polymerising inhibitor Cytochalasin D led to the prediction that recruitment of Tec to the phagosome precedes actin polymerisation during Fc- γ R-dependent phagocytosis (Atmosukarto, 2001). Actinin-4 was similarly shown to redistribute to the phagosome during phagocytosis of latex beads by macrophages (Araki *et al.*, 2000). Studies with the PI3K inhibitor Wortmannin indicate PI3K may be involved in signal transduction for Actinin-4 recruitment and/or F-actin-binding (unpublished data cited by Araki *et al.*, 2000). Therefore, Tec together with Actinin-4 is expected to bridge PI3K activation and actin cytoskeleton restructuring during phagocytosis and, potentially, in analogous signalling systems downstream of a variety of cellular receptors that have previously implicated Tec kinase.

In conclusion, the interaction of Tec and Actinin-4 was confirmed in mammalian cells. An assay system to test the binding of Tec and Actinin-4 variants was prepared. Electrostatic polarization may be involved in binding of Tec to Actinin-4. Further studies are required to probe the involvement of Tec binding to Actinin-4 during cytoskeletal remodelling in processes such as Fc- γ R-dependent phagocytosis of IgG-opsonised particles. Progress in dissecting the role of the interaction is addressed in the following chapter.

CHAPTER

5

Actinin-4 Repeat-3 Protein Structure

5.1 Introduction

5.1.1 Background

In cancer cells, the subcellular localisation of Actinin-4 correlates with sites of active cell movement and Actinin-4 is, therefore, implicated in the metastatic potential of human cancers (Honda *et al.*, 1998a). If Actinin-4 function at the leading edge of cells is influenced or regulated by Tec kinase activity, then preventing the Tec induced release of Actinin-4 from the actin cytoskeleton could restrict cell movement and prevent infiltration of cancer cells during metastasis. Potentially, the Tec:Actinin-4 interaction could be modulated through the addition of factors that either prevent binding by steric hindrance, prevent the consequence of binding, such as, prevent the release of Actinin-4 from actin, or dramatically increase the binding affinity by stabilisation. If Actinin-4 is a substrate of Tec, the interaction could be modulated through inhibition of Tec kinase activity either directly or indirectly through its regulatory domains.

Contemporary techniques use small molecular weight drugs for interference of protein interactions or activity. Advantages over peptide ligands include absorption through cell membranes, cost/ease of production and, in clinical trials if the drug proves useful, oral rather than intravenous delivery and avoidance of host immune system.

Structure-based drug design has provided a crucial link in analysis of biological function with respect to protein structure. It requires intimate knowledge of the three dimensional structure of the protein domain of interest and the interface through which it interacts with other proteins or ligands. Thus, recombinant protein production is generally required to obtain the protein domain structure.

Structure based drug design is used to design specific ligands for specific protein motifs and identify predicted high affinity ligands through computer simulation of binding. Ligands can be designed to interfere with or enhance interactions mediated with the protein of interest. Synthesis of the designed compound enables its use in experiments that test the biological function of the protein of interest. For the Tec protein, the compound could be used to test the involvement of Tec with Actinin-4 during phagocytosis. It is expected that different conformations exist for the bound and unbound Tec domains in order to signal to other parts of the Tec protein and influence the regulatory domains and/or enzyme activity.

The three dimensional structure has been resolved for the PH domains of Btk (Hyvonen and Saraste, 1997, Baraldi *et al.*, 1999), dynamin (Downing *et al.*, 1994, Ferguson *et al.*, 1994, Timm *et al.*, 1994, Fushman *et al.*, 1995), PLC- δ (Ferguson *et al.*, 1995),

pleckstrin (Yoon *et al.*, 1994), Sos (SOS; Koshiba *et al.*, 1997, Zheng *et al.*, 1997), β -spectrin (Macias *et al.*, 1994) and β -ARK1 (Fushman *et al.*, 1998). They each have a similar fold consisting of a seven-stranded β -sandwich closed at one end with a C-terminal α -helix (see Figure 1.4A; Blomberg *et al.*, 1999). β -strands 1-4 form one β -sheet, while the other is formed by β -strands 5-7 (Yoon *et al.*, 1994). The hydrophobic core of the protein includes a tryptophan residue that is conserved in all PH domains. This tryptophan, which makes contacts with many other core hydrophobic amino acids, is thought to have an integral structural role (Zhang *et al.*, 1995). PH domains have structural homology to the phosphotyrosine-binding (PTB) domain (Zhou *et al.*, 1995, Eck *et al.*, 1996), Enabled/VASP homology (EVH1) domain (Prehoda *et al.*, 1999) and Ran-binding domain (RanBD) (Vetter *et al.*, 1999), however, these domains bind different ligands.

The greatest sequence variability is in the loops between the β -strands and is thought to contribute to binding-specificity of different PH domains. The PH domain surface has strong electrostatic polarisation and it is, therefore, not surprising that PH domain ligands include complementary charged surfaces as predicted by Ferguson *et al.*, 1994. Most PH domains bind phosphatidylinositol lipids and Tec-family PH domains specifically bind 3-phosphorylated inositol derivatives, products of phosphatidylinositol-3-kinase (Blomberg *et al.*, 1999). Indeed, the structure of the Btk PH domain was determined in complex with inositol 1,3,4,5-tetraphosphate (see Figure 1.4B; Baraldi *et al.*, 1999). Recently, PI 3,4,5-P₃ was shown to directly regulate Btk kinase activity (Saito *et al.*, 2001).

Overlapping binding sites have been identified for Btk PH domain ligands (see Figure 3.1). For example, competition experiments suggest that PI 4,5-P₂ competes with both PKC- and F-actin-binding of Btk PH domain but F-actin has no effect on PKC-binding (Yao *et al.*, 1997, Yao *et al.*, 1999). Amino acids 11-20 of the PH domain, which are present in the first β -strand, represent the minimum actin-binding site while a region incorporating the β -strands 2-3 binds PKC.

The crystal structure of the rod domain of Actinin-2 was recently determined. Initially, the structure of repeats 2 and 3 was solved (Djinovic-Carugo *et al.*, 1999) and this has been built upon with the more recent structure of repeats 1-4 (Ylanne *et al.*, 2001). Individual spectrin repeats consist of three α -helices that form a triple helical bundle structure and are connected by helical linkers. Electrostatic interactions are important for the formation of the antiparallel symmetrical dimers. From one end of the rod domain to the other there is an

extensive torsional twist. The rod domain contains a conserved acidic surface and is considered a potential interaction site for intracellular signalling molecules.

5.1.2 Structural Determination by NMR Spectroscopy

The three dimensional structure of proteins can be determined from samples in solution using nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy uses the magnetic spin properties of atomic nuclei, such as ^1H , ^{15}N , and ^{13}C , to identify physically close atoms (Howard, 1998). The nuclei, when oriented by a magnetic field, absorb radiation at a particular frequency depending on their local molecular environment (Roberts, 1993). The frequency at which nuclei resonate is measured as chemical shift or parts per million (ppm) relative to a reference signal. In a one-dimensional (1D) ^1H spectrum the intensity of the signal is measured and plotted against chemical shift.

Protons contained within similar molecular structures, such as functional groups, resonate at similar frequencies. In unstructured polypeptide chains, protons in the following functional groups resonate at characteristic frequencies: methyl group (CH_3) at ~ 1 ppm; methylene group (CH_2) at ~ 2 -3 ppm; α -protons at 4-5 ppm; aromatic groups at 6-7.5 ppm; and, finally, amide groups at 7-11 ppm (Wuthrich, 1986). However, the frequency of proton resonance can be shifted upfield (lower ppm value) or downfield when the polypeptide chain is folded and contacts are formed between different residues.

Spectral overlap is observed when numerous protons exist in similar environments. Therefore, 1D spectra need to be extended into two or more dimensions in order to determine full protein structures by NMR spectroscopy. In multidimensional experiments, the interaction between the same (homonuclear i.e. ^1H - ^1H) or different (heteronuclear eg. ^1H - ^{15}N) types of nuclei are observed. These experiments include correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and nuclear Overhauser enhancement spectroscopy (NOESY) experiments. COSY and TOCSY experiments reveal information about adjacent nuclei that have through-bond linkages (Wuthrich, 1986). NOESY experiments detect interactions between nuclei that are close together in space (less than 5 Å apart) but not necessarily close in the linear sequence of the peptide (Wuthrich, 1986).

To resolve a protein structure, each resonance needs to be mapped to its corresponding nucleus using sequence-specific resonance assignments. After building a list of distance restraints between nuclei, the protein structure is resolved by creating a model in which each distance restraint is satisfied.

5.2 Aims

The aim of the work described in this chapter was to express and purify Tec PHTH domain (PH domain and Btk motif of the TH domain) and Actinin-4 spectrin repeat 3 proteins for structural determination in the ligand-bound state to identify residues of the interaction interfaces. This will enable the future design and testing of small molecular weight drug ligands to probe the biological function of the Tec:Actinin-4 interaction.

5.3 Approaches

Several protein chemistry techniques were used to purify recombinant Tec and Actinin-4 protein domains for structural studies. Two bacterial expression systems, pGEX and pET, were used to express and purify PHTH and Rpt3 proteins as fusions with a protein partner. These systems manipulate bacterial cells into expressing high levels of soluble protein that can be purified through a moiety of the fusion partner. In some instances, the proteins are not soluble but instead form aggregates or inclusion bodies. The BL21(DE3) strain of *Escherichia coli* was used for recombinant protein expression. This strain lacks the Lon protease and the ompT outer membrane protease and, therefore, reduces the occurrence of protein degradation (Grodberg and Dunn, 1988). The DE3 host contains a chromosomal copy the T7 polymerase gene, which provides better regulation of protein induction when using the pET vector protein expression system. Optimal bacterial expression of fusion proteins was assessed using SDS-PAGE.

The pGEX bacterial expression system was used to separately express and purify PHTH and Rpt3 proteins as fusions with GST. GST fusion proteins are purified from the bulk of the other *E. coli* proteins using the single-step glutathione agarose chromatography. The pET system was used in an initial attempt to obtain R817 protein (the clone identified in the yeast two-hybrid assay) as a fusion with Thioredoxin (Trx) and the N-terminal 6-His tag. The C-terminal 6-His tag-encoding sequence was not translated due to the in frame stop codon at the end of the R817 sequence. Trx fusion proteins are purified using the 6-His tag and nickel-IDA agarose chromatography.

Affinity chromatography was used to purify the GST- or Trx- fusion protein away from bacterial proteins. This step was dependent on generation of soluble fusion protein. Sonication was initially used to lyse cells, however, pressure-induced lysis was later found to be more efficient and, as a result, released higher yields of soluble fusion protein. Yields were calculated from Bradford assay estimates of purified fusion protein concentration. SDS-PAGE

was used to monitor the purification of fusion proteins. The dimerisation of Actinin-4 protein fragments was assessed by non-denaturing size exclusion chromatography using the Smart System (Pharmacia).

Thrombin protease was used to separate fusion proteins into their GST and Rpt3 or GST and PHTH components. Progress of enzymatic cleavage reactions was measured using SDS-PAGE. Size exclusion chromatography was used to purify Rpt3 protein away from carrier GST protein. The structure of purified Rpt3 protein was examined by preliminary 1D and 2D NMR spectroscopy analysis.

5.4 Results

5.4.1 Recombinant PHTH Domain Protein Expression and Purification

Protein expression and purification experiments were performed in a preliminary step to investigate the association interface of Tec and Actinin-4. PHTH domain purification was based on experiments performed by Pursglove, 2001. PHTH domain-encoding sequence cloned into the pGEX2T vector (Amersham-Pharmacia Biotech) and transformed into *E. coli* BL21(DE3) bacterial cells was obtained from S. Pursglove and used to express GST-PHTH fusion protein. Zinc sulphate (30 μ M) was included in the media to aid in the proper folding of the Btk motif of the TH domain, which, in Btk, was shown to coordinate a zinc ion (Hyvonen and Saraste, 1997).

Bacteria transformed with pGEX2T-PHTH plasmid were grown at 37°C to an OD_{600nm} of 0.6 and GST-PHTH protein expression was induced with 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 4.5 h. Cells were harvested by centrifugation and lysed by sonication in Triton-X-100 Tris buffered saline (TTBS) supplemented with the protease inhibitor phenylmethylsulfonylfluoride (PMSF, 1 mM) (Section 2.3.2.1). Samples set aside at each step of the purification process were analysed by SDS-PAGE and an induced band at the expected size for GST-PHTH protein (44 kDa) was identified (Figure 5.1A). The majority of the GST-PHTH fusion protein partitioned in the Tween-20 insoluble pellet (P) fraction compared with the soluble supernatant fraction (S). As a result, the overall GST-PHTH protein purification was inefficient. The soluble fusion protein was purified using glutathione agarose affinity chromatography (Section 2.3.2.2). When fractions of eluted GST-PHTH protein (Figure 5.1B) were pooled, a yield of 4.5 mg of fusion protein per litre of culture was obtained. This yield made production of isotope-labelled fusion protein an unfeasible experiment.

Figure 5.1 GST-PHTH Domain Fusion Protein Expression and Purification

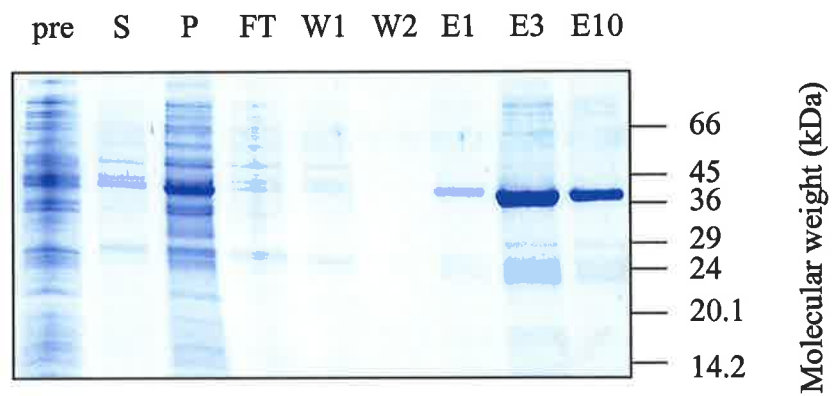
Escherichia coli BL21(DE3) cells transformed with pGEX2T-PHTH plasmid were kindly provided by Sharon Pursglove (Dr Grant Booker laboratory). GST-PHTH protein was expressed and purified as described in Section 2.3.2.1. Briefly, a 500mL culture was grown to an OD_{600nm} of 0.6 and a sample (pre) was taken before cells were induced with 0.2 mM IPTG for 4.5 hours to express GST-PHTH domain fusion protein. Cells were harvested by centrifugation and lysed by sonication in TTBS supplemented with PMSF (1 mM). Supernatant (S) and pellet (P) fractions were obtained by centrifugation. Glutathione agarose affinity chromatography was used to purify GST-PHTH protein from the supernatant fraction. Proteins that did not bind the column (equilibrated in TBS and TTBS) were collected as flow-through (FT). The column was washed extensively with TTBS (W1) and TBS (W2) and GST-PHTH protein was eluted in fresh 10 mM reduced glutathione in TBS (pH8.0). Ten 1 mL fractions of the eluate (E1-E10) were collected.

- A SDS-PAGE of samples set-aside during the expression and purification process. Samples were resolved by SDS-PAGE of a 12.5% Tris-tricine gel and stained with Coomassie blue.
- B Graph of GST-PHTH protein elution profile during glutathione agarose affinity chromatography (Section 2.3.2.2). Bradford Assay (Section 2.3.2.4) was used to estimate the concentration of protein in each of the ten eluate fractions.
- C Thrombin digest time trial of GST-PHTH protein. GST-PHTH protein (0.2 mg/mL) was digested with Thrombin (5 U/mL) at 37°C and 15 µL samples were taken at 30 minute intervals and stored at -20°C. Samples were resolved by SDS-PAGE on a 12.5% Tris-tricine gel and stained with Coomassie blue.

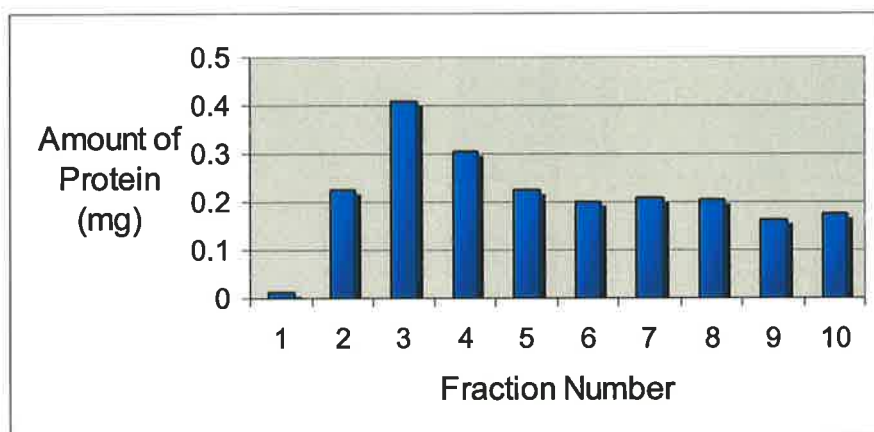
SDS-7 markers were used for size comparison of protein bands in SDS-PAGE; the positions of molecular weight standard proteins are indicated.

GST: glutathione-S-transferase
PHTH: pleckstrin homology domain and Btk motif of the Tec homology domain

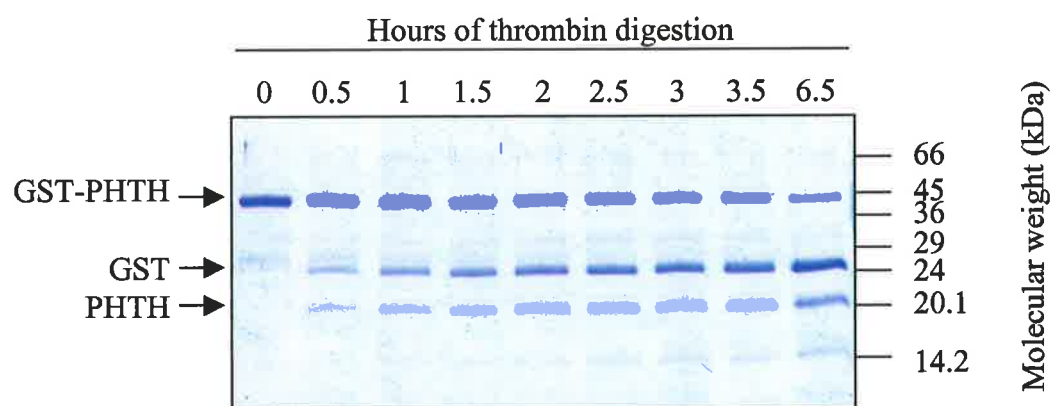
A



B



C



PHTH domain was cleaved from GST carrier protein using thrombin digestion (Section 2.3.2.7). Figure 5.1C shows a time trial of thrombin digestion of purified GST-PHTH protein to identify the optimal time for the cleavage reaction. After 6.5 h, the majority of GST-PHTH (44 kDa) was cleaved into GST (26 kDa) and PHTH (18 kDa). An extra band of approximately 14 kDa, most likely corresponding to further cleaved PHTH domain, was also present. Experiments performed concurrently by Pursglove identified precipitation of purified PHTH domain at 1 mM concentration required for NMR spectroscopy (Pursglove, 2001). Therefore, in the scope of this PhD, there was insufficient soluble PHTH protein for structural analysis.

To increase solubility of PHTH domain, it was expressed in mammalian cells in its more natural environment. As described in Section 4.4.6, PHTH was cloned and expressed as a fusion with EGFP in COS-1 cells. The fusion protein contained the PHTH domain at the N-terminus separated from the C-terminal EGFP moiety by an engineered thrombin cleavage site. The majority of this fusion protein partitioned in the Triton-X100 soluble fraction and could be immunopurified with rabbit anti-GFP antibody. Due to time constraints, large-scale purification of PHTH-EGFP was not carried out. However, it is anticipated that PHTH-EGFP expressed in mammalian cells could be used in surface plasmon resonance experiments that test the binding strength of the PHTH and Rpt3 proteins. For these experiments, it is important that the PHTH domain, which occupies the extreme N-terminus of Tec protein, is native. Thus a C-terminal fusion partner for the PHTH domain, such as that expressed from pEGFP-N2 vector, is desirable. A monoclonal antibody directed at GFP would be suitable to immobilise the PHTH-EGFP fusion protein as it should eliminate interference with the PHTH domain that occurs with other immobilisation methods such as amide coupling.

5.4.2 Recombinant Trx-R817 Protein Expression and Purification

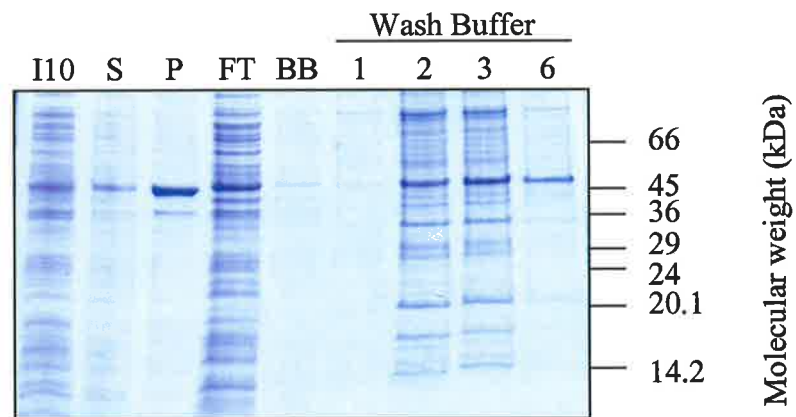
Two expression systems were used to create Actinin-4 protein for structural analysis. In an initial attempt to express and purify the protein encoded by the R817 clone that was identified in the yeast two-hybrid assay, R817 was cloned into the pET32a⁺ vector and transformed into *E. coli* BL21(DE3) cells. Expression of the Thioredoxin-R817 (Trx-R817) fusion protein was induced with 0.1 mM IPTG for 1 h. Cell lysates were prepared as for GST-PHTH and samples set aside at each step of the purification process were analysed by SDS-PAGE (Figure 5.2A). The majority of the Trx-R817 fusion protein partitioned in the Tween-20 insoluble pellet (P) fraction and not in the soluble supernatant fraction (S).

Figure 5.2 Trx-R817 Fusion Protein Expression and Purification

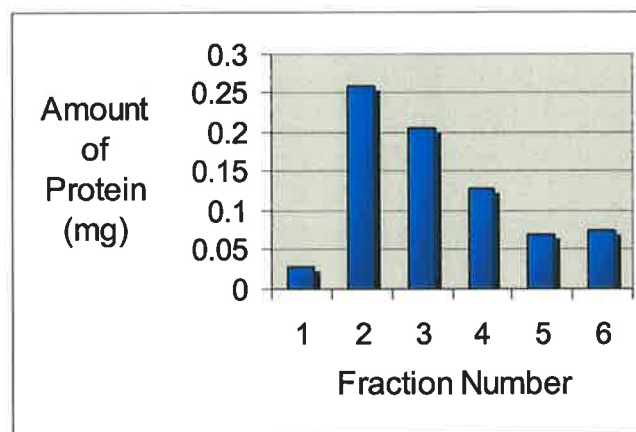
pET32a+R817 was cloned by ligation of the 731 bp *EcoRI/XhoI* fragment of R817 into pET32a+ plasmid and verified by sequencing. After transformation of the plasmid into *Escherichia coli* BL21(DE3) cells, Thioredoxin-R817 (Trx-R817) fusion protein (43 kDa) was expressed and purified as described in Section 2.3.2.1 and Section 2.3.2.3. Briefly, a 50 mL culture was grown to an OD_{600nm} of 0.6 and induced with 0.1 mM IPTG for 3 hours to express Trx-R817 fusion protein. A sample of the culture was taken 10 min after induction (I10). Cells were harvested by centrifugation and lysed by sonication in Ni-IDA Binding Buffer supplemented with PMSF (1 mM). Supernatant (S) and pellet (P) fractions were obtained by centrifugation. Nickel-IDA agarose affinity chromatography was used to purify Trx-R817 protein from the supernatant fraction. Proteins that did not bind the column (equilibrated in Ni-IDA binding buffer) were collected as flow-through (FT). The column was washed extensively with Ni-IDA Binding Buffer (BB) and Ni-IDA Wash Buffer and Trx-R817 protein was eluted in Ni-IDA Elute Buffer. Six 1 mL fractions of the Wash Buffer and ten 1 mL fractions of the Elute Buffer were collected.

- A SDS-PAGE of samples set-aside during the expression and purification process. Samples were resolved by SDS-PAGE of a 12.5% Tris-tricine gel and stained with Coomassie blue. Trx-R817 protein elutes from the column in Ni-IDA Wash Buffer along with many other proteins. SDS-7 markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.
- B Graph of Trx-R817 protein prematurely eluting in Wash Buffer during Nickel-IDA agarose affinity chromatography. Bradford Assay (Section 2.3.2.4) was used to estimate the concentration of protein in each of the six Wash Buffer fractions.
- C Graph of Trx-R817 protein eluting in Elute Buffer during Nickel-IDA agarose affinity chromatography. The scale of the graph is the same as in part (B) for comparison of protein amount in fractions. Bradford Assay (Section 2.3.2.4) was used to estimate the concentration of protein in each of the ten Elute Buffer fractions. Most of the Trx-R817 had eluted during the wash step and, therefore, not much was collected in Elute Buffer.

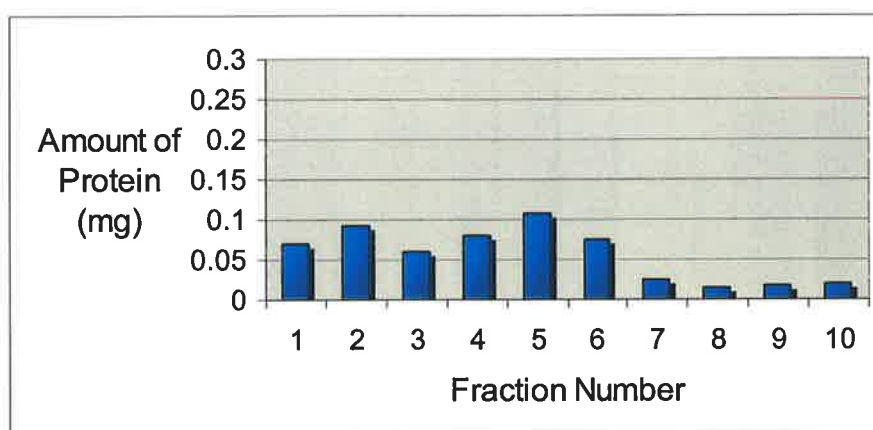
A



B



C



Nickel-IDA agarose affinity chromatography was attempted to purify the fusion protein from the soluble fraction (Section 2.3.2.3). However, most of the Trx-R817 eluted during the wash step (60 mM imidazole) before the elution step (1M imidazole) (compare Figure 5.2B and C). The binding, wash and elution buffers used in the Ni-IDA agarose purification system differ in imidazole concentration, which correlates with stringency. Therefore, in further experiments, less stringent wash steps were attempted to increase the amount of desired fusion protein retained on the column. However, Trx-R817 still eluted in the wash step, so a more efficient expression and purification system was sought.

Similar Trx-fusion protein purification experiments were performed on a subclone of R817 that spanned I498-S696 of the native Actinin-4 sequence (see Figures 3.11 and 3.14A). This peptide lacked residues of R817 that are attributed to cloning artefact from construction of the human liver cDNA library in pB42AD vector. The Trx-I498-S696 fusion protein has a predicted molecular weight of 41 kDa which was calculated using pI/MW software (Section 2.2.2). Sufficient soluble Trx-I498-S696 fusion protein was purified for dimerisation analysis.

5.4.3 Cloning of GST-Rpt3 For Expression and Purification

In a second attempt to express and purify Actinin-4, the pGEX vector system was used. Based on the yeast two-hybrid deletion series data, a fragment encompassing spectrin repeat-3 that spanned residues K518-Q645 of native Actinin-4 was predicted to contain the shortest fragment of R817 that bound Tec PHTH domain. For ease of cloning, the coding region of residues T519-Q645 (denoted Rpt3) was, therefore, amplified by polymerase chain reaction using primers #170 and #171 and subcloned into the pGEX4T2 expression vector (Amersham-Pharmacia Biotech) as described in (Figure 5.3) and verified by automated DNA sequencing. pGEX4T2-Rpt3 plasmid was transformed into *E. coli* BL21(DE3) bacterial cells. Preliminary experiments indicated that GST-Rpt3 protein partitioned in the Tween-20 soluble fraction and, therefore, expression of GST-Rpt3 was optimised.

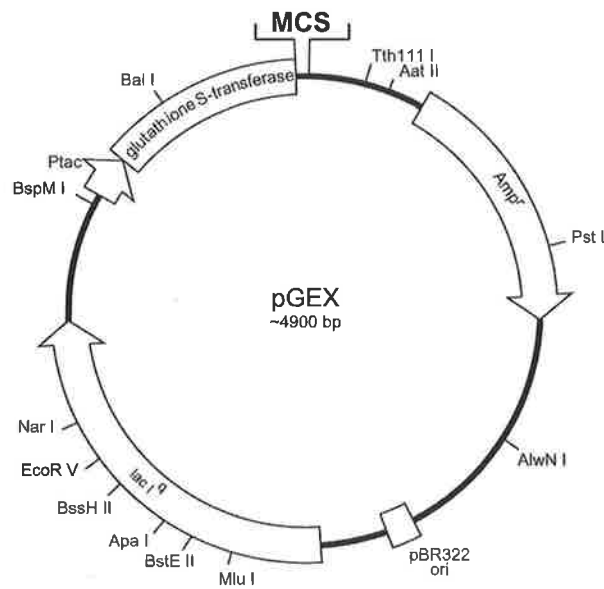
5.4.4 Optimisation of GST-Rpt3 Expression

SDS-PAGE analysis of whole cell lysates from six different induced clones and, subsequently, three different induction conditions of clone #5 was used to investigate the conditions for maximal GST-Rpt3 fusion protein expression. Fusion protein expression was investigated in MinA minimal medium (Section 2.1.8, Miller, 1972) in preparation for isotopic labelling experiments in which the only nitrogen source is $^{15}\text{NH}_4\text{Cl}$. This is important as the incorporation of a heteronucleus such as ^{15}N allows the reduction of spectral overlap

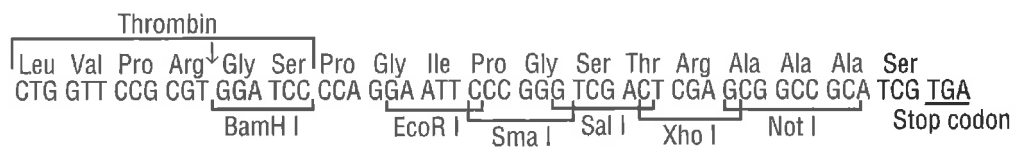
Figure 5.3 Cloning of GST-Rpt3 Expression Plasmid

- A Vector map of the plasmid pGEX4T2 that expresses the GST protein from the IPTG inducible promoter (Ptac) and has the 4T2 variant of the multiple cloning site (Amersham-Pharmacia Biotech).
- B Sequence and restriction map of the multiple cloning site of the 4T2 variant of pGEX plasmid (Amersham-Pharmacia Biotech).
- C Diagrammatic representation of the Actinin-4 third spectrin repeat encoding sequence that was cloned in frame into the *Bam*HI/*Xho*I restriction sites of pGEX4T2. The coding region of residues T519-Q645 (denoted Rpt3) was amplified by PCR using primers #170 and #171 (Section 2.1.22) which introduced *Bam*HI and *Xho*I restriction sites. The sequence of the insert was confirmed by automated DNA sequencing.
- D Linear representation of the GST-Rpt3 fusion protein used in this study. The GST tag is at the N-terminus of the protein and can be cleaved from Rpt3 by thrombin protease digestion. Predicted molecular weight was calculated using pI/MW software.

A

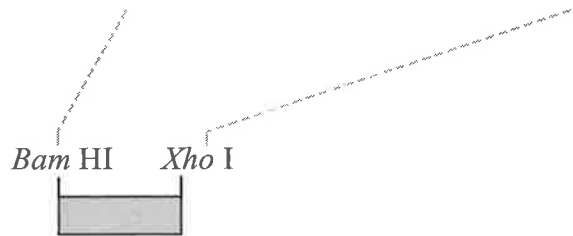


B



C

Rpt3 (390 bp)



D

GST-Rpt3 (41 kDa)



Thrmbin cleavage site

through the use of 3D NMR experiments to facilitate the spectral assignment stage of structure determination of the Rpt3 protein. MinA minimal medium contains salts and lacks the yeast extract present in Luria broth medium (Miller, 1972). Expression was optimised with respect to OD_{600nm} of bacterial culture immediately before induction, length of induction time and concentration of IPTG inducing agent (Figure 5.4A). An induced band at the expected size for the GST-Rpt3 protein (41 kDa) was identified in all induced samples. Generally, samples induced with 0.2 mM IPTG showed increased fusion protein expression compared to samples induced with 0.1 mM IPTG. Expression was greater in samples induced at an OD_{600nm} of 0.6 compared with at 0.5 or 0.55. Increased amounts of fusion protein were detected in samples induced for 4 h compared with 2 h, and this was not further increased by induction for 16 h. Therefore, in subsequent experiments bacteria transformed with pGEX4T2-Rpt3 were grown at 37°C to an OD_{600nm} of 0.6 and GST-Rpt3 protein expression was induced with 0.2 mM IPTG for at least 4 h.

5.4.5 Optimisation of GST-Rpt3 Fusion Protein Purification

Two different methods were used to lyse bacterial cells induced to express the GST-Rpt3 fusion protein. Sonication was used to effectively lyse cells in small scale experiments but was inefficient at lysing larger volumes of sample that were generated in large scale expression experiments. This was judged by SDS-PAGE of respective supernatant and pellet fractions obtained after lysis. Pellet fractions from large-scale experiments had relatively more protein, including GST-Rpt3, than that from small-scale experiments, and this was attributed to incomplete lysis of cells. The French press was identified as an effective alternative procedure to lyse the cells.

Glutathione agarose affinity chromatography (Section 2.3.2.2) was used to purify the GST-Rpt3 protein from the soluble fraction of lysates generated by sonication or the French press (Figure 5.5A and B, respectively). SDS-PAGE was used to analyse samples set aside at each step of the expression and purification process when sonication or The French press was used to lyse cells (Figure 5.4B and C, respectively). The yield of GST-Rpt3 protein recovered from the soluble fraction of cells lysed by both procedures was approximately 15 mg.L⁻¹ culture and 100 mg.L⁻¹ culture for sonication and The French Press, respectively, indicating an approximately 7-fold difference in yield. The purified GST-Rpt3 was collected and prepared for further analysis by buffer exchange into Tris buffered saline (TBS) to remove the reduced glutathione eluting agent.

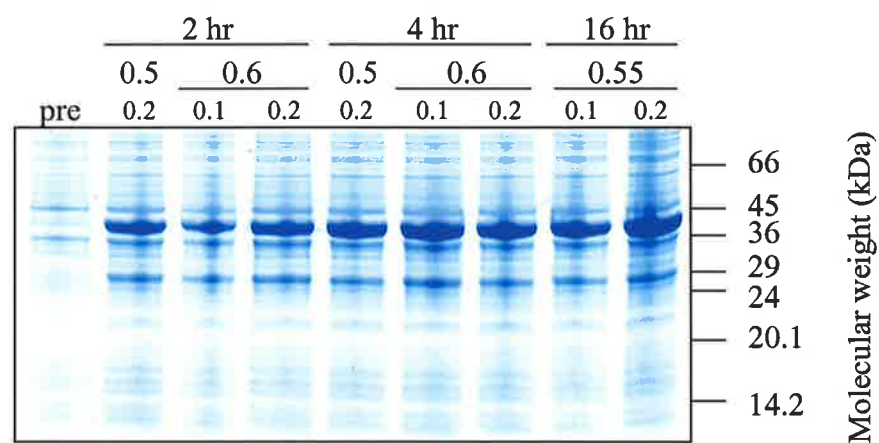
Figure 5.4 Optimisation of GST-Rpt3 Fusion Protein Expression and Purification

- A Optimisation of GST-Rpt3 protein expression. GST-Rpt3 fusion protein (40kDa) was expressed in *Escherichia coli* BL21(DE3) cells as described in Section 2.3.2.1. Combinations of different conditions were tested to identify those that provide maximum induction of GST-Rpt3 protein expression. Briefly, 50mL cultures were grown to an OD_{600nm} of 0.5-0.6 and induced with 0.1 or 0.2 mM IPTG for up to 16 hours to express GST-Rpt3 fusion protein. Samples of culture were taken before induction (pre), at 2, 4 and 16 h and resolved by SDS-PAGE on a 12.5% Tris-tricine gel that was stained with Coomassie blue.
- B SDS-PAGE of samples set-aside during the expression and purification process when cells were lysed by sonication. GST-Rpt3 protein was expressed and purified as described in Section 2.3.2.1. Briefly, a 2L culture in minimal media was grown to an OD_{600nm} of 0.6 and a sample (pre) was taken before cells were induced with 0.2 mM IPTG for 4 hours to express GST-Rpt3 domain fusion protein. A post-induction sample (post) was taken prior to cell harvest. Cells were harvested by centrifugation and lysed by sonication in TTBS supplemented with PMSF (1 mM). Supernatant (S) and pellet (P) fractions were obtained by centrifugation. Glutathione agarose affinity chromatography was used to purify GST-Rpt3 protein from the supernatant fraction (see Figure 5.5A). Proteins that did not bind the column (equilibrated in TTBS) were collected as flow-through (FT). The column was washed extensively with TTBS and TBS and GST-Rpt3 protein was eluted (E) and collected in fresh 10mM reduced glutathione in TBS (pH8.0).
- C SDS-PAGE of samples set-aside during the expression and purification process when cells were lysed by the French press. Samples were prepared as in part (B) except cells were lysed by 3 passes through the French press at 1000 PSI instead of by sonication. A post induction sample (post) was taken before cells were lysed and a sample of the filtered supernatant (filt) was taken before glutathione agarose affinity chromatography (see Figure 5.5B). The eluted GST-Rpt3 protein was cleaved into GST and Rpt3 components by Thrombin (T) digestion and samples (1 µL and 5 µL) were resolved by SDS-PAGE on a 12.5% Tris-tricine gel.

SDS-7 markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

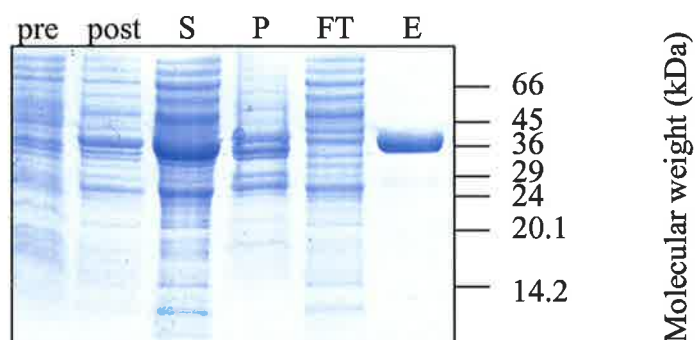
GST: glutathione-S-transferase

A



B

Sonication
Yield 15 mg.L⁻¹



C

French Press
Yield 100 mg.L⁻¹

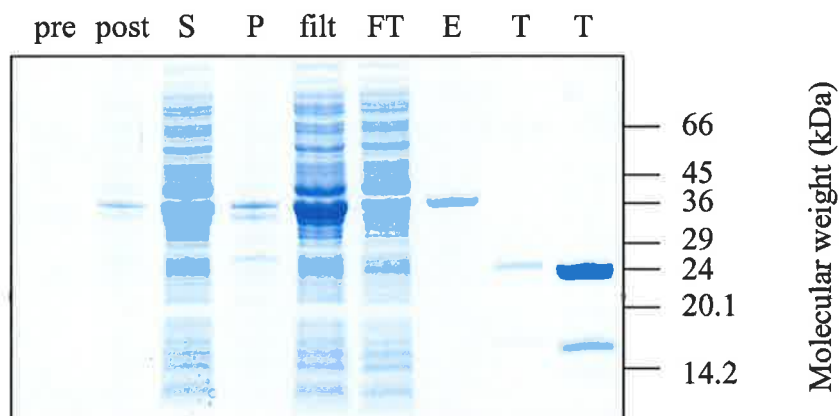
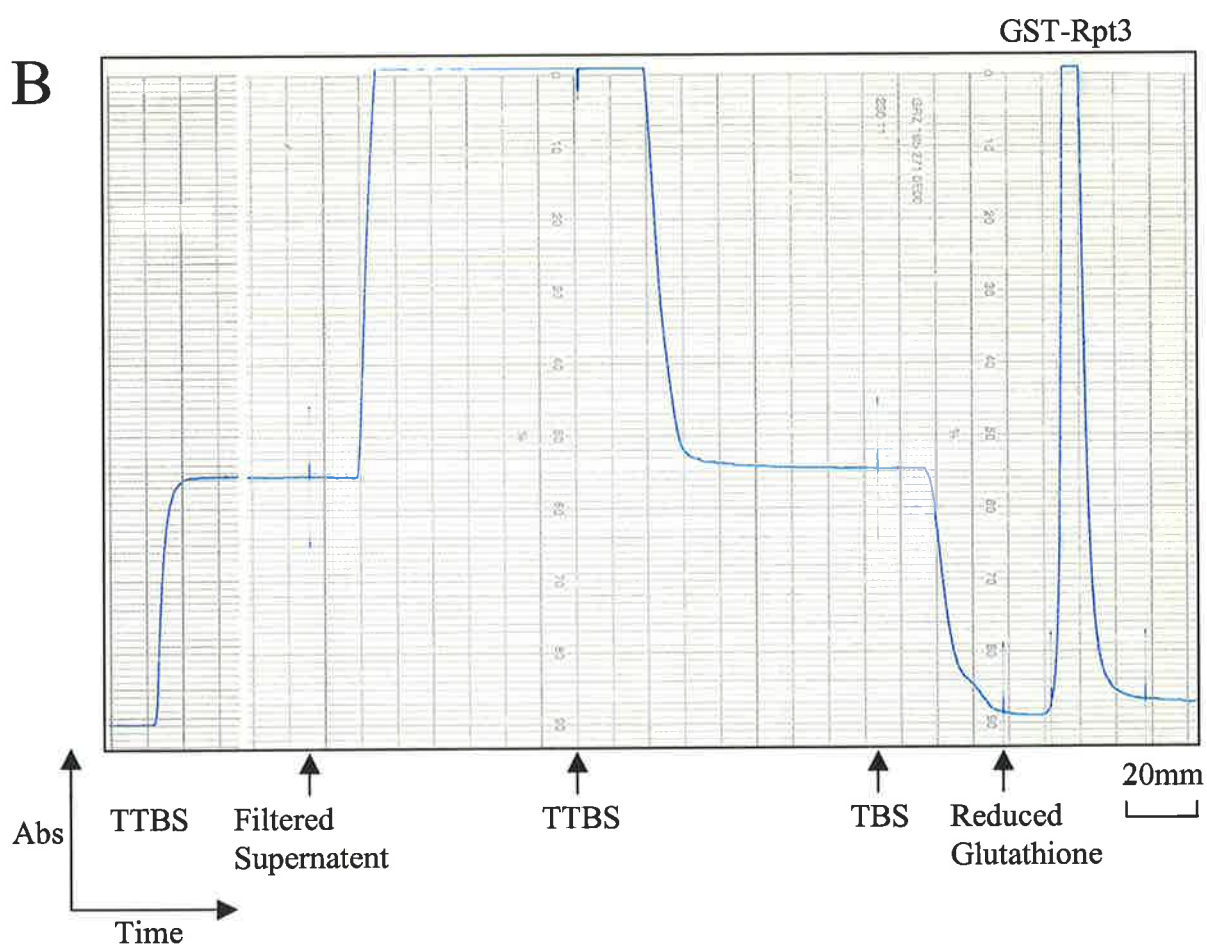
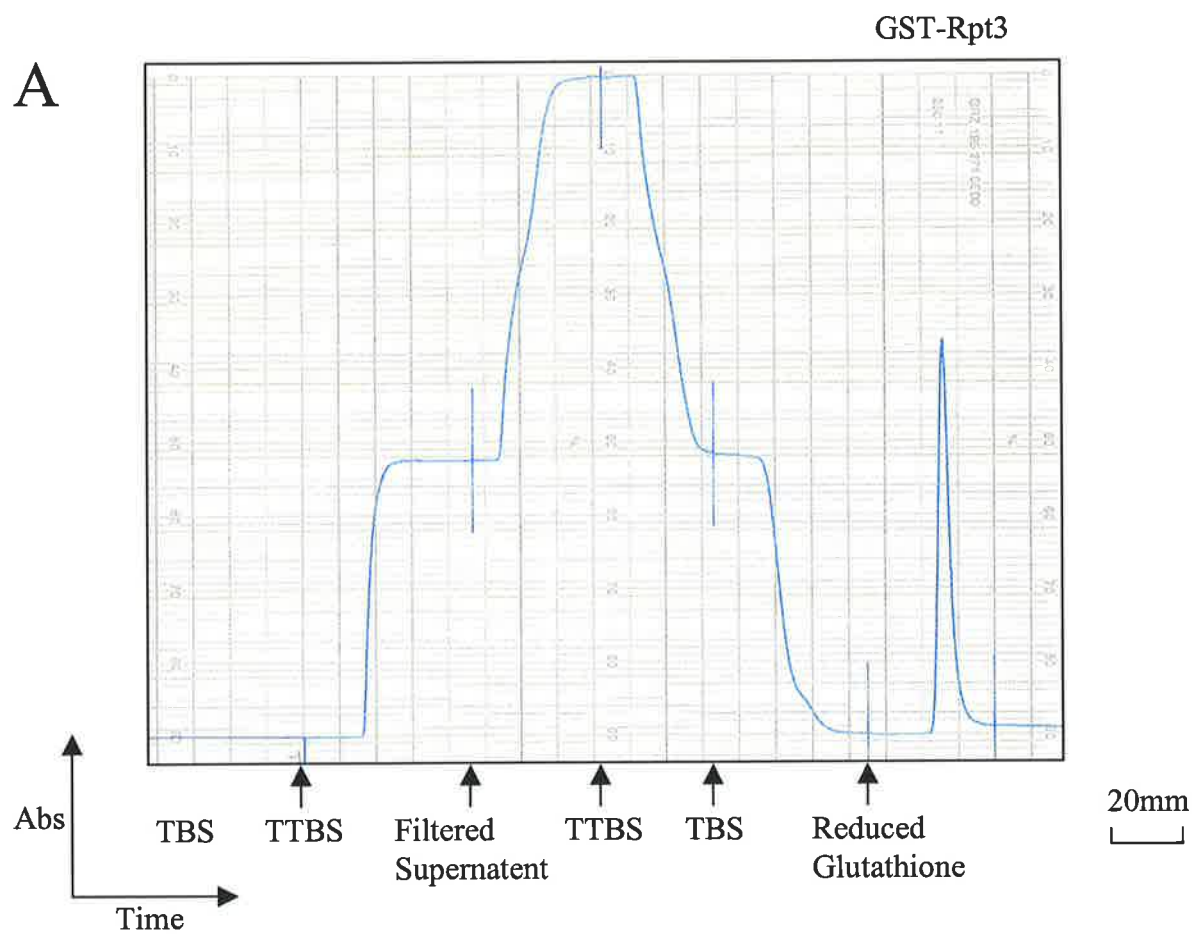


Figure 5.5 Purification of GST-Rpt3 Fusion Protein

Chart recordings of glutathione agarose affinity column purification of GST-Rpt3 fusion protein. Absorbance at 280 nm is plotted against time. GST-Rpt3 protein was expressed in *Escherichia coli* BL21(DE3) cells grown in four 500 mL cultures in minimal media as described in Figure 5.4B. The fusion protein was purified as described in Sections 2.3.2.1 and 2.3.2.2 from cells lysed by sonication or the French press. The column had a bed volume of 40 mL. After column equilibration in TBS (4 column volumes) and TTBS (4 column volumes), filtered supernatant containing the GST-Rpt3 fusion protein was loaded onto the column and the flow-through was collected. The column, with bound fusion protein, was washed extensively with TTBS and equilibrated in TBS before the GST fusion protein was eluted with fresh 10 mM reduced glutathione in TBS (pH8.0). Arrows indicate the times of change in buffer, or sample, being loaded onto the column. There is an approximately 8-minute delay time for column traversal before the detector at the end of the line records the change. In each case the flow rate was 4 mL/min, the chart speed was 2 mm/min and the absorbance units range of the detector was AU=2.

- A GST-Rpt3 purification from GST-Rpt3 expressing *Escherichia coli* BL21(DE3) cells lysed by sonication.
- B GST-Rpt3 purification from GST-Rpt3 expressing *Escherichia coli* BL21(DE3) cells lysed by the French Press.



5.4.6 Optimisation of GST-Rpt3 Thrombin Digestion

Thrombin protease digestion (Section 2.3.2.7) was used to cleave the GST carrier protein away from the desired Rpt3 protein. Optimal cleavage conditions were determined by SDS-PAGE analysis of GST-Rpt3 samples that were digested with thrombin at different temperatures and for different lengths of time (Figure 5.6A-D). When GST-Rpt3 at 1.0 mg.mL^{-1} was digested with thrombin at 10 U.mL^{-1} , the optimal cleavage reaction identified was 18^+ h at 37°C (Figure 5.6D 18 h). The effect of fusion protein concentration and thrombin enzyme concentration on the reaction was also investigated. A ten-fold decrease in thrombin concentration resulted in less complete fusion protein cleavage (Figure 5.6E lane 2) whereas a two-fold increase took the reaction to completion (Figure 5.6E lane 3). When the protein sample was concentrated (Section 2.3.2.6) prior to thrombin cleavage and the protein:enzyme ratio was maintained at 1 mg:20 U, the reaction proceeded to completion in less time and produced an over digested species of 14 kDa. This is probably a result of Rpt3 containing many lysine residues and a potential pseudo-thrombin cleavage site or auto-catalysis of thrombin leading to partial loss of specificity.

5.4.7 Purification of Rpt3 from GST

Superdex-75 size exclusion chromatography (Section 2.3.2.8) was used to separate GST and Rpt3 proteins after thrombin digestion. Figure 5.7A shows a chart recording of the chromatography step with spikes corresponding to each 5 mL fraction in PBS/0.01% NaN_3 . As shown in Figure 5.7B, in which samples corresponding to each peak were resolved by SDS-PAGE, the GST protein (26 kDa) elutes before the Rpt3 protein (14.8 kDa), as expected. The recovery of GST and Rpt3 proteins in separate peaks suggests that Rpt3 is monomeric and not dimeric as it does not co-elute with a protein approximately twice its size. No protein was identified in the third peak, which probably contained salts.

The amount of GST-Rpt3 fusion protein or separated GST and Rpt3 proteins was determined for samples taken at various steps during the purification process. These calculations were based on Bradford assay (Section 2.3.2.4) estimates of protein concentration. On average, when 50 mg of GST-Rpt3 was digested with thrombin, 30 mg of GST and 9.4 mg of Rpt3 were recovered. Based on the molecular weights of GST and Rpt3, recovery of 31.8 mg and 18.1 mg were expected, respectively. Therefore, approximately half of the Rpt3 protein was lost during the thrombin cleavage reaction and/or the size exclusion chromatography step while only 5% of GST protein was lost.

Figure 5.6 Optimisation of Thrombin Digestion of GST-Rpt3 Fusion Protein

Optimisation of GST-Rpt3 Thrombin digestion. Purified GST-Rpt3 fusion protein was digested with Thrombin as described in Section 2.3.2.7 at different temperatures and different protein and enzyme concentrations to identify the conditions that provide maximum cleavage of GST-Rpt3 protein.

Effect of temperature on Thrombin digestion of GST-Rpt3. Briefly, 1 mg/mL aliquots were digested with 10 U/mL Thrombin for up to 18 hours at the following temperatures. Samples were taken before addition of enzyme (0 h) and after 1, 2.5, 4, 5, 6, 16 and 18 h and resolved by SDS-PAGE on 10% Tris-tricine gels that were stained with Coomassie blue.

A 4°C

B 16°C

C 25°C

D 37°C

E Effect of concentration on Thrombin digestion of GST-Rpt3. Briefly, 1mg/mL aliquots were digested with 1 U/mL or 20 U/mL Thrombin for 48 hours at 37°C. In another sample, the protein concentration was increased five fold to 5 mg/mL using an Amicon stirred cell and YM10 membrane (10 kDa MWCO) and the sample was treated at 37°C for 27 hours with 100 U/mL Thrombin to maintain protein to enzyme ratio of 20 U/mg. Samples were resolved by SDS-PAGE on a 12.5% Tris-tricine gel and stained with Coomassie blue.

SDS-7 markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

GST: glutathione-S-transferase

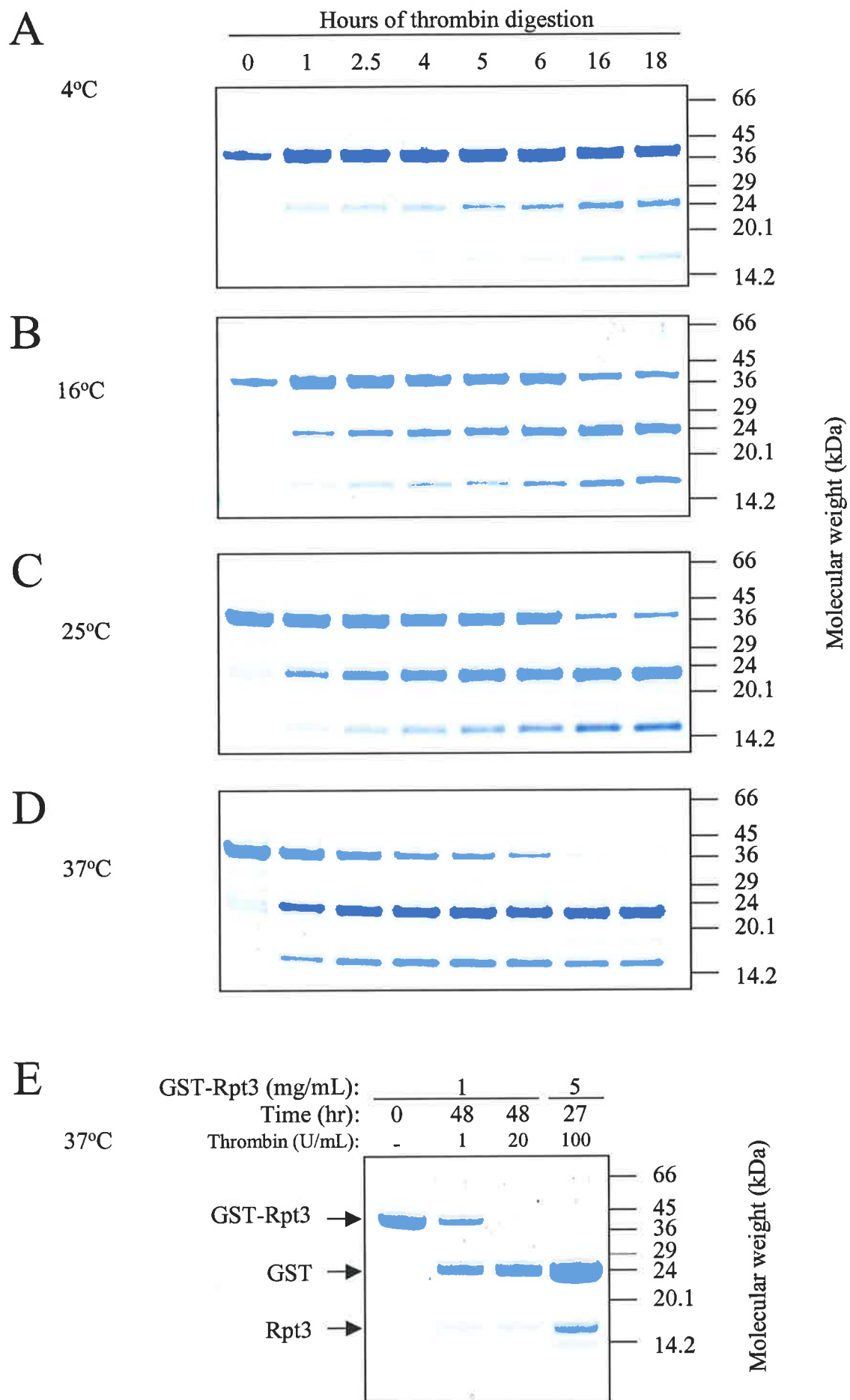


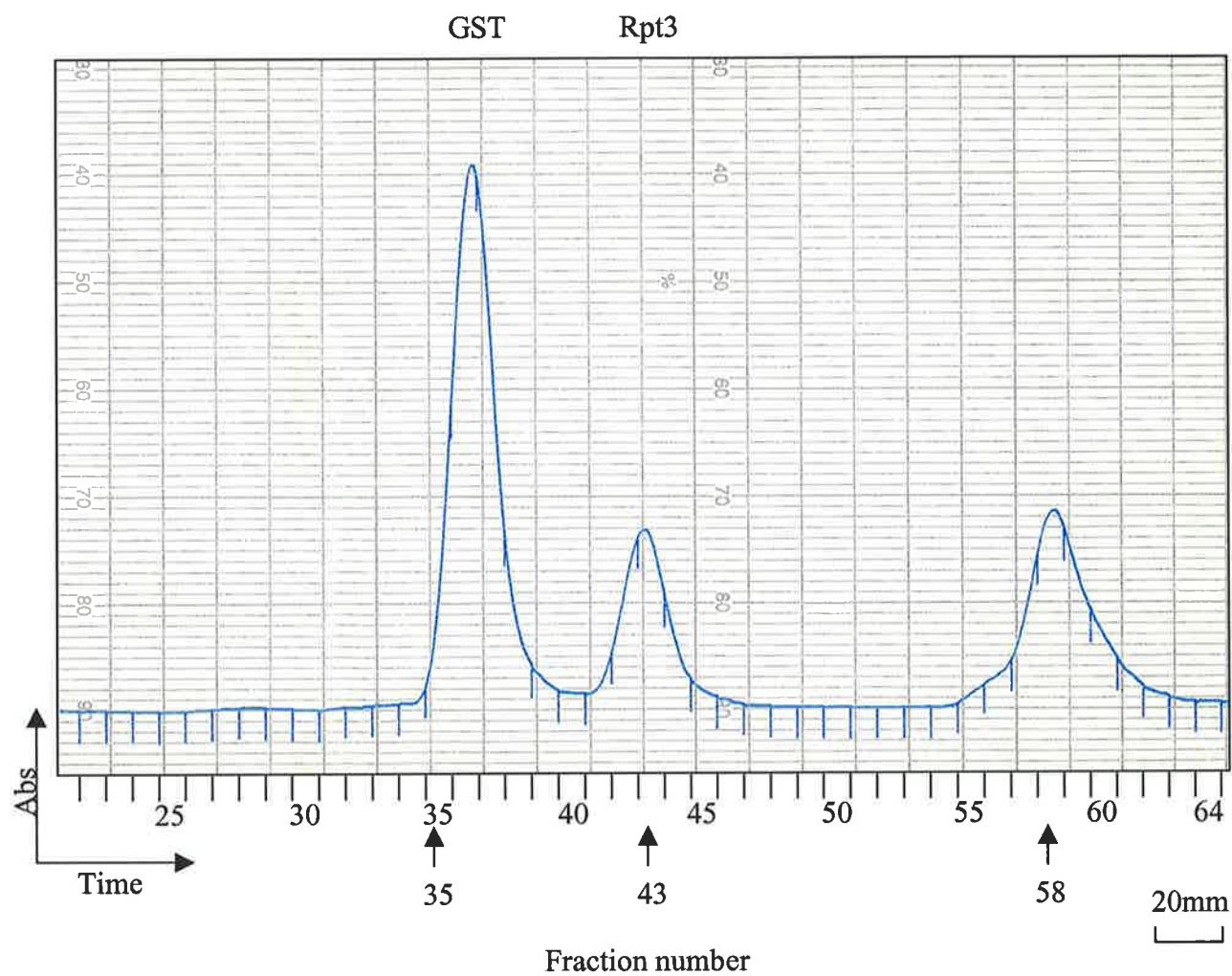
Figure 5.7 Purification of Rpt3 Protein From GST Protein

Chart recording of size exclusion chromatography purification of Rpt3 protein. Absorbance at 280 nm is plotted against time. After cleavage of GST-Rpt3 protein with Thrombin, GST and Rpt3 proteins were separated and purified by size exclusion chromatography. Purified GST-Rpt3 protein (5 mg/mL) was digested with Thrombin (100 U/mL) at 37°C for 4 hours. The GST and Rpt3 components were separated by size exclusion chromatography using a Superdex-75 column with bed volume of 200 mL. After column equilibration in PBS/0.01% NaN₃ (4 column volumes), the filtered, Thrombin cleaved GST-Rpt3 protein was loaded onto the column and 5 mL fractions were collected. The flow rate was 2 mL/min, the chart speed was 2 mm/min and the absorbance units range of the detector was AU=1. Larger proteins elute first. Arrows indicate fractions analysed by SDS-PAGE.

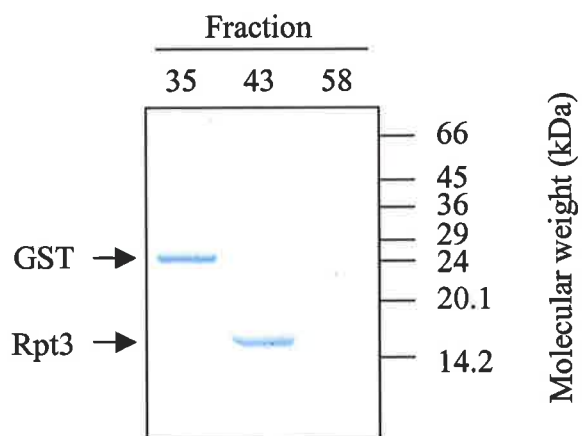
- A Chart recording of Superdex-75 size exclusion chromatography column purification of GST and Rpt3 proteins.
- B SDS-PAGE analysis of fractions corresponding to each peak in the chart recording in part (A). SDS-7 markers were used for size comparison of protein bands; the positions of molecular weight standard proteins are indicated.

GST: glutathione-S-transferase

A



B



5.4.8 Dimerisation Analysis of Actinin-4 Protein Fragments

The dimerisation of Actinin-4 protein fragments was assessed by non-denaturing size exclusion chromatography using the Smart System (Pharmacia) in conjunction with Jackie Cawthray. Apparent protein size inversely correlates with elution time. As shown in Figure 5.8A-C, the elution profiles of Rpt3 protein (14.8 kDa) and fusion proteins containing Actinin-4 fragments: Trx-I498-S696 (41 kDa) and GST-Rpt3 (41 kDa); were compared with that of monomeric standard proteins of known molecular weight: BSA (67 kDa), Ovalbumin (43 kDa) and GST (26 kDa).

GST-Rpt3 eluted at a similar time as Ovalbumin (Figure 5.8A and C) indicating that these two proteins have similar apparent molecular weight and, thus, that GST-Rpt3 is monomeric. Rpt3 protein eluted 10 min later than GST (Figure 5.8C), indicating that Rpt3 alone is not dimeric as it does not elute coincident with a protein approximately double its size. Therefore, Rpt3 alone is also monomeric. Trx-I498-S696 eluted before BSA indicating that the apparent molecular weight of Trx-I498-S696 is larger than 67 kDa (Figure 5.8A and B). The elution profile is consistent with Trx-I498-S696 having an apparent molecular weight of 82 kDa, which suggests that this protein is a dimer. Therefore, the extra residues in Trx-I498-S696 compared with Rpt3 (T519-Q645) promote dimerisation.

5.4.9 Rpt3 Protein Sample Preparation For NMR Spectroscopy Analysis

Nine milligrams of purified Rpt3 protein in PBS/0.01% NaN₃ was prepared for NMR spectroscopy analysis. In replica experiments, a Rpt3 protein sample for NMR spectroscopy analysis was prepared by Dr Kowalski and used to obtain the spectra illustrated in Figures 5.9 and 5.10. Rpt3 protein concentration was increased using an Amicon stirred cell with 3 kDa MWCO filter (Section 2.3.2.6). The sample was exchanged into water using a PD10 desalting column pre-equilibrated in water and Rpt3 containing fractions were pooled and adjusted to pH8. Rpt3 protein was concentrated by evaporation and supplemented with phosphate buffer (10mM), NaCl (100 mM), D₂O (10% v/v) and NaN₃ (0.01% w/v). The pH was adjusted to 6.75 with 1 M HCl. The final concentration of the 500 µL Rpt3 protein sample was 1.0 mM and contained 10% (v/v) D₂O. The sample was transferred into a 5 mm high-resolution thin-walled glass NMR tube.

Figure 5.8 Actinin-4 Repeat-3 is Monomeric

Chart recordings of size exclusion chromatography of standard proteins and Actinin-4 Repeat-3 containing proteins using the Smart System (Pharmacia). Absorbance at 280 nm is plotted against time in minutes. Larger proteins elute first. Arrows indicate elution time of peak maxima.

- A Chart recording of BSA (67 kDa, red) and Ovalbumin (43 kDa, aqua) monomeric standard proteins.
- B Chart recording of Trx-I498-S696 (41 kDa, green) protein.
- C Chart recording of GST-Rpt3 (41 kDa), GST (26 kDa) and Rpt3 (14.8 kDa) proteins. Prior to chromatography, the samples were buffer exchanged into TBS using a PD10 column to remove any reduced glutathione that would promote dimerisation of GST protein.

BSA: bovine serum albumin
GST: glutathione-S-transferase
Trx: thioredoxin

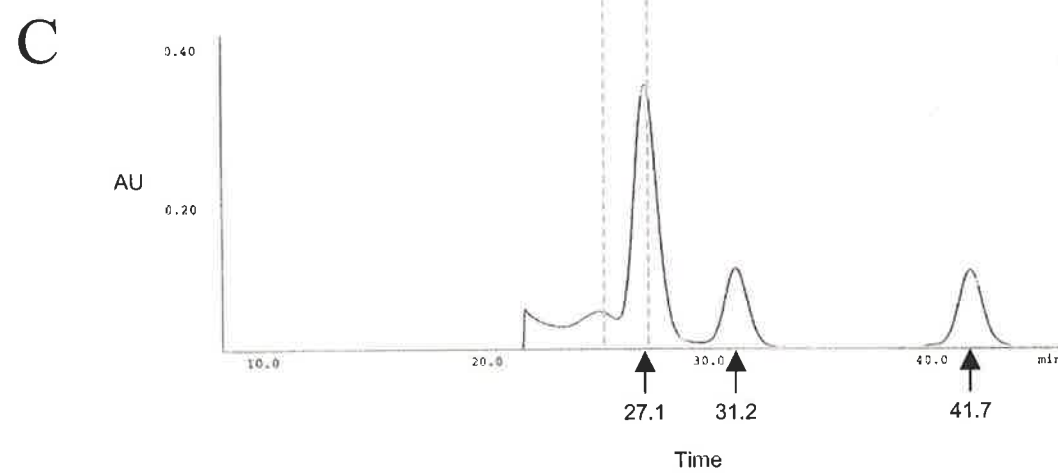
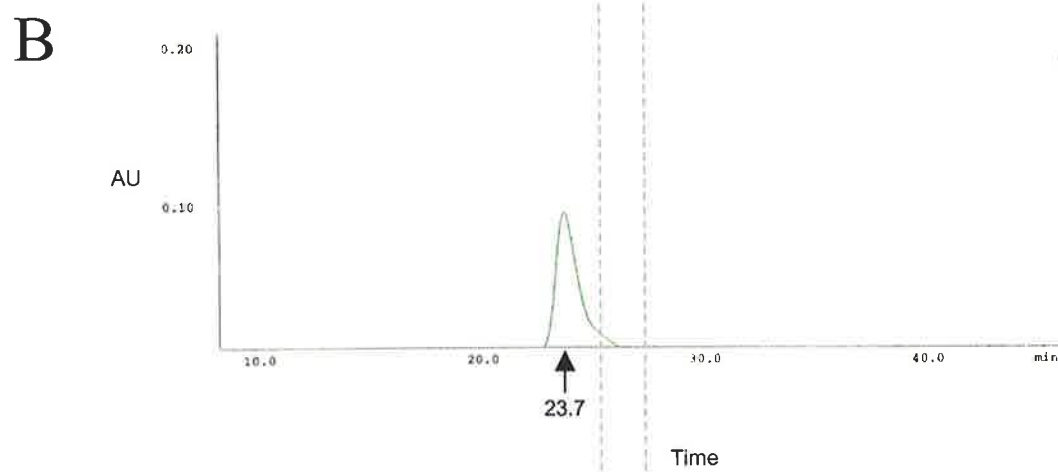
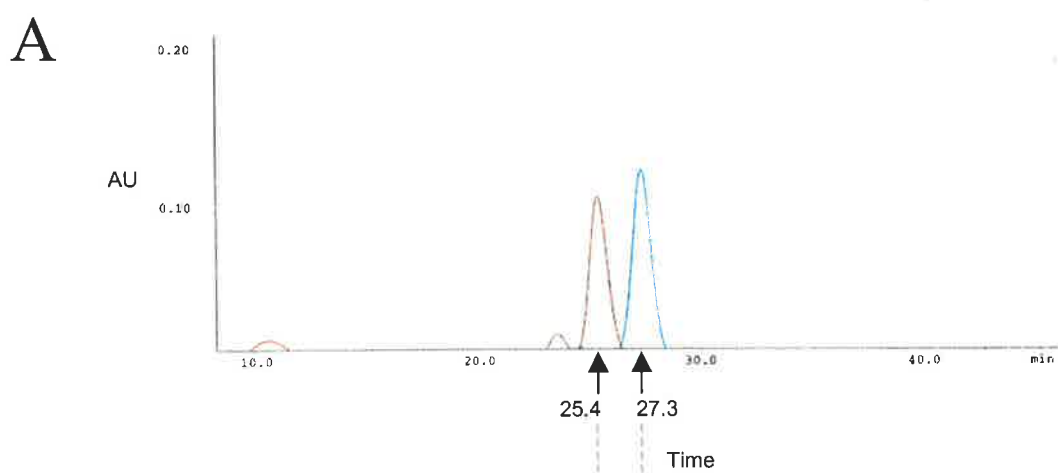
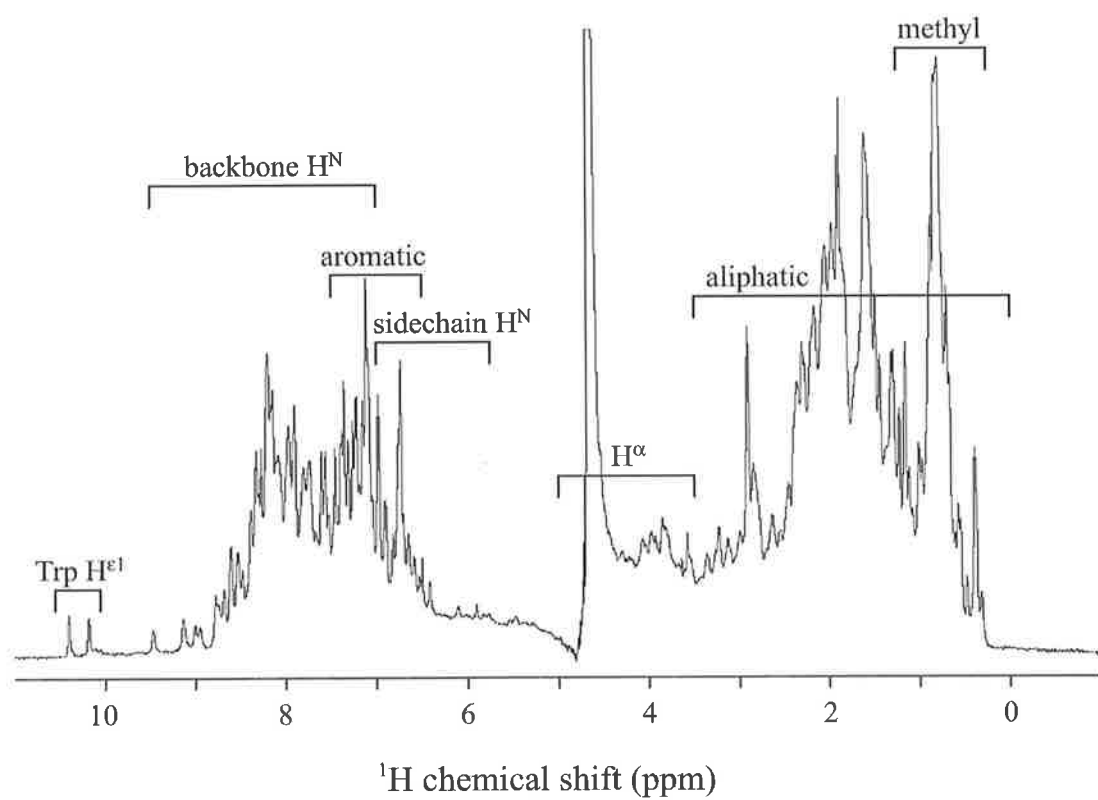


Figure 5.9 **1D ^1H NMR Spectra of Actinin-4 Rpt3**

Plot of one dimensional ^1H NMR spectra of Rpt3 protein. ^1H chemical shift in parts per million (ppm) is plotted against signal intensity. The characteristic frequencies of protons in different functional groups and backbone or sidechain positions of the polypeptide chain are indicated above the spectra. These reflect the different molecular environments surrounding the protons. The spectrum was acquired using a 1 mM sample at 25°C. Dr Kasper Kowalski performed sample preparation and NMR spectra acquisition.



5.4.10 1D NMR Spectroscopy Experiments

Preliminary NMR experiments were conducted using a Varian Inova 600 spectrometer in the Department of Chemistry, Adelaide University with the assistance of Dr Booker and Dr Kowalski. All data sets were recorded at 25°C using a 5 mm inverse triple resonance $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ PFG probe. The carrier frequency was centred on the H_2O signal. 1D presaturation experiments were used to test the purity and signal strength of the replica samples. Data were acquired using the VNMR software. As shown in Figure 5.9, the signal to noise ratio was high and the spectra showed wide dispersion in the ^1H dimension indicating that the Rpt3 protein is stably folded. As there are very few downfield-shifted backbone NHs, the majority of the protein is expected to be helix. The presence of sharp peaks indicated that Rpt3 protein is monomeric. This is consistent with the identification of Rpt3 protein as a monomer using size exclusion chromatography (see Sections 5.4.7 and 5.4.8).

5.4.11 2D NMR Spectroscopy Experiments

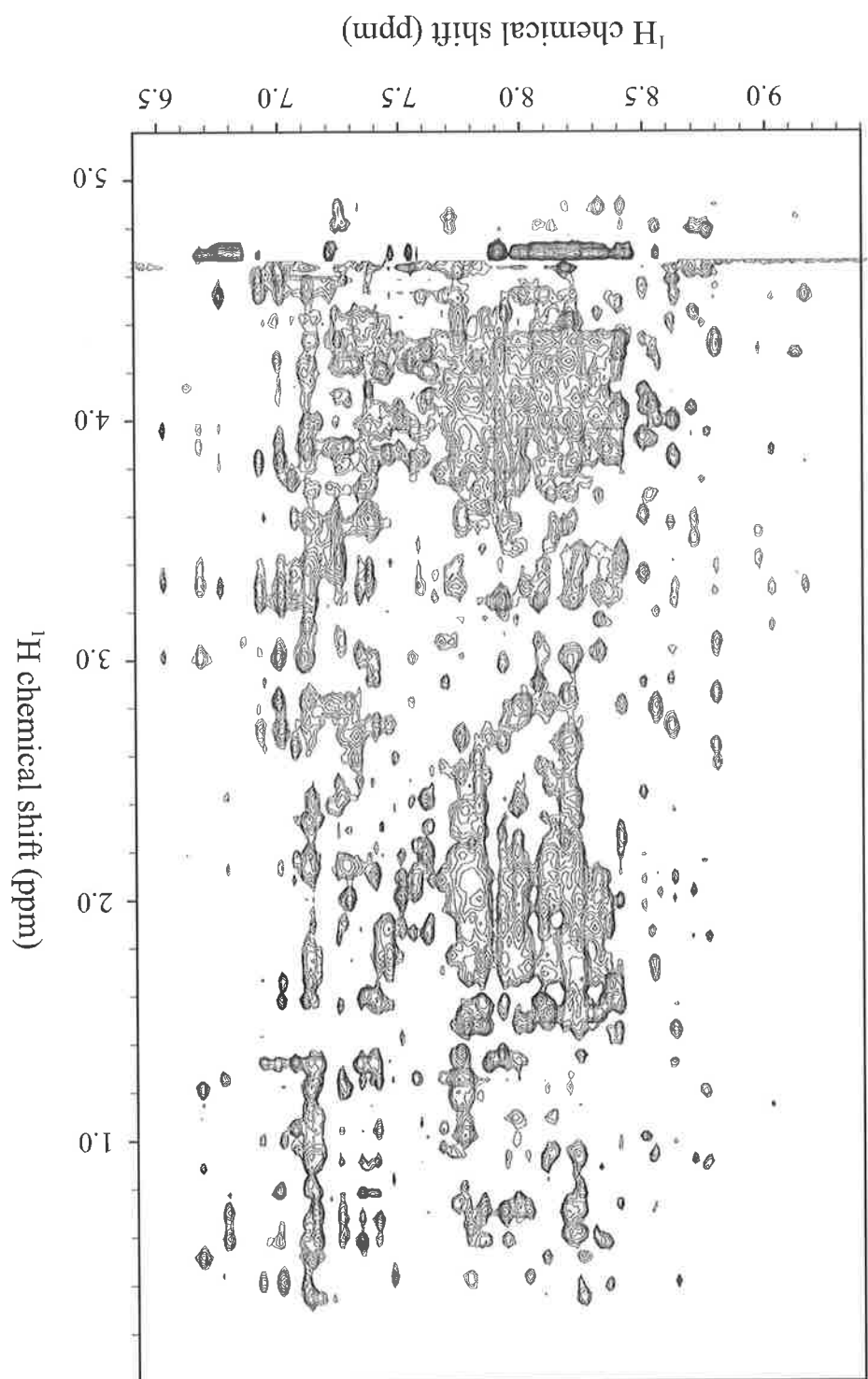
Initial two dimensional homonuclear experiments were recorded. These included 2D ^1H double-quantum-filtered J -correlated spectroscopy (DQF-COSY), ^1H TOCSY and ^1H NOESY experiments. The NMR data was recorded and processed by Dr Booker and Dr Kowalski. The 2D spectrum from the ^1H NOESY experiment is shown in Figure 5.10. ^1H NOESY experiments reveal information about through-space interactions between different protons. In these 2D contour plots, each proton is represented as a peak on the diagonal. A cross peak (off the diagonal) is observed when two protons are within approximately 5 Å of each other and this can be extrapolated to the diagonal to identify the interacting protons (Clowes *et al.*, 1995) The presence of a large number of $d_{\text{NN}(i,i+1)}$ NOEs is consistent with the structure of Rpt3 protein containing a significant degree of α -helix. Analysis of the fingerprint region and aromatic region enabled preliminary assignment of tryptophan and tyrosine side chain resonances.

5.4.12 Predicted Structure of PHTH and Rpt3 Proteins

In the absence of experimentally determined structures of the Tec PHTH and Actinin-4 Rpt3 domains, models were made based on the structures of closely related proteins. Dr Kowalski modelled the structure of Actinin-4 Rpt3 domain and the adjacent Rpt2 domain on that of Actinin-2 using SwissModel (Section 2.2.2). Residues of Actinin-4 were mapped onto the corresponding residues of the Actinin-2 Rpt2-3 crystal structure

Figure 5.10 2D ^1H NMR Spectra of Actinin-4 Rpt3

Plot of two dimensional homonuclear ^1H NOESY spectra of Rpt3 protein. The interaction between different ^1H nuclei that are close together in space (less than 5 Å apart) are observed. The ^1H chemical shift in parts per million (ppm) of each nuclei in each interacting pair are plotted against each other. The spectrum was acquired using a 1 mM sample at 25°C with a mixing time of 150 ms. Sample preparation and NMR spectra acquisition were performed by Dr Kasper Kowalski.



(Djinovic-Carugo *et al.*, 1999) (also see Section 3.4.9 and Figure 3.13A). Each spectrin repeat clearly adopted the triple helical bundle conformation although minor variations in surface structure exist for the different isoforms. As shown in Figure 3.13A, the surface of the central rod domain of Actinin-4 is expected to display numerous charged and polar residues.

The Tec PHTH domain structure was similarly modelled on that of the closely related Tec-family member Btk by Dr Kowalski (see Figure 3.13A). Apart from the extra residues in the loop between β -strands 1 and 2 of Btk compared with Tec, the two structures adopt the same overall fold. Additionally, after this PhD project was commenced a report was published in which molecular modelling was used to predict the structure of the Tec, Itk and Bmx PH domains. Differences in binding regions were identified despite overall similar scaffolding and electrostatic polarisation (Okoh and Vihinen, 1999).

5.5 Discussion

The structures of several PH domains and spectrin repeats have been reported in the literature. While different PH domains have limited primary sequence homology, their diverse sequences, surprisingly, adopt remarkably similar topology. The PH domain is composed of two orthogonally aligned β -sheets that are capped at one end with an α -helix (Blomberg *et al.*, 1999). PH domains have strong surface electrostatic potential (Blomberg *et al.*, 1999). PH domains of Tec-family kinases share greater identity to each other than to other PH domains. α -actinin genes are thought to be ancestors of genes that encode spectrin repeat domains (Pascual *et al.*, 1997). Therefore, it is not surprising that different spectrin repeat domains adopt a similar triple helical bundle topology. Spectrin repeat domains form a rigid rod-like structure that dimerises through specific electrostatic interactions. The Tec:Actinin-4 interaction may be based on electrostatic interactions mediated between the Tec PHTH domain and the Actinin-4 central rod domain.

5.5.1 Production of Recombinant PHTH domain and Rpt3 Domain Proteins

This section of work aimed to produce purified recombinant Tec PHTH domain and Actinin-4 Rpt3 proteins for structural analysis. Since NMR spectroscopy was chosen for structural analysis, approximately 500 μ L amounts of millimolar concentration purified soluble protein were required. This was achieved for the Rpt3 domain protein but not for the PHTH domain protein.

Most of the PHTH domain-containing fusion protein produced using the pGEX bacterial expression system partitioned in the Tween-20 insoluble fraction of cells. However, increased amounts of soluble PHTH protein can be purified from larger bacterial culture volumes (Pursglove, 2001). The scale of experiment required to produce 1 mM PHTH domain protein is not suitable for creating isotopically-labelled PHTH domain. But, unlabelled protein is suitable for use in titration experiments with labelled Rpt3 protein to identify Rpt3 residues involved in the binding interface. Other researchers have had difficulty in purifying PHTH domain protein for structural analysis. For example, the Btk PH domain and Btk motif crystal structure was resolved for the R28C mutant of the PH domain due to an inability to produce high quality crystals of the wildtype version (Hyvonen and Saraste, 1997).

Preliminary steps were taken to produce PHTH domain in mammalian cells as an N-terminal fusion of EGFP (Section 4.4.6). This is expected to minimise the likelihood of incorrect folding because the PHTH domain protein is in its natural environment. Unlike bacterial expression systems, where the protein of interest is a C-terminal fusion of a carrier protein, the mammalian expression system enabled the positioning of the PH domain at the extreme N-terminus of the fusion protein, its natural location. The majority of PHTH-EGFP protein partitioned in the Triton-X100 soluble fraction of lysed, transfected COS-1 cells (data not shown). Protein produced using this expression system can be used in experiments that test the interaction of Tec with ligands. For example, PHTH-EGFP fusion protein could be immobilised by the EGFP fusion partner in surface plasmon resonance experiments. For this experiment, 2-10 μ g of purified fusion protein would be required and it is estimated that this could be obtained from one 10 cm dish of transiently transfected COS-1 cells (assuming that PHTH-EGFP represents 1% of total soluble protein). Ideally, a monoclonal antibody directed at GFP could be used to immobilise the PHTH-EGFP protein. One such commercially available antibody is ab1218-100 (Abcam,). This would provide the potential to analyse the binding strength of N-terminal PHTH domain with either Rpt3 alone or GST-Rpt3 analyte that has a larger mass.

The Actinin-4 Rpt3 domain protein was expressed in bacteria and purified to homogeneity using a combination of affinity chromatography and size exclusion chromatography. Dimerisation data indicated that Rpt3 protein is monomeric while larger proteins that, in comparison, have N- and C-terminal extensions are dimeric.

The expression of Rpt3 protein was investigated in minimal media in preparation for production of isotopically-labelled protein, which can be achieved by growing bacteria in a defined minimal medium, such as Min A (Section 2.1.8, Miller, 1972), containing $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source. This is important to reduce spectral overlap in multidimensional spectra of Heteronuclear Single Quantum Coherence (HSQC)-based NMR spectroscopy experiments during the structural determination of the Rpt3 protein. Optimal conditions for expression of GST-Rpt3 and purification were defined and yields of 100 mg.L^{-1} in minimal media were obtained. Rpt3 protein was soluble at concentrations required for NMR spectroscopy analysis. Therefore, we are now in a position to create isotope-labelled Rpt3 domain and investigate its structure using heteronuclear NMR spectroscopy experiments and test its interaction with the Tec PHTH domain using HSQC experiments.

5.5.2 Structural Analysis of Actinin-4 Spectrin Repeat-3 Domain

A series of preliminary homonuclear NMR spectroscopy experiments, including 1D ^1H and 2D ^1H COSY, ^1H TOCSY and ^1H NOESY experiments, were used to investigate the structure of the Actinin-4 Rpt3 domain protein. These experiments indicate that the Rpt3 protein is suitable for structural analysis by NMR spectroscopy. In particular, Rpt3 is predicted to be correctly folded and monomeric, and stable for long periods at room temperature needed for recording NMR spectra. Preliminary spectral analysis indicates that Rpt3 has predominantly an α -helical structure. This is consistent with the structure of related spectrin repeat domains.

In order to generate a complete list of chemical shifts and distance restraints for the Rpt3 protein, a ^{15}N isotope-labelled protein is required in order to perform $^{15}\text{N}/^1\text{H}$ -HSQC-based heteronuclear 3D experiments. These experiments facilitate the assignment of chemical shifts to specific nuclei by extending the homonuclear ^1H 2D experiments into a third, ^{15}N , dimension and, thus, reducing spectral overlap. The unambiguous assignment of distance restraints uses an iterative approach and will be required to produce a high quality 3D structure of the Rpt3 protein.

It is envisaged that residues of the interaction interface will be identified by comparison of spectra obtained from the PHTH domain bound and unbound to Rpt3. Unlabelled PHTH domain protein would be titrated into a sample of ^{15}N -labelled Rpt3 protein and the movement of affected Rpt3 residues would be examined in 2D by ^1H - ^{15}N -HSQC experiments. It is anticipated that at least two molar equivalents of PHTH protein will be

required in order to observe changes in the HSQC spectra of ^{15}N -labelled Rpt3 protein upon titration with PHTH. Since final concentrations of 100 μM Rpt3 are adequate for these HSQC experiments, and sample volume is approximately 500 μL , a final concentration of 200 μM PHTH would be achieved by adding 1.7 mg of lyophilised PHTH protein. Purification of this amount of PHTH protein is achievable. Ultimately, this will enable the future design and testing of small molecular weight drug ligands to probe the biological function of the Tec:Actinin-4 interaction. Furthermore, once identified, the residues of the interaction interface could be altered by site-directed mutagenesis and the function of the mutant proteins could be tested in the biological system.

Theoretically, the results of the HSQC experiments may be pre-empted by molecular docking studies that use the computer modelled PHTH and Rpt3 protein structures and simulate their binding. The preponderance of surface-exposed charged residues in the predicted structures of PHTH and Rpt3 proteins supports the idea of a charge-based interaction between these domains of the intracellular Tec and Actinin-4 proteins. Surface structure variations in the PHTH domains of Tec-family members and Rpt3 domains of α -actinins are expected to determine their individual binding-specificities and whether or not Tec-family kinases commonly bind α -actinins.

In conclusion, the Actinin-4 spectrin repeat 3 domain protein can be made in amounts suitable for NMR spectroscopy experiments. This is a major step toward identification of the interaction interface between Actinin-4 and Tec and, therefore, toward dissecting the signalling pathway involving Tec and Actinin-4 proteins. Although purification of bacterially expressed PHTH protein was inefficient compared to that of Rpt3, sufficient soluble PHTH protein can be purified for use in HSQC NMR spectroscopy experiments in combination with labelled Rpt3 protein. Furthermore, PHTH protein expression in mammalian cells was identified as an alternative source of soluble PHTH fusion protein. Future experiments are expected to capitalise on the groundwork presented here and further comment on this is provided in the following chapter.

CHAPTER

6

Final Discussion And Future Directions

6.1 Final Discussion

Responding to environmental stimuli is fundamentally important in the life of cells. Communication is the key ingredient in this process. Cellular growth and differentiation are tightly regulated and rely on accurate interpretation and transmission of signals between and within cells. Protein tyrosine phosphorylation is a major biochemical mechanism by which extracellular signals are conveyed through cells (Pawson, 1995). Activation of tyrosine kinase enzymatic activity is stringently controlled to prevent aberrant signalling that can cause disease. Regulatory mechanisms used to control protein tyrosine kinase activity were recently shown to include subcellular compartmentalisation and conformational change in addition to protein interactions.

Modular protein interaction motifs play key roles in the activation of Tec kinases. Members of this family of proteins, in general, contain PHTH, SH3, SH2 and tyrosine kinase domains. Intramolecular and intermolecular interactions mediated within and between these domains have been demonstrated to contribute to kinase domain activity. A variety of different mutations in these domains of Btk have been identified in XLA patients and have proved useful in elucidating the function of the individual domains in regulating the kinase activity.

6.1.1 Mechanism of Activation of Tec-family Kinases

Much of the knowledge learned about Tec-family kinase function has been gained from studies on Btk and Itk. In the inactive form of the Tec-family kinase, intramolecular interactions between the SH3 domain and adjacent proline-rich region of the TH domain are thought to hold the molecule in a closed conformation (Andreotti *et al.*, 1997, Pursglove *et al.*, 2002). The apposition of the two kinase domain lobes sterically prevents kinase domain activation. Ligands of the regulatory domains, such as PI 3,4,5-P₃ and G protein $\beta\gamma$ subunits that bind the PHTH domain, are thought to disrupt the intramolecular interactions and promote intermolecular interactions that are associated with altered kinase domain conformation and increased enzymatic activity (Scharenberg *et al.*, 1998, Saito *et al.*, 2001). These domains are, thus, described as allosteric regulators of kinase activity.

PH domain-dependent membrane translocation of Tec kinases facilitates the phosphorylation of Tec kinases by Src kinases (Rawlings *et al.*, 1996). Auto-phosphorylation of the SH3 domain of Tec kinases provides a docking site for SH2 domain-containing proteins and is required for full enzymatic activity (Park *et al.*, 1996). Docking of Tec kinases

to SLP/BLNK-family adaptor proteins, which is dependent on the SH2 domain of Tec kinases, juxtaposes them with their kinase substrate PLC- γ (Kurosaki *et al.*, 2000, Rodriguez *et al.*, 2001). Over the last few years, Btk and Itk kinases have been suggested to play an important role in the maximal activation of PLC- γ in BCR and TCR signalling, respectively. The biological role of Tec is less well defined.

Numerous signalling pathways reportedly include Tec or other Tec-family members (see Section 1.5.2). These kinases may, thus, be a general link between receptor-initiated stimuli and gene transcriptional control that requires sustained calcium signalling.

6.1.2 Isoforms of Tec

The presence of two Tec isoforms, that arise by alternative splicing of the 66 base pair exon 8 and differ in the C-terminus of the SH3 domain, suggests that the isoforms' activity may be differently regulated (Merkel *et al.*, 1999). The Tec3 protein lacks the C-terminal 22 amino acids of the SH3 domain present in Tec4 but retains the auto-phosphorylated tyrosine residue equivalent to Y223 in Btk. The incomplete ligand-binding site of the Tec3 SH3 domain is not expected to endorse the TH-SH3 domain intramolecular interaction. Therefore, Tec3 may be intrinsically more active than Tec4. Deletion of the entire Tec SH3 domain yields hyperphosphorylated and activated Tec kinase (Yamashita *et al.*, 1996). Interestingly, the removal of exon 8 in Btk transcripts, which removes 21 residues of the SH3 domain C-terminus and provides a Tec3-like version of Btk, was identified in an XLA patient, indicating that this deletion has functional consequences in Btk (Zhu *et al.*, 1994). This deletion weakens ligand-binding of Btk SH3 domain (Tzeng *et al.*, 2000). However, in that XLA patient, only the truncated form and none of the full-length form was present. This is in contrast to Tec, where both isoforms are expressed in some cell types while in others only one was detected (Atmosukarto, 2001).

Although not described in this thesis, significant time during this PhD was spent characterising the mouse Tec locus in order to create Tec knockout mice and better understand the five reported Tec isoforms. It was envisaged that the isoforms, which differ in both the regulatory (PH and SH3) and catalytic domains would have altered ligand-binding properties that would confer different activities and regulation to the molecules. In conjunction with studies by Ines Atmosukarto, those experiments were published (Merkel *et al.*, 1999; a reprint of that article is included in the Appendix). It was also important for the current work of defining Tec regulatory domain ligands that the prevalence of each isoform

be established. Using RNase protection and RT-PCR analysis, Atmosukarto demonstrated that the predominant isoforms in mouse tissues are Tec3 and Tec4 (Merkel *et al.*, 1999, Atmosukarto, 2001). The Tec1 isoform, which lacks a PH domain, was not identified.

6.1.3 Signalling of Tec Kinases to the cytoskeleton

It has recently been discovered that, upon receptor-initiated stimuli, the regulatory domains of Tec kinases modulate protein association with the cytoskeleton in addition to interactions with proteins involved in signal transduction (Yao *et al.*, 1999, Chen *et al.*, 2001). In broad terms, the cytoskeleton is composed of a filamentous network of F-actin, microtubules and intermediate filaments that are each composed of distinct chemical subunits. The scaffold has high negative electrostatic charge density and an enormous surface area upon which proteins and cytoplasmic components can dock, and provides a dynamic connection between nearly all cellular structures. The cytoskeleton is linked to transmembrane receptors and is rapidly reorganised in response to extracellular stimuli. Cell activation by antigens, cytokines, growth factors and hormones induces rapid and drastic changes in actin organisation and involves actin-binding proteins and lipids. Cellular processes in which the actin cytoskeleton plays fundamental roles include the maintenance of cell morphology, cell-cell and cell-matrix adhesion, cell division and phagocytosis.

Many components of Tec kinase signalling pathways have previously been linked to the cytoskeleton (reviewed in Janmey, 1998). Src-family kinases were among the first non-receptor tyrosine kinases found to translocate to the cytoskeletal fractions after activation of cells (Ballmer-Hofer *et al.*, 1988). Lipid kinases and phospholipases are also recruited to the cytoskeleton by signals that promote cytoskeletal-binding of the protein kinases. Most of the PLC activity localised to the cytoskeleton is due to the PLC- γ isoform (Banno *et al.*, 1996). Furthermore, intracellular calcium concentration levels have also been linked to cytoskeleton structure and the cytoskeleton plays roles in signalling to the nucleus.

Several experiments implicate Tec kinases in rapid cytoskeletal rearrangements that occur upon receptor-binding. Itk has been implicated in signalling events that regulate integrin-mediated T-cell adhesion (Woods *et al.*, 2001). Enhanced integrin-mediated adhesion occurs upon TCR signalling to β -1 integrins and involves coordinated recruitment and activation of Itk by Src-family tyrosine kinases and PI3K (Woods *et al.*, 2001). Chen *et al.*, 2001, presented evidence that Bmx is also involved in integrin signalling and promotes cell migration. Tec and Btk were previously shown to participate in platelet signalling

downstream of integrin activation (Laffargue *et al.*, 1997, Hamazaki *et al.*, 1998, Laffargue *et al.*, 1999). Translocation of Btk to the cytoskeleton was detected in thrombin-stimulated platelets and was coincident with tyrosine phosphorylation of Btk (Mukhopadhyay *et al.*, 2001a). Btk was suggested to be a component of a signalling complex containing specific cytoskeletal proteins in the activated platelets (Mukhopadhyay *et al.*, 2001a). Stimulation of the platelet non-integrin collagen receptor, glycoprotein VI, evoked a signalling response similar to that induced by antigen receptor activation in B and T lymphocytes and was also shown to activate Btk and Tec (Quek *et al.*, 1998, Oda *et al.*, 2000). In conjunction with the work presented in this thesis, these findings lead to the attractive hypothesis that Tec kinases and α -actinins are co-ordinately involved in the regulation of cytoskeletal remodelling.

6.1.4 The Role of the PTH Domain in Tec Signalling

The PTH domain of Tec-family kinases has a dual role in protein targeting and allosteric regulation of enzyme activity. Both of these roles are intertwined and governed by interactions with proteins and/or cellular factors such as PI 3,4,5-P₃. These roles have been documented for other PH domains in the literature (reviewed in Blomberg *et al.*, 1999).

6.1.5 Actinin-4 is A Novel Binding Protein of Tec PTH Domain

Of all the ligands described for Tec-family PTH domains, two proteins, F-actin and FAK, revealed direct cytoskeletal links for Tec-family kinases. As described in this thesis, Actinin-4 was identified as a novel binding partner of the Tec PTH domain and provides another potential direct link between Tec-family tyrosine kinases and the regulation of cytoskeletal structure.

To investigate the ligands of the Tec PTH domain, yeast two-hybrid assay studies described in Chapter 3 were used to screen a human liver cDNA library with LexA-PTH fusion protein as bait. Of the ten clones identified, five were found by DNA sequencing to encode a fragment of Actinin-4. This newly isolated isoform of non-muscle α -actinin has been implicated in actively moving regions of cells and the metastatic potential of human cancers (Honda *et al.*, 1998a, Araki *et al.*, 2000). Non-muscle α -actinins are widely expressed and play a role in stress-fibre formation, cell adhesion, cell shape and motility.

Not surprisingly, little is known about the function of Actinin-4 since it was only relatively recently discovered (Honda *et al.*, 1998a). New evidence suggests that defects in Actinin-4 are linked to the human disease focal and segmental glomerulosclerosis (FSGS),

which is a common, non-specific renal lesion that often leads to end-stage renal failure in affected patients (Kaplan *et al.*, 2000). Mutant Actinin-4 was identified in three families with an autosomal dominant form of FSGS. Compared with wild-type Actinin-4, the mutant form was found to bind F-actin more strongly. The mutations in Actinin-4 were, therefore, implicated in causing the disease. Furthermore, downregulation of Actinin-4 may increase aggressiveness of cancers as expression was correlated with substrate adhesivity in many adherent tumor cell lines of diverse tissue origins (Nikolopoulos *et al.*, 2000). Actinin-4 was, thus, suggested to exhibit tumour suppressor activity. Combined with the links to the metastatic potential and invasiveness of human cancers (Honda *et al.*, 1998a), these experiments reveal Actinin-4 as a biologically important protein.

Characterisation studies described in Chapter 3 indicated that the entire third spectrin repeat of Actinin-4 plus flanking sequences were required for the interaction with Tec PHTH domain. The flanking sequences were found to confer dimerisation. The possible requirement for dimerisation is not surprising since the *in vivo* actin crosslinking function of α -actinins relies on their head to tail dimerisation to form a bridge between adjacent strands of F-actin.

Yeast two-hybrid studies were previously used to identify ligands of Tec kinase domain but not Tec PH domain. In these studies, SOCS-1, PI3K subunits, Vav, Grb10 and BRDG-1 were found to bind Tec (Ohya *et al.*, 1997, Takahashi-Tezuka *et al.*, 1997 Mano *et al.*, 1998, Ohya *et al.*, 1999). In other yeast two-hybrid studies, Actinin-4 was identified as a putative ligand of BERP, a novel ring finger protein; CLP-36, a PDZ-LIM family adaptor protein; and the intracellular segment of densin-180, a transmembrane protein that is postulated to function as a synaptic adhesion molecule (El-Husseini *et al.*, 2000, Vallenius *et al.*, 2000, Walikonis *et al.*, 2001). Actinin-4 may function in linking cell-signalling to cytoskeletal organisation. The link may be direct, through characterised signalling molecules such as Tec, or indirect and mediated through adaptor molecules such as CLP-36 or BERP. Thus, important functions of Actinin-4 could involve its link with Tec.

In vitro IP techniques are routinely used to dissect signalling pathways and identify physical links between proteins. Combined with imaging techniques that define the intracellular localisation of specific proteins, detailed knowledge of individual protein functions can be gained. As described in Chapter 4, several mammalian cell lines were shown by Western blot to express both Tec and Actinin-4 proteins and immunofluorescence studies showed their colocalisation. The results presented here are in agreement with the previously reported cytoplasmic localisation of Actinin-4 (Honda *et al.*, 1998a). However, unlike cells in

the experiments of Honda *et al.* (1998a), the cells used in these experiments lacked obvious stress fibres and, therefore, localisation in those structures was not detected. Since the formation of stress fibres within cells can be induced by particular cell culture conditions and may represent an artefact of growing conditions, their formation was not pursued.

Araki *et al.* (2000) demonstrated Actinin-4 localisation to cell cortical regions and redistribution to phagocytic cups during phagocytosis of latex beads. These results complement the results of Atmosukarto (2001), in which Tec was found to relocate to the phagosome like Syk kinase, a known effector of phagocytosis. Therefore, Tec and Actinin-4 may function together during phagocytosis, a process employed by macrophage cells to scavenge and destroy exogenous particles or pathogens and protect organisms from infection. The key to this process, highly localized and rapid actin rearrangement, may be influenced by the concerted actions of Tec and Actinin-4.

Co-IP of Tec and Actinin-4 proteins was inefficient although it did establish their interaction in mammalian cells. Other researchers have found it difficult to IP α -actinin from cell lysates (Izaguirre *et al.*, 1999). Therefore, epitope-tagged versions of the proteins were expressed in mammalian cells and co-immunoprecipitated from cell lysates. Interestingly, the majority of full-length Tec fused to EGFP and expressed in COS-1 cells was sequestered to the insoluble cytoskeletal compartment and heavily tyrosine phosphorylated. Phosphorylation of Tec-family kinases was recently shown to correspond with translocation to the insoluble fraction of cells (Mukhopadhyay *et al.*, 2001a). Furthermore, activated Src-family kinases translocate into insoluble cellular components (Ballmer-Hofer *et al.*, 1988). Therefore, when activated, these kinases may become components of signalling complexes that contain specific cytoskeletal proteins. There is also a possibility that translocation to detergent-insoluble, cholesterol-rich regions of the membrane, known as lipid RAFTs or glycolipid-enriched microdomains, which play a crucial role in T cell signalling, results in insolubility of Tec. Actinin-4 could likewise be targeted to these insoluble microdomains through Tec- and/or PI 3,4,5-P₃-mediated interactions.

To investigate the involvement of particular residues in binding of the two proteins, truncation and substitution mutants of Actinin-4 and Tec were created. Replacement of a HIKE-like motif (a candidate PH-domain ligand; Alberti, 1998) in the BC loop of Repeat-3 with alanine residues did not prevent binding of Actinin-4 to the Tec PTH domain in the yeast two-hybrid assay, indicating that these residues are not critical PTH domain-binding determinants of Actinin-4.

Actinin-4 lacking the actin-binding domain co-immunoprecipitated from mammalian cell lysates with a predicted actin-binding-mutant of Tec, indicating that the binding of the two proteins in cells is not indirect and mediated by actin. This is consistent with yeast two-hybrid analysis identifying the third spectrin repeat of Actinin-4 and not the actin-binding domain as the site of Tec interaction. It is unlikely that the Actinin-4 and actin-binding sites of Tec overlap, as the substitution of three predicted actin-binding residues in the Tec PH domain, K18A/K19A/K20A, did not impair binding of Actinin-4 to Tec. This mutation removed positively charged residues and would have altered the electrostatic polarisation of the PH domain surface. Electrostatic interactions may be important in the binding of Tec and Actinin-4.

A cytoskeletal restructuring assay was planned to demonstrate an example where the Tec:Actinin-4 interaction may be important and, hence, set a platform from which to elucidate the functional relevance of the interaction. To this end, the Fc- γ -receptor-dependent phagocytosis assay was commenced, but further work is required to optimise the immunofluorescent staining component of this experiment. From the immunofluorescent staining of Tec and Actinin-4 by Atmosukarto (2001) and Araki *et al.* (2000), respectively, it is expected that Tec and Actinin-4 will colocalise at the phagosome during phagocytosis.

Intimate knowledge of intermolecular protein interactions can be used in the design of therapies to combat the effects of inappropriate signalling that arise in disease states. In experiments described in Chapter 5, structural information about the interaction interface of Tec and Actinin-4 was sought to design small molecular weight drug compounds that interfere with the Tec:Actinin-4 interaction.

The purification of Actinin-4 Rpt3 protein for NMR spectroscopy experiments was optimised, although time constraints did not permit elucidation of the structure. *In vitro* experiments using the Rpt3 fragment of Actinin-4 fused to GST confirmed its interaction with the Tec PHTH domain. This was despite this fragment of Actinin-4 only weakly binding the Tec PHTH domain in the yeast two-hybrid assay. In future experiments, Rpt3 protein with several extra carboxyl terminal residues could be used in structural analysis provided that it binds to Tec PHTH domain in the yeast two-hybrid assay.

It is expected that unlabelled PHTH domain protein will be acquirable for use in the HSQC titration experiment to identify specific Rpt3 residues involved in PHTH domain-binding. Preliminary steps to estimate the binding strength of the Tec:Actinin-4

interaction were not achieved due to the current lack of sufficient folded soluble PTH domain protein for use in surface plasmon resonance experiments.

6.1.6 The Implications of Tec and Actinin-4 Binding

The work described in this thesis has provided a foundation for the analysis of Tec involvement in signalling to the cytoskeleton. Potentially, activated Tec at the cell membrane could be involved in cell restructuring through interaction with and phosphorylation of underlying cytoskeletal components in processes such as migration, phagocytosis, and the formation of membrane projections such as filopodia, lamellipodia or membrane ruffles. These are cellular processes that are critical for survival, especially in immune system cells.

The ability of Tec to bind to Actinin-4, a putative scaffolding protein, provides a potential link between Tec and as yet undefined multiprotein complexes. The association of Tec with multiprotein complexes is not unprecedented, as Tec was identified to form a stable complex with Dok-1, Lyn and two unidentified phosphoproteins of 56 kDa and 140 kDa (van Dijk *et al.*, 2000). The adapter protein Dok-1 presumably nucleates the formation of this complex during PI3K-dependent c-Kit-mediated signalling pathways activated by stem cell factor. Interactions with scaffold proteins would explain the involvement of Tec in a wide range of signal transduction mechanisms and identifies the scaffolding proteins as attractive regulatory targets in signal transduction. The suggestion that Actinin-4 regulates the actin cytoskeleton and increases cellular motility makes it an ideal target for cancer therapy (Honda *et al.*, 1998a).

The possibility that Actinin-4 is a substrate of Tec is appealing. Phosphorylation of the related non-muscle α -actinin isoform, Actinin-1, was identified upon TCR activation of T cells and in lysates of PMA stimulated platelets (Egerton *et al.*, 1996, Izaguirre *et al.*, 1999). Tyrosine phosphorylation of Actinin-1 in platelets by FAK decreases the binding to F-actin and increases the fluidity of the cytoskeleton, suggesting that phosphorylation of α -actinin facilitates actin cytoskeleton reorganisation (Izaguirre *et al.*, 2001). Increased detergent solubility of Actinin-1 was also identified upon tyrosine phosphorylation consistent with a report that PDGF treatment, which induces migration, also causes redistribution of Actinin-1 from insoluble to soluble fractions of cells (Greenwood *et al.*, 2000).

Tec kinases may coordinate with α -actinins in phosphoinositide and calcium signalling pathways. The subcellular distribution and interactions of both proteins are

influenced by PI 3,4,5-P₃ indicating that both families of proteins may be downstream effectors of PI3Ks.

6.2 Future Directions

While the aims set out in Section 1.11 were significantly achieved, the groundwork described in this thesis has, importantly, opened the doors to further exciting study. The cytoskeleton may be a target of Tec-family kinases. Further characterisation of signalling pathways involving both Tec-family kinases and α -actinins is necessary to establish a role for Tec kinases in cytoskeletal remodelling. The areas that require further analysis include (1) investigating the possibility that α -actinins are substrates of Tec kinases, (2) analysing the co-redistribution of Tec-family kinases and α -actinins upon stimulation of specific cell surface receptors, (3) identifying the specific binding determinants involved in Tec and Actinin-4 binding and (4) testing inhibitors of Tec and Actinin-4 interaction in cytoskeletal restructuring assays.

6.2.1 Are α -actinins Substrates of Tec-family Kinases?

It is not known whether Actinin-4 is a substrate of Tec kinase domain. It would be reasonable to expect that activation of Tec is a prerequisite for testing Tec-mediated phosphorylation of Actinin-4. This implicates the involvement of PI 3,4,5-P₃, which is also known to affect α -actinin function. Otherwise, constitutively active and kinase-dead versions of Tec could be created and assayed for *in vitro* Actinin-4 phosphorylation. Studies on Actinin-1 identified Y12 as a substrate of FAK (Izaguirre *et al.*, 2001). Phosphorylation of Y12 decreased the binding to actin as determined by sedimentation assays. Therefore, it would be interesting to examine the actin-binding ability of Actinin-4 in conjunction with kinase assays. The actin-binding ability of Tec could also be examined in the inactive and active states.

6.2.2 Do Tec Kinases and α -actinins Co-redistribute in Stimulated Cells?

As previously mentioned, Tec kinases and α -actinins are rapidly redistributed upon cytoskeletal remodelling. Independent research has identified common relocation of Tec and Actinin-4 proteins during phagocytosis (Atmosukarto, 2001, Araki *et al.*, 2000). Further characterisation is required to identify co-redistribution during cellular movement in processes such as migration, phagocytosis, cell division, and membrane ruffling. These

experiments would incorporate stimulation of appropriate receptors of the specific signalling pathways, such as the PDGF receptor for migration. The localisation of other previously implicated proteins, including actin, could be examined simultaneously. It would also be interesting to investigate the localisation of Actinin-4 in these processes in cells with a Tec null background.

6.2.3 What are the Determinants of Tec and Actinin-4 Binding?

The precise binding determinants involved in Tec and Actinin-4 interaction need to be further characterised. The conservation of those residues in other Tec-family kinases and α -actinin isoforms could be evaluated to predict whether Tec kinases commonly interact with α -actinins. The interaction of Tec with other α -actinin isoforms or other spectrin repeat-containing proteins could be tested in the yeast two-hybrid assay described in Chapter 3 or the IP system described in Chapter 4. Other Tec-family members could similarly be tested for binding to Actinin-4 or other α -actinin isoforms. However, sequence differences in the third and fourth spectrin repeats suggest that the two human non-muscle α -actinins may bind different targets (Nikolopoulos *et al.*, 2000). Since Bmx and Actinin-1 are both involved in focal adhesion structures and FAK signalling, there is a possibility that they interact. The binding of human Tec and Actinin-4 proteins needs to be assessed, as does the binding of the respective mouse proteins. The contribution of cofactors such as PI 3,4,5-P₃ could be assessed in promoting the interaction of the two proteins.

6.2.4 What is the Effect of Preventing Tec Signalling to Actinin-4?

Interrupting the Tec:Actinin-4 interaction could provide a means to evaluate its role in intracellular signalling. Although Tec^{-/-} mice have been created and are viable, information concerning myeloid cell, and thus macrophage, presence and proper function are yet to be evaluated (Ellmeier *et al.*, 2000). Potentially, other Tec family kinases play compensatory roles in the absence of Tec.

To probe the role of the Tec:Actinin-4 interaction, the burgeoning field of computer aided drug design could be incorporated to design small molecular weight ligands that compete for binding sites on Tec or Actinin-4. These ligands are also expected to prevent binding of related family members to the targeted binding site and, thus, reduce redundancy. Structure based drug design has provided valuable tools for analysis of protein-mediated interactions and their biological function. Microarray analysis of drug treated cells versus

untreated cells could be used to determine the signalling pathways in which Tec and Actinin-4 interact.

6.3 Concluding Remarks

Most research involving intracellular signalling downstream of Tec kinase has focussed on gene expression changes, in particular, c-fos transcription (Yamashita *et al.*, 1998). The research presented here provides important information on how the cell's physical properties can be regulated through cytoskeletal rearrangement downstream of Tec kinase. It therefore, provides a direct link between receptor initiated signalling cascades with cell phenotype changes. Furthermore, this research highlights the prospect of identifying new interactions between kinases and structural proteins to further elucidate regulation of cytoskeletal rearrangement.

Since change in cell morphology is a key aspect in oncogenesis, it is important to understand and characterise the cytoplasmic signals that regulate cytoskeletal architecture. While identification of such interactions promotes research into drug design and distinguishes new drug targets, importantly, it may provide targets for control of cell phenotype. Future studies can then manipulate the cytoplasmic signals to avert tumour formation or metastasis.

In conclusion, to fully appreciate the function of a cellular enzyme, it is necessary to identify its upstream activators and downstream effectors. The binding of Tec to Actinin-4 and the colocalisation of both proteins imply a role for Tec in modulating the function of Actinin-4. This result signifies a potential to control cytoskeletal regulation during migration and metastasis of cancer cells. The next challenge will be to modulate signalling from Tec kinases to the cytoskeleton, possibly through actin-binding proteins such as Actinin-4.

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Appendix

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