'THE INTERACTION OF THE α2 CHIMAERIN SH2 DOMAIN WITH TARGET PROTEINS'

A Thesis by

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Submitted to

University College London

for the

Degree of Doctor of Philosophy, Ph.D

1999

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1

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Dedicated to my parents, Noreen and Giovanni Ferrari, and

to my brother, Roberto

Acknowledgements

I wish to express my gratitude to Professor Louis Lim for giving me the opportunity to study for a Ph.D in his London laboratory as a member of the Glaxo-IMCB group. I wish to thank both him and my co-supervisor Dr. Christine Hall for their guidance and encouragement over the period in which the work presented here was carried out and for the advice they have offered whilst I have been writing this thesis. I have also had the opportunity to travel to Singapore, a new and interesting experience. I gratefully acknowledge the support of the Glaxo-Singapore Research Fund.

I would like to thank all those members of the laboratory who have helped, in their various ways, to further my work by offering practical advice, providing reagents and by contributing to an enjoyable working environment. Special mention should be made of Christine Hall, Clinton Monfries, Kate Marler, Shanta Cariese, Ric Passey, and particularly Elena Prigmore.

My friends and family have been most supportive during the period I have spent writing this thesis, and deserve my sincere thanks. Tony, who has tolerated much and indulged me greatly, should know how much I appreciate his generosity and understanding.

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Abstract

Rac is a member of the Rho subfamily of low molecular weight GTPases (p21s) and is involved in diverse cellular processes. GTPase-activating proteins (GAPs) regulate p21 activity by increasing intrinsic GTPase activity. The chimaerins are a family of p21-Rac GAPS with distinct patterns of tissue and developmental distribution. $\alpha 2$ chimaerin contains an amino-terminal SH2 domain and is selectively expressed within the nervous system. SH2 domains bind specific phosphotyrosine-containing sequences and the presence of this domain may place $\alpha 2$ chimaerin within tyrosine kinase signalling pathways. Comparisons between SH2 domains suggest that the mechanism of target interaction of the chimaerin SH2 domain may be distinct from that of others.

Affinity chromatography was used to detect potential $\alpha 2$ chimaerin SH2 domain target proteins in rat brain extracts; some of these proteins were tyrosine-phosphorylated. Tubulin and actin were isolated as targets and peptide sequence information was obtained for three other potential target proteins, two of which appeared to be novel sequences. Several different kinase activities bound $\alpha 2$ chimaerin SH2 domain affinity columns; one of these phosphorylated full length $\alpha 2$ chimaerin.

Full length $\alpha 2$ chimaerin and its isolated SH2 domain bound a phosphotyrosine column. Amino acid residue substitutions were made in the $\alpha 2$ chimaerin SH2 domain at sites essential for function in other SH2 domains; certain point mutations affected phosphotyrosine-binding. $\alpha 2$ Chimaerin probes bound two previously identified putative $\alpha 2$ chimaerin target proteins of molecular mass 13kDa and 64kDa; these interactions were phosphotyrosine-independent. The interactions of the 13kDa and 64kDa proteins with $\alpha 2$ chimaerin differed in their sensitivity to point mutation of the chimaerin SH2 domain. Specific antibodies have been raised to these proteins to facilitate further studies. Results suggest that substrates of the $\alpha 2$ chimaerin SH2 domain may include both tyrosine-phosphorylated and non-tyrosine phosphorylated proteins.

TABLE OF CONTENTS

9

10

11

14

FIGURES & ILLUSTRATIONS

Chapter Four

Chapter Five

Chapter Six

Abbreviations

A	Adenosine
AA	Arachidonic acid
AG	Affigel-10
ACK	Activated Cdc42-associated kinase
AEBSF	4-(2-Aminoethyl)benzenesulphonyl fluoride
Amp	Ampicillin
APS	Ammonium persulphate
ARF	ADP-ribosylation factor
B13	13 KDa subunit of the mitochondrialComplexI NADH oxidreductase
(p13	Recombinant B13)
Bcr	Breakpoint cluster region gene product
BL-21	A protease-deficent strain of E.coli
BSA	Bovine serum albumin
C	Cytidine
CRMP	Collapsin response mediator protein
DAG	Diacylglycerol
ddH$_2$O	Deionised, purified water
DH	Dbl homology domain
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
E.coli	Escherichia coli
EDTA	Ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	Ethylene glycol-0,0'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	Extracellular signal-regulated kinase

F-actin	Filamentous actin
FGF	Fibroblast growth factor
G	Guanosine
GA	Glutathione agarose
G-actin	Globular (monomeric) actin
GAP	GTPase-activating protein
GDI	Guanine-nucleotide dissociation inhibitor
GEF	Guanine-nucleotide exchange factor
G-protein	Guanosine-nucleotide-binding protein
GST	Glutathione S-transferase
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
GTPase	Enzyme that catalyses the hydrolyis of GTP to GDP
Hsp	Heat shock protein
IF	Intermediate filament
IP$_3$	Inositol 1,4,5 triphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
JAK	Janus kinase
JNK/SAPK	c-Jun amino-terminal kinase/ stress activated protein kinase
kDa	Kilo dalton
Klenow	E. coli DNA polymerase I (large fragment)
LB	Luria-Bertani medium
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MAPKAP	MAP-activated protein kinase
MCS	multiple cloning site
MEK	MAPK kinase
MES	4-morpholinepropanesulphonic acid
MLCK	myosin light chain kinase
MRCK	Myotonic dystrophy related Cdc42-binding kinase
N	A, C, G, or T
NF	Neurofilament

NADH	Reduced nicotinamide adenine dinucleotide
NF1	Neurofibromatosis type 1gene product
NGF	Nerve growth factor
NP-40	Nonidet P40; ethylphenyl-polyethylene glycol
NTP	Nucleotide 5'-triphosphate
dNTP	Peoxynucleotide 5'-triphosphate
PAGE	Polyacrylamide gel electrophoresis
p21	Member of the Ras family of low molecular weight GTPases
p38	Hog1-related MAPK
p85	The regulatory subunit of PI-3-kinase
p110	The catalytic subunit of PI-3-kinase
PAK	$p21^{Cdc42/Rac}$-activated kinase
PBS	Phosphate buffered saline
PDGF	platelet-derived growth factor.
PH	Pleckstrin homology domain
PI	Phosphatidylinositol
PIP	Phosphatidylinositol 4-phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PI 3-K	Phosphatidylinositol 3-kinase
PI 4P-5K	Phosphatidylinositol 4 phosphate -5-kinase
PIX	Pak-interacting nucleotide exchange factor
PK-A	cAMP-dependent kinase
PK-C	Protein kinase C
PL-A	Phospholipase A
PL-C	Phospholipase C
PL-D	Phospholipase D
PMA	Phorbol 12-myristate 13 acetate
PMSF	Phenylmethylsulphonyl fluoride
PTB	Phosphotyrosine binding domain
mRNA	Messenger RNA
RNase A	Ribonuclease A

ROK	RhoA-binding kinase
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
Sev	Sevenless
SDS	Sodium dodecyl sulphate
SH2	Src homology 2 domain
SH3	Src homology 3 domain
Sos	Son of sevenless
SRE	serum response element
SRF	serum response factor
STAT	Signal transducer and activator of transcription
SV40	simian virus 40
T	Thymidine
TCF	Ternary complex factor
TEMED	N,N,N',N'-tetramethylethylene diamine
TGF	Transforming growth factor
TOAD-64	'Turned-on-after-division', a neuronal phosphoprotein
(p64	Recombinant TOAD-64)
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
Triton X-100	Octylphenoxypolyethoxyethanol
Tween-20	Polyoxyethylenesorbitan monolaurate
WASP	Wiskott-Aldrich syndrome protein
XL-1B	XL-1Blue, a strain of E.coli

CHAPTER ONE

INTRODUCTION

Cells exist in complex environments to which they must be able to respond. The cues they receive may originate from the extracellular matrix, adjacent cells or soluble factors such as growth factors and neurotransmitters and the appropriate response may be as diverse as differentiation or death, may lead to the initiation of transcription or to the rearrangement of pre-existing cellular molecules or structures. The series of molecular interactions that connect the cue and the response constitute a signal transduction pathway which can be described biochemically by elucidation of the individual protein-protein interactions. At any one time the cell may be receiving signals from multiple sources; these may not act evenly over the entire cell surface but be localised to specific regions and the response to any single signal may be dependent upon others. Alternatively, a single signal may mediate multiple responses. A series of discrete linear signalling cascades would not offer a sufficiently flexible system for the co-ordination of multiple responses; there must be points at which different pathways can interact and regulate each other downstream of their initial activators, molecules that act as focal points for the integration of the cellular response to its environment. This is facilitated by the modular design of many signal transduction molecules.

Variety in the potential cellular stimuli demands diversity in the array of receptors at the cell surface and there are a number of different classes of these proteins , for example the cytokine receptors and the seven-transmembrane receptors. Many of the signalling molecules that regulate cell growth and development activate transmembrane receptor tyrosine kinases (RTKs) which can then initiate intracellular signalling pathways via their cytoplasmic domains. When growth factor binds the extracellular domain of an RTK, receptor dimerisation is induced. This activates the intracellular tyrosine kinase domain, leading to autophosphorylation. Phosphorylated tyrosine residues are high affinity binding sites for the Src Homology 2 (SH2) domains of cytoplasmic signalling molecules which are consequently recruited to the membrane where they can promote the activation of their downstream target proteins, so propagating the signal that was received at the cell surface. The proteins that bind to the activated receptor may themselves contain catalytic domains but there also exist adaptor proteins that are not direct effectors of the RTK but, by their ability to interact with more than one protein, permit the formation of multimeric signalling complexes.

The activation of RTKs can regulate diverse processes that include phospholipid

metabolism, the activation of the Ras family of low molecular mass GTPases and protein phosphorylation of cellular substrates and malignant transformation can be induced by mutation that causes constitutive activation of the RTK or its downstream targets. The correct response of the cell to its extracellular environmentwill depend upon the integrity of the molecular step subsequent to receptor activation. The phenotypic effects that are observed upon RTK stimulation will be the net product of a complex intracellular signalling network and the greater the number of individual interactions that are elucidated, the better the understanding of the co-ordination of the cellular response to an extracellular cue.

1A Receptor Tyrosine Kinases

The ligand binding site of a receptor tyrosine kinase is found on its extracellular domain, separated from the conserved cytoplasmic tyrosine kinase domain by a single transmembrane region. There are also regulatory sequences in the cytoplasmic domain that are subject to autophosphorylation and may act as binding sites for specific downstream effectors. Extracellular domains typically contain combinations of conserved modules, including immunoglobulin-like domains, fibronectin type-III type domains and cysteine rich domains. The different receptors are classified into families on the basis of similarities in their structural characteristics and at the present time more than sixteen such families have been identified (Schlessinger, 1997). Examples of such families include the Platelet-Derived Growth Factor Receptors (PDGFRs), the Epidermal Growth Factor Receptors (EGFRs), the Fibroblast Growth Factor Receptors (FGFRs), the neurotrophin receptors (Trk A, B and C) and the Eph receptor family.

1A.1 The Activation of Receptor Tyrosine Kinases by Soluble Ligands

A model originally proposed for the mechanism of activation of the EGFR is of general applicability to RTKs (reviewed by Lemmon & Schlessinger, 1994). With the exception of the insulin receptor, which pre-exists as a dimer of α/β pairs, all known receptors with tyrosine kinase activity undergo dimerisation upon binding of the cognate ligand. Autophosphorylation and the subsequent recruitment of target proteins to the membrane ensues.

There are variations in the precise mechanisms of receptor dimerisation. In certain

cases, e.g. PDGF, the ligand is itself dimeric and can simultaneously interact with two receptor molecules, so inducing receptor dimerisation. Other ligands, including EGF and FGF, are monomeric in solution and it has been proposed that ligand binding stabilises, rather than induces the formation of, a dimeric form of the receptor. EGFR dimerisation even in the absence of ligand has been demonstrated in A431 cells (a cell line that over-expresses this receptor), supporting this model (Gadella & Jovin, 1995). FGF monomers require the co-operation of soluble or membrane-associated heparin sulphate proteoglycans to bind them into a multivalent complex that is competent to induce FGFR dimerisation (Spivak-Kroizman et al, 1994). Human growth factor (hGF), although a monomer, can simultaneously bind two receptor molecules (de Vos et al, 1992). A requirement for an accessory molecule at the level of the receptor rather than the ligand has also been observed. The Ret receptor can bind and be activated by glial derived nerve growth factor (GDNF) binds to only when it is itself complexed with a membrane-attached lipid-binding sub-unit (Jing et al, 1996). Thus, even if tyrosine kinase receptor ligands are not themselves dimeric, the evidence suggests that they can function divalently to cross-link two separate receptor molecules.

The combination of different isotypes of a receptor, heterodimerisation rather than homodimerisation, is a mechanism that effectively expands the range of distinct functional forms of a receptor, allowing an increase in the potential diversity of signalling. This phenomenon was first described during studies of the PDGFR. Two forms of the PDGFR exist, α and β, and also two forms of PDGF; AA PDGF binds only the $\alpha\alpha$ receptor isotype and AB the $\alpha\alpha$ and $\alpha\beta$, whereas BB PDGF binds all three possible isotypes (Heldin et al, 1989). There may be specific differences in signalling through these alternative ligand/receptor complexes may be seen and different cell types may be restricted in the receptor isotypes they express, so limiting their response to a particular signal.

Tyrosine kinase activation follows receptor dimerisation. The subsequent phosphorylation that follows is not 'true' autophosphorylation for it is believed that one member of the dimer phosphorylates the other (Ullrich & Schlessinger, 1990). Phosphorylation is restricted principally to two distinct sets of tyrosine residues. In many cases, for example those of the PDGFR and the insulin receptor (IR), a single conserved tyrosine (Tyr-857 in the PDGFR-β) or multiple conserved residues in the kinase domain are

phosphorylated. This precedes further phosphorylation events and can up-regulate the kinase activity. Other receptors, for example the EGFR, are not regulated by phosphorylation within the kinase domain (Heldin, 1995). The second set of tyrosine residues that are autophosphorylated are normally located outside the kinase domain and act as substrate binding sites for effector molecules containing phosphotyrosine-binding motifs.

1A.2 The Activation of Receptor Tyrosine Kinase by Non-Diffusable Ligands

Although the physiological ligands of growth factor receptors were conventionally considered to be soluble factors, a number of membrane-bound ligands have now been described. These activate cognate receptors that are expressed on the surface of neighbouring cells, providing a mechanism for communication between adjacent cells, short range rather than long range communication. Such ligands include the Ephrins, membrane-bound or transmembrane proteins that bind and activate Eph receptors (reviewed by Orioli & Klein, 1997). Like other RTKs, the Eph receptors autophosphorylate in response to ligand binding but significant activation occurs only if the ligand is membrane-bound; ligands released into the culture medium have little effect (Davis et al, 1994). The majority of Eph receptors are specifically expressed in the nervous system; the expression patterns are spatially restricted and extremely dynamic in the developing embryo. The binding of ligand to these receptors causes a repulsive signal that is essential for correct axonal guidance. Interestingly, an ephrin/Eph receptor interaction can lead to phosphorylation of the cytoplasmic domain of ephrin, suggesting that a bi-directional signal is being transduced (Holland et al, 1997).

A number of receptors have been isolated on the basis of sequence similarities but have no known physiological ligand; these have been termed orphan receptors. The extracellular matrix protein collagen has recently been identified as the novel ligand for two receptors that had previously fallen into this category, DDR1 and DDR2 (Shrivastava et al, 1997 and Vogel et al, 1997). Tyrosine phosphorylation of these receptors can be stimulated either by both soluble and immobilised fibrillar collagen. The kinetics of activation are unusual: soluble growth factors can activate their receptors within seconds whereas stimulation of DDR1/2 is seen only after prolonged treatment with collagen (30-60 minutes), possibly suggesting a novel activation mechanism.

1B Substrates of Activated Receptor Tyrosine Kinases

Src Homology 2 (SH2) domains and phosphotyrosine binding (PTB) domains can both interact with phosphotyrosine-containing peptide target sequences that are generated upon activation of a receptor tyrosine kinase; the specificity of the interactions are determined by the amino acid context of the phosphorylated amino acid residues (reviewed by Pawson, 1995 and Pawson & Scott, 1997). These protein modules will be discussed in greater depth later in this chapter. They are found either in molecules that have a catalytic function and can act as direct effectors, such as the Src tyrosine kinase, or in adaptor or docking proteins such as Grb2 and Shc that contain specialised protein-protein interaction domains but have no catalytic activity and serve to promote the formation of multimeric signalling complexes. Phosphorylation of such molecules can effectively expand the range of potential binding sites available to effector proteins upon RTK activation. This category of proteins also includes two major substrates of the insulin receptor, IRS-1 and IRS-2 (Yenush et al, 1996). In some cases, for example that of Grb2, translocation to the membrane is sufficient for activation of downstream signalling, whereas in others, for example Shc, the protein must be phosphorylated by the receptor tyrosine kinase to allow effector binding and the activation of downstream signalling pathways (Rozakis-Adcock et al, 1992). In the case of direct effectors, interaction with the receptor may lead to up-regulation of catalytic activity. Hck, a Src family kinase, shows a ten-fold increase in its kinase activity in the presence of the optimal phosphopeptide substrate for its SH2 domain (Pellicena, 1998). Modulation of catalytic activity may be dependent upon phosphorylation, as in the case of phospholipase-$C\gamma$ (Nishibe et al, 1990) or, as suggested for phosphatidylinositol-3-kinase (PI-3K), may be dependent upon a conformational change that occurs upon interaction with the target (Van Horn et al, 1994). A schematic representation of possible mechanisms by which the activation of RTKs may be coupled to the activation of downstream signalling pathways is shown in Figure 1.1.

1B.1 Receptor Tyrosine Kinases can Bind Multiple Substrates and Activate Multiple Signalling Pathways

The intracellular domain of an activated RTK contains multiple potential substrate binding sites. For example, eleven sites have been identified in the cytosolic domain of the PDGFR-β
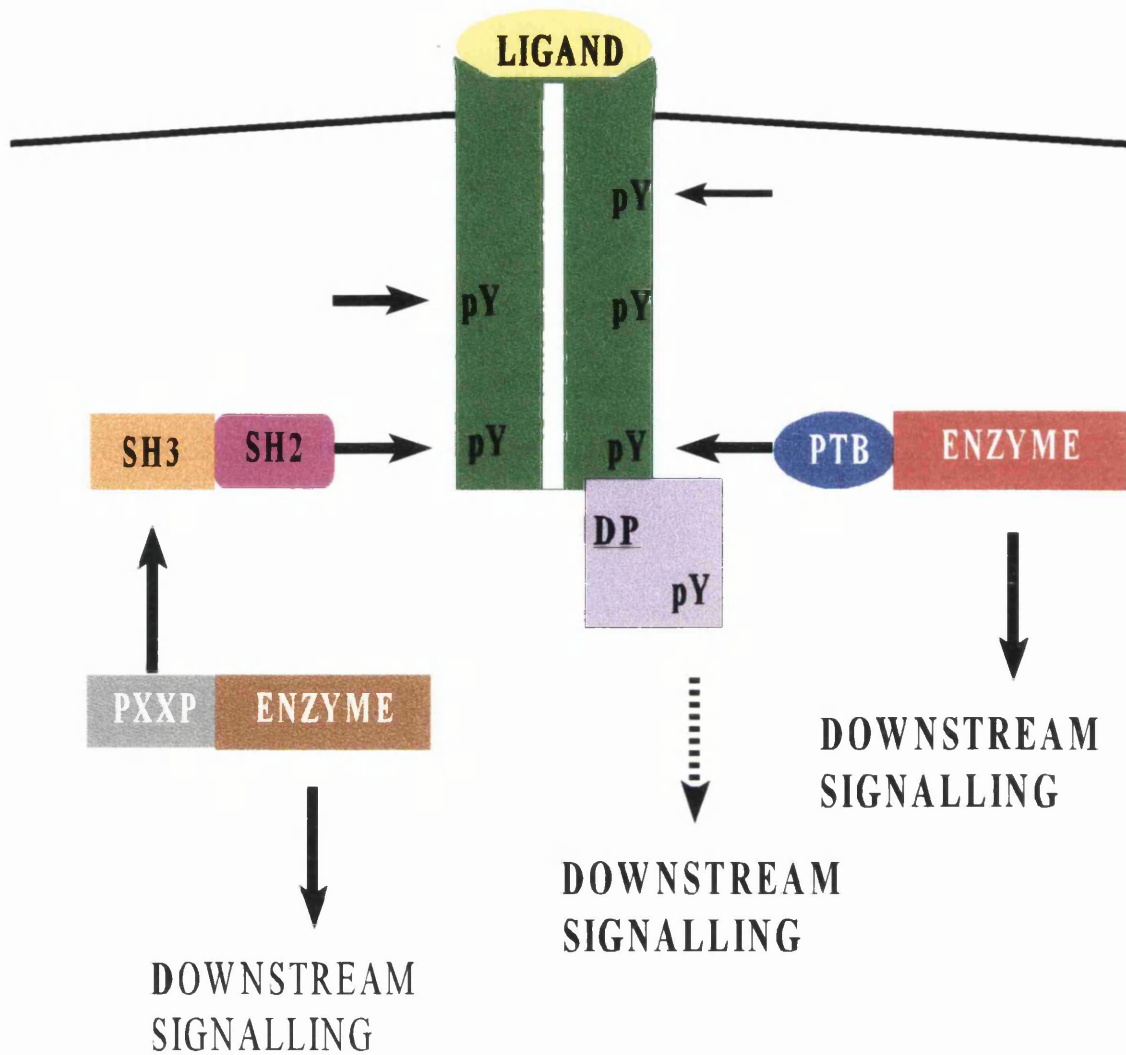
Figure 1.1: Activation of Signalling Pathways Downstream of Receptor Tyrosine Kinases

This figure illustrates three possible mechanisms by which receptor activation may be coupled to downstream signalling events. Upon activation of a receptor tyrosine kinase by ligand binding, autophosphorylation occurs on intracellular tyrosine residues (pY), creating multiple binding sites for SH2 domain and PTB domain-containing proteins. Binding proteins may contain other non-catalytic modules, e.g. SH3 domains, which can recruit and activate downstream effectors. Alternatively, a protein with catalytic activity may be directly activated by the interaction of its SH2/PTB domain with the receptor. A 'docking protein' (DP) may be phosphorylated upon receptor activation of the receptor, creating further binding sites for downstream signalling molecules.

and at least ten different molecules have been shown to interact, amongst them Src kinase, the adaptor proteins Grb2, Nck and Shc, PI-3K and the tyrosine phosphatase SHP-2, initiating signalling pathways that lead to cell growth and motility (Heldin, 1997). The functional diversity of the potential effectors suggests that stimulatory and inhibitory pathways may be initiated in parallel. Multiple effectors may bind to a single site and there can be considerable overlap between the sets of effector proteins associating with different sites on an activated RTK. The adaptor protein Nck, the phosphatase SHP-2 and the lipid kinase PI-3-K can all bind to the Y1213 phosphorylation site on the Flt-1 RTK upon stimulation with vascular endothelial growth factor (VEGF) and Nck can also bind the Y1333 site (Igarashi et al, 1998). Different signalling pathways may utilise common components and this can lead to alternative signalling responses: for example, Crk, an adaptor protein that can associate with the activated PDGFR or with the insulin receptor substrate IRS-1, has distinct modulatory effects on PDGF- and insulin-dependent signalling pathways (Sorokin et al, 1998).

The existence of a novel protein-binding domain that can interact with RTKs in a kinase-dependent manner has recently been reported (He et al, 1998). The BPS module (between PH and SH2 domains) is a fifty amino acid residues of Grb10, an adaptor protein of unknown function whose SH2 domain has been implicated in insulin receptor signalling. The sequence is highly conserved in the related proteins, Grb7 and Grb14. The IR bound both the SH2 and BPS domains of Grb10 whereas the EGFR, to which Grb10 binds less avidly than to the IR, interacted strongly with only the SH2 domain. This suggests that, in certain cases, multidomain interactions may modulate the specificity with which a single target protein interacts with different receptors.

1B.2 The Duration of Signalling can Determine Signalling Output

Certain receptors may induce differing biological effects by initiating biochemically similar signalling cascades. The rat pheochromocytoma cell line, PC12, has been used as a model system for the study of neuronal differentiation; differentiation and neurite outgrowth are induced in response to NGF (Greene & Tischler, 1976). The TrkA receptor and the EGFR mediate differentiation and proliferation respectively. These responses appear not to be defined entirely by differences in the downstream pathways utilised but partly by the duration

of the action of these pathways: NGF induces a prolonged stimulation of the MAP kinase pathway whereas the stimulation of this pathway in response to EGF is transient. The TrkA and EGF receptors have different affinities for the phospholipase Cγ SH2 domain and exchange of the binding site sequences between the two receptors results in a modified signalling pattern that reflects the original source of the SH2 domain binding site. This suggests that different affinity RTK/substrate interactions may affect the signalling output (Obermeier et al, 1996).

1C Phosphatases can Transduce Positive and Negative Signals

The central role of specific phosphorylation in the control of cellular signalling implies that dephosphorylation must also be precisely regulated. A common feature of intracellular phosphatases is the presence of non-catalytic domains that mediate protein-protein interactions. For example, the mammalian tyrosine phosphatases SHP-1 and SHP-2 contain two amino terminal SH2 domains that both regulate phosphatase activity and enable recruitment to complexes of specific tyrosine-containing proteins (Sun & Tonks, 1994). The interaction between an RTK and its substrate may directly inactivate the receptor. SHP-1 has been shown to negatively regulate growth factor and cytokine-induced signalling pathways: it can bind to and down-regulate the activation of the c-Kit RTK following stimulation with stem cell factor (Koslowski et al, 1998) and is also a critical negative regulator of interleukin-3 (IL-3) signalling in haemopoietic cells (Yang et al, 1998). The level and duration of RTK-mediated tyrosine phosphorylation can be greatly increased by treatment with phosphatase inhibitors (Gordon, 1991). Inhibition of an IR-associated phosphatase correlates with the up-regulation of Insulin signalling (Band et al, 1997), and expression of tyrosine phosphatases is implicated in the reversal of Src-mediated transformation (Woodford-Thomas et al, 1992). Phosphatase signalling is not necessarily inhibitory: the mammalian tyrosine phosphatase SHP-2, related to the Drosophila phosphatase Corkscrew, is required for positive signalling and couples activation of the PDGFR-β to the low molecular mass GTPase Ras (Bennett et al, 1994).

Protein serine kinases are also essential cellular regulators. The cyclin-dependent kinases regulate cell-cycle transitions, PP1 plays a central role in the insulin-induced switch from glycogen catabolism to glycogen synthesis and serine phosphatases are the targets of

many microbial toxins (reviewed by Hunter, 1995).

1D The Activation of Downstream Signalling Pathways

The molecular components of a number of intracellular signalling pathways have been described. Multiple signalling pathways may be activated by a single receptor type. The induction of epithelial tubules by the hepatocyte growth factor involves firstly the activation of Rac and PI-3-K, secondly the stimulation of the Ras-dependent MAP kinase cascade and lastly activation of cytosolic STAT (signal transducer and activator of transcription), enabling translocation to the nucleus and induction of transcription (Boccaccio et al, 1998). Additional complexity may be introduced by 'cross-talk' between different pathways: different signalling pathways have have common components and hence the activation of one may cause the activation of another at a stage downstream of the receptor.

1D.1 MAP Kinase Cascades

The binding of growth factors, hormones and cytokines to their receptors regulates signalling cascades that control diverse cellular functions. MAP (Mitogen Activated Protein) Kinase cascades link the activation of RTKs to cytoplasmic and nuclear events. These cascades are best characterised in yeast, where distinct MAPK pathways regulate processes downstream of G protein-coupled receptors including mating type, cell wall biosynthesis and osmotic sensitivity; the mammalian and yeast kinases are well conserved (Herskowitz, 1995). The central component of these pathways is a three kinase cascade. A serine/threonine 'MAP kinase kinase kinase' (MAPKKK) which phosphorylates and activates a dual specificity 'MAP kinase kinase' (MAPKK) which in its turn phosphorylates threonine and tyrosine residues and activates a serine/threonine MAP kinase (MAPK), the effector of the pathway. The amino acid residues phosphorylate by the MAPKK are present in a 'TXY' motif and MAPKs can be classified on the identity of the 'X' amino acid residue, as indicated in Figure 1.2.

1D.1A The ERK Family of MAPKs

In the ERK pathway, implicated in regulation of cell growth, differentiation and protection from apoptosis (Seger & Krebs, 1995), the three kinases of this signalling cascade are Raf

(a MEKK), MEK1/MEK2 and ERK1/ERK2 (Extracellular signal-regulated kinases of 42 and 44 kDa). MEK1/2 can also be activated by Mos, a serine/threonine kinase that, unlike Raf, has a restricted tissue distribution. Signalling is initiated by the activation of Ras (reviewed by Treisman, 1996). Receptor activation recruits the Ras exchange factor Sos to the membrane where it activates Ras by promoting the transition from the inactive GDP-bound state to the active GTP-bound state (Rozakis-Adcock, 1993). Ras-GTP binds Raf and causing the translocation of Raf to the membrane but studies suggest that the Ras/Raf1 interaction has a role in Raf1 activation that is distinct from membrane recruitment (Mineo et al, 1997).

Downstream targets of ERKs are present both in the cytosol, for example $p90^{rsk}$ kinase (Blenis, 1993), and in the nucleus where the phosphorylation of transcription factors leads to immediate-early gene induction. Two nuclear substrates, Elk-1 and SAP-1 are Ternary Complex Proteins (TCP). These bind the serum response element (SRE), a sequence present in the promoters of many growth factor-regulated genes, to form a ternary complex with serum response factor (SRF). ERKs phosphorylate the TCPs *in vitro* and the kinetics of their modification correlate well with those of ERK *in vivo* (reviewed by Treisman, 1994). Activation of the ERK cascade has been shown to be sufficient for cellular transformation (Mansour et al, 1994).

1D.1B The JNK and p38 Families of MAPKs

The c-jun amino terminal kinases (JNKs) and the related family of stress activated proteins kinases (SAPKs) are a second family of MAP kinases. They are activated in response to chemical and heat stress, ultraviolet radiation and tumour necrosis factor. Unlike the ERKs, there are reports of their activation by many upstream kinases, including MEKKs, MLKs (mixed lineage kinases), PAKs (p21 activated kinases) and TAKs (TGFβ- activated kinases). Some of these kinases phosphorylate MKK4, a specific activator of JNK, *in vitro,* suggesting that their effects may be mediated through an alternative MEKK-like protein (reviewed by Fanger et al, 1997). The JNKs bind to the amino terminal of c-jun, phosphorylate it on serine residues and activate transcription.

The p38 kinase, activated in response to hyperosmotic shock and inflammatory cytokines (Raingeaud et al, 1995), is a third MAPK. It is the mammalian homologue of the
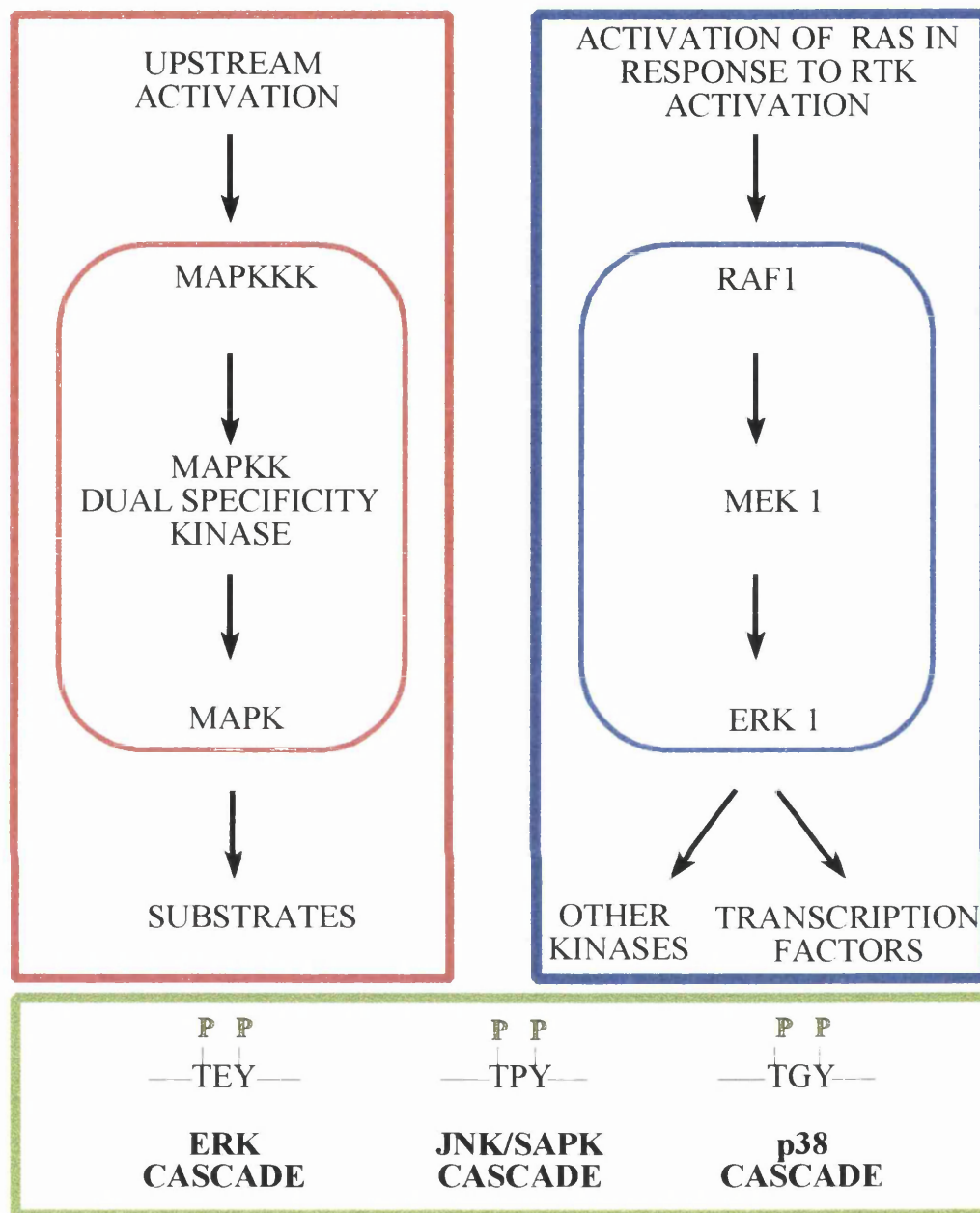
Figure 1.2: The Map Kinase Signalling Cascade

A generic MAP kinase signalling cascade is detailed in the red panel and its central MAP kinase module (MAPKK →MAPKK →MAPK) is outlined. A specific example of such a cascade, leading to activation of the extracellular signal-regulated kinase, ERK, is described in the blue panel. The second kinase of the MAPK module phosphorylates the MAPK on threonine and tyrosine residues. The peptide motif phosphorylated differs according to the MAPK; these different motifs are indicated in the green panel. The abbreviations used are detailed in Section 1D.6.

yeast kinase HOG1 which is involved in protection against osmotic shock (Brewster et al, 1993). Ras has not been shown to directly activate either the JNK or p38 cascades but the Ras-related Rho family of low molecular mass GTPases can do so (Minden et al, 1995; Coso et al, 1995 and Hill et al, 1995). Their role in the activation of transcription through these pathways is discussed at later point.

The number of potential regulators of the JNK pathways encourage speculation that a specific MKKK/MKK/JNK cascade may exist for the transduction of each particular upstream signal. A slightly different interpretation which would account for the rather fewer known modulators of the ERK cascades is that the signalling output seen in response to stimulation of a cell will depend rather on the balance of all the MAPK pathways active at that time.

1D.2 Phospholipid Signalling

Two sets of enzymes can generate biologically active lipid molecules that act as second messengers in signalling pathways: the phospholipases and the phosphatidylinositol kinases. Certain inositol phospholipid products of the these kinases are substrates for hydrolysis by the phospholipases. These different enzymes can be targets for activated receptors.

1D.2A Phospholipase C and Phospholipase D

Phospholipase-C (PL-C) exists in three isoforms, β, γ and δ, and hydrolyses phosphatidyl inositol phospholipids to produce diacyl glycerol (DAG), an activator of protein kinase-C (PK-C). The second reaction product depends on the specific substrate: if it is phosphatidyl inositol (PI), inositol-1-phosphate (IP_1) is generated, if PI-phosphate (PIP), inositol-1,4-bisphosphate (IP_2) and, if PI-4,5 bisphosphate (PIP_2), then inositol trisphosphate (IP_3). The latter product mediates the release of calcium from intracellular stores (Berridge, 1993). All three PL-C isoforms contain a pleckstrin homology domain which may regulate membrane association. PL-Cβ are activated downstream of heterotrimeric G protein-linked receptors whereas PL-Cγ has SH2 domains which mediate its association with activated receptor tyrosine kinases, for example the EGFR, Trk receptors, the PDGFR and the FGFR. The receptor/substrate interactions lead to the up-regulation of PL-Cγ phospholipase activity (reviewed by Lee & Rhee, 1995). Over-expression of PLC-γ in fibroblasts is associated with

cellular transformation (Smith et al, 1998). A phosphatidylinositol transfer protein (PITP) has been identified; it appears to be required for sustained PL-C signalling (Kaufmann Zeh et al, 1995). The activity of PL-Cγ is decreased and PL-Cδ is increased in the brains of patients suffering from Alzheimers Disease (Shimohama et al, 1998), suggesting that these enzymes are potentially involved in the pathophysiology of the disease.

Phospholipase-D (PL-D) hydrolyses phosphatidylcholine (PC) to phosphatidic acid and choline. Phosphatidic acid has been implicated in the regulation of diverse signalling molecules (Foster, 1993), and choline. The activity of one PL-D isoform is dependent upon the small GTPase Arf and PIP$_2$, indicating that co-factors may be play an essential role in regulating the activity of these enzymes (Brown et al, 1995). PL-D can be activated by v-Src by a mechanism that is dependent on the activation of the GTPases Ras (Jiang et al, 1995a) and Ral (Jiang et al, 1995b).

1D.2B Phosphatidyl Inositol kinases

Phosphatidyl inositol kinases phosphorylate the D-3, D-4 and D-5 positions of the inositol ring of phosphatidyl inositol (PI) to generate PI-3P, PI-4P, PI-4,5P$_2$, PI-3,4P$_2$ and PI-3,4,5P$_3$. Multiple isoforms of each of these enzymes have been identified (reviewed by Carpenter & Cantley, 1996).

1D.2B.1 Phosphatidyl Inositol 3-Kinase

The products of PI-3K phosphorylation accumulate in the cell in response to agonist stimulation. Diverse cellular functions have been ascribed to these lipids, some of which have been shown to specifically interact with PH domains. The first PI-3-K described was a mammalian enzyme formed of a p110 catalytic subunit and a p85 regulatory subunit. p85 has tandem SH2 domains that can associate with activated protein tyrosine kinases and also a putative RhoGAP domain (Carpenter et al, 1990). Other family members have since been identified, some of which have restricted substrate specificity, for example the yeast Vps34p, a PI-specific kinase (Schu et al, 1993). Activation may be mediated by different cell surface receptors, including the $\beta\gamma$ subunit of heterotrimeric G proteins (e.g. Kurosu et al, 1997) and receptor tyrosine kinases: for example, the p85 subunit SH2 domain binds and is phosphorylated by the activated PDGFR (Kavanaugh et al, 1994). Alternatively, PI-3-K can

be activated by interaction between the p110 subunit and activated Ras (Rodriguez-Vicania et al, 1994).

Amongst the cellular activities in which PI-3-K has been implicated are mediation of an anti-apoptotic pathway via the growth factor-regulated serine/threonine kinase PK-B (reviewed by Downward, 1998a), the regulation of cell motility and cytoskeletal rearrangement (van Weering et al, 1998 and Hawkins et al, 1995), mediation of the effector functions of Ras and vesicular trafficking (Cremona & De Camilla, 1997). The activation of PL-C can down-regulate PI-3K activity (Batty et al, 1997). Signalling through the lipid products of PI-3-K has recently been reviewed by Toker & Cantley (1997).

1D.2B.2 Phosphatidyl Inositol 4-Phosphate 5-Kinase

PI-4-P-5K interacts with and is modulated by small GTPases of the Rho subfamily. This leads to production of PI-4,5P$_2$ which has been identified as a regulator of the actin cytoskeleton (Hartwig et al, 1995). This lipid can also interact with the PH domain of the exchange factor Sos, inhibiting its activation of Ras, possibly suggesting a negative regulatory link between the Rac and Ras signalling pathways (Jefferson et al, 1998).The involvement of Rho family proteins and lipid kinases will be discussed later in this chapter.

1E Other Cell Surface Receptor/Signalling Systems

Different classes of cytokine receptors mediate signal transduction upon stimulation with interleukins, interferons, colony-stimulating factors and hormones and undergo homo- or hetero-oligomerisation when exposed to their cognate ligands. The intracellular domains of these receptors lack intrinsic kinase activities but, when activated, can bind and activate specific Janus tyrosine kinases (JAKs). JAKs autophosphorylate and phosphorylate receptor components, creating binding sites for STATs which are recruited to the membrane and activated by phosphorylation upon which they dimerise, translocate to the nucleus and participate in transcriptional activation (reviewed by Heldin, 1995). The phosphorylation of cytokine receptors can also create binding sites for SH2 domain-containing proteins. These may then activate signalling molecules that are considered to lie downstream of receptor tyrosine kinases, for example Ras (Boulton et al, 1994). Cytokine receptors and RTKs may also share certain substrates: the JAK2-binding protein, SH2-B β, which binds can also with

the activated PDGFR (Rui & Carter-Su, 1998).

Cell surface receptors with intrinsic serine/threonine kinase activity have also been identified; the majority transduce signals for members of the Transforming Growth Factor-β (TGF-β) superfamily which includes bone morphogenic proteins and growth differentiation factors (Josso & di Clemente, 1997).

1F The Cytoskeleton

The ability of cells to adopt diverse morphologies and to move in a co-ordinated and directed manner depends on the integrated activities of three sets of protein filaments, defined in terms of their major protein component, which together comprise the cytoskeleton: actin fibres, microtubules and intermediated filaments. The cytoskeleton is a highly dynamic structure which can rearrange rapidly in response to both intracellular and extracellular stimuli; such reorganisation is mediated through the rapid polymerisation/depolymerisation of actin fibres and microtubules. The proteins that form these fibres and proteins that associate with them are potential targets for cell signalling molecules.

1F.1 The Actin Cytoskeleton

Each filament (filamentous actin, F-actin) consists of a helical arrangement of uniformly orientated actin monomers (G-actin) and is a polar structure with a relatively inert minus or pointed end and a faster-growing plus or barbed end. Polymerisation is ATP-dependent: a conformational change occurs upon polymerisation of ATP-bound actin monomers leading to ATP hydrolysis. The physiological environment is rich in ATP and it is believed that F-actin and G-actin exist in a dynamic equilibrium in which, in the absence of any regulatory factor, the addition of actin monomers at the barbed end is matched by the dissociation of actin from the pointed end, a process described as treadmilling (Wegner, 1976). To maintain this steady-state balance, the cell employs different regulatory proteins. Monomer sequestering proteins such as profilin which can control polymerisation by regulating monomer availability (Sun et al, 1995). Capping proteins, such as gelsolin, that regulate polymerisation onto at the ends of pre-existing filaments (Schafer & Cooper, 1995).

1F.1A Actin Filaments Can be Cross-Linked to Create Different Structures

Actin monomers of 43 kDa can assemble into different arrangements of filaments, including filamentous bundles or meshworks. These manifest themselves at the leading edge of a cell, a major site of actin polymerisation, as slender protrusions of the plasma membrane, termed filopodia, and sheet-like projections, termed lamellipodia. A third arrangement of actin filaments, stress fibres that traverse the cell and are associated with adhesion complexes at the cell periphery, is formed in response to the activation of the small GTPase Rho but the physiological relevance of these structures is not fully established. These fibres demonstrate an ATP-dependent contractile activity. Actin binding proteins that cross-link the individual filaments give rise to these different arrangements. The largest class of cross-linking proteins includes fimbrin, α-actinin, actin binding protein-120 (ABP-120) and spectrin. Members of this family are characterised by the presence of a conserved 27kDa actin-binding domain (Matsudaira, 1994). This consists of two tandem calponin homology (CH) domains, although it has been demonstrated that a single CH domain can support binding to F-actin (Way et al, 1992). These proteins either contain two actin-binding domains (e.g. fimbrin) or dimerise to produce a functional unit that contains two binding sites (e.g. α-actinin). The relative proximity of the two F-actin binding modules will determine the filamentous structure formed: if the modules are close together they will direct the formation of rigid, tightly-packed structures and if they are more distant, loose assemblies will be produced. The cross-linkers are potential targets for signalling molecules which may lead to the modulation of cellular actin structures. For example, both fibrin and α-actinin-mediated cross-linking is regulated by calcium binding to the EF hand motifs present in these molecules (Puius et al, 1998). The interaction between actin and its binding proteins may also be regulated by PIP_2: for example, the binding of vinculin to talin and actin is promoted by PIP_2 (Gilmore & Burridge, 1996).

1F.1B The Interaction of Actin and Myosin

The interaction between actin filaments and myosin is the basis of cellular contractility, a mechanism well characterised in skeletal muscle but which is also an essential feature of such processes as the action of the contractile ring in separating two daughter cells during mitosis in all cell types. Recent evidence suggests that myosin movement on microfilaments is

required for diverse cellular processes including organelle movement, endo/exocytosis, neurosensation and RNA transport (Mermall et al, 1998). Fourteen classes of myosins have been identified to date and they act as motor proteins, translocating along actin filaments in an ATP-dependent fashion (Baker & Titus, 1998). These proteins contain well-conserved head domains at their amino termini which contain the actin-binding site and divergent carboxyl terminal domains which are suggested to target the myosin to its site of action and may have a distinct biological activity. The unconventional myosins myr 5 and myosin IXb have GTPase activating domains for the Rho family of proteins at their carboxyl termini (Muller et al, 1997 and Wirth et al, 1996 respectively).

1F.1C Regulation of Actin Polymerisation

Polymerisation can be initiated by the extension of pre-existing filaments or by nucleation of new filaments. Members of the gelsolin and cofilin families of proteins can sever existing filaments, so creating free barbed ends; gelsolin can also cap free ends, inhibiting polymerisation (Schafer & Cooper, 1995). Capping protein (CP) is a ubiquitously expressed protein that can bind to free barbed ends; both it and gelsolin proteins are subject to regulation by phosphoinositide lipids which promote dissociation of the protein from the filament, so revealing a site for polymerisation (Ayscough, 1998). It has been reported that high concentrations of PIP_2 can modulate actin filament growth by three different mechanisms: the inhibition of capping, severing pre-existing filaments and the removal of capping proteins (DiNubile & Huang, 1997). Gelsolin proteins are further regulated by calcium, which promotes their capping activity. Monomer-binding proteins such as profilin sequester G-actin in the cytosol but there is increasing evidence that their effects on the dynamics of actin polymerisation *in vivo* are rather more complex than the simple inhibition that is observed *in vitro*. Net polymerisation is achieved when the combined action of the different regulatory proteins is such as to produce a localised increase in the effective G-actin concentration, shifting the steady-state equilibrium towards increased polymerisation. The mechanisms by which actin polymerisation is nucleated *in vivo* are not well understood at the present time, although advances have been made by the study of pathogenic model systems: invading *Listeria monocytogenes* and *Shigella flexneri* rearrange and recruit the host cell actin cytoskeleton, nucleating actin polymerisation from a single protein expressed

42

on their cell surfaces (reviewed by Higley & Way, 1997).

1F.1D ERM Proteins Cross-Link the Actin Cytoskeleton and the Membrane

Actin microfilaments differ from the other filament types of the cytoskeleton is that they are intimately associated with the plasma membrane. Proteins of the ezrin/radixin/moesin (ERM) family are believed to play a general role in mediating this association; their interactions with actin and with the membrane are regulated by signal transduction pathways (reviewed by Tsukita et al, 1997). The ERM proteins are approximately 80 kDa in size and share a highly conserved amino terminal membrane-binding domain with similarity to that found in the erythrocyte membrane protein, Band 4.1 (Arpin et al, 1994). This domain interacts specifically with membrane-associated proteins including CD44 (Tsukita et al, 1994). The actin binding site, localised to 34 amino acid residues at the carboxyl termini (Turunen et al, 1994), is also highly conserved. ERM proteins are substrates for both tyrosine and serine/threonine phosphorylation (Arpin et al, 1994). The potential impact of this is not well understood although it has been suggested to be a possible signal for translocation to the cell periphery and activation (Chen et al, 1995). At physiological ionic strength full length ERM proteins have a relatively low affinity for CD44; this is not the case for the isolated amino terminal of the protein, implying that the carboxyl terminal may regulate the interaction (Hirao et al, 1996). Specific interactions between ERM proteins and the inositol phospholipids PIP and PIP_2 which can increase the affinity of the proteins for their membrane targets have been reported. This may be a mechanism for the activation of these proteins.

There is evidence indicating that the Rho family of low molecular mass GTPases may be an upstream regulator of ERM protein activation and hence of cross-linking actin filaments and the plasma membrane. The interaction between ERM proteins and CD44 in the presence of cell extract is enhanced by GTPγS, a non-hydrolysable GTP analogue that, when bound to Rho would prevent its inactivation. This effect is abolished by C3 toxin, a specific inhibitor of Rho (Hirao et al, 1996). The Rho guanine nucleotide dissociation inhibitor (RhoGDI), which inhibits the activation of Rho family proteins, can bind ERM proteins (Takahashi et al, 1997). This interaction down-regulates GDI function, and so promotes the activation of Rho. The Rho family proteins are known regulators of the actin

cytoskeleton and involvement in the regulation of the ERM proteins might contribute to this activity.

The regulation of the actin cytoskeleton is mediated by interactions with many proteins. The activity of different binding proteins can be modulated by different signals allowing restructuring of the filamentous organisation in response to cellular signalling processes.

1F.2 The Microtubule Network

Microtubules are cylindrical structures of varying length found in almost all eukaryotic cells. Their diameter is 25nm and they have a hollow core of approximately 5nm, making them the largest of the cytoskeletal filaments (Brinkley, 1997). They are required for diverse cellular functions, including chromosome separation during mitosis, organelle transport and the maintenance of cellular morphology. α and β tubulin, the components of microtubules, are highly homologous proteins of approximately 55kDa. Each exists in different isotypic forms that can undergo a variety of post-translational modifications and may be differentially expressed. The different isotypes are not necessarily functionally interchangeable, although they are generally still able to form tubules. The carboxyl terminal sequences of different isoforms are diverse and it has been proposed that they mediate isotype-specific activities (reviewed by Luduena, 1998)

1F.2A The Assembly of Microtubules

Microtubules are formed by the self-assembly of $\alpha\beta$-tubulin dimers into a proto-filament; around thirteen of these protofilaments then bundle along their length to form a hollow tubule. Like actin filaments, microtubules are polar structures, a consequence of the interaction of the $\alpha\beta$ heterodimers in a head-to-tail arrangement (reviewed by Wade & Hyman, 1997). Polymerisation occurs preferentially at the 'plus' end whilst the 'minus' end remains tethered in the interior of the cell. Nucleation of the tubule is directed out from the centrosome and mediated through a specialised form of tubulin, γ-tubulin, which binds the minus end of the tubule (Zheng et al, 1995). It is expressed in rings around the centromere.

Microtubule assembly is a GTP-dependent process and there are GTP-binding sites on both the α and β subunits but it is only the GTP bound to β-tubulin that is hydrolysed

44

(Downing & Nogales, 1998). This occurs following the addition of a dimer to the end of the growing microtubule and consequently β-tubulin is associated with GDP when assembled into tubules. The stability of the tubule is maintained by a β-tubulin-GTP cap at the end; upon loss of this cap the tubule can disassemble. Microtubules are highly dynamic structures, undergoing cycles of elongation and cutting-back (treadmilling), but the *in vitro* kinetics of this cycling differs depending on whether cell extracts or purified tubulin is tested, indicating that the cytoplasm may contain factors that regulate this process *in vivo* (Matus, 1988). Microtubules can form highly organised networks that differ according to cell type: for example, in the axons of neuronal cells the microtubules are arranged longitudinally with the plus end pointing away from the cell body whereas in the majority of cell types the microtubules radiate from the centre of the cell, the plus end pointing towards the cell periphery.

1F.2B Microtubule Associated Proteins and Regulation of Microtubule Dynamics

The regulation of microtubule dynamics, microtubule spacing and microtubule organisation has been ascribed to a set of microtubule associated proteins (MAPs, reviewed by McNally, 1996) and, more recently, to motor proteins of the kinesin and dynein families which are involved in the transport of cellular organelles along microtubules (reviewed by Hirokawa et al, 1998). Tubulin has also been shown to interact *in vitro* with the low molecular mass GTPase Rac1 in its GTP-bound state, although Rac1 was not shown to affect tubulin polymerisation (Best et al, 1996). The Rac1 binding site is located in the amino-terminal region of the tubulin molecule. Recently, the βγ subunit of a heterotrimeric G protein has been shown to modulate microtubule assemblies (Roychowdhury & Rasenick, 1997). The regulatory proteins must be able to control both the assembly and maintenance of stable structures, such as those found in cilia and flagella, and the transient rearrangements that are necessary for cellular activities, including reorganisation of the mitotic spindle to allow segregation of duplicated chromosomes.

MAPs have been identified on the basis of their ability to interact with tubulin *in vitro*. This family includes large proteins (200-300 kDa) such as MAP-1A-C, MAP-2 and MAP-4 and smaller proteins such as Tau and MAP-2C which share an ability to interact with the carboxyl termini of tubulins and stabilise microtubules (Maccioni & Cambiazo, 1995).

Expression of these proteins can be developmentally regulated and some MAPs are selectively distributed: for example, in a single neuron Tau is expressed predominantly in the axon whereas MAP-2 is associated with dendritic structures. Differences in the amino terminal regions of MAPs have been associated with the formation of differential organisations of microtubule bundles (Hirowaka, 1994). Tau is abnormally hyperphosphorylated in the brains of patients with Alzheimer's disease and it is believed that competition of this pathological form of Tau with tubulin for binding to normal Tau inhibits the correct assembly of microtubules in affected neurons, a feature of this neurodegenerative disorder (Grundke-Iqbal & Iqbal, 1989).

1F.3 Intermediate Filaments

So called because, at 10nm, their diameter is greater than that of actin filaments (6 nm) and less than that of microtubules (23nm), intermediate filaments (IFs) extend throughout the cytoplasm and are formed by the packing of tetramers of fibrous molecules, such as keratin, vimentin and the neurofilament proteins which are characteristic of different cell-types. These proteins have an amino terminal head domain and a carboxyl terminal tail domain linked by a central α-helical domain that promotes the formation of coiled-coil dimers between two parallel molecules. Two such dimers assemble to produce the repeating unit of the filament, an antiparallel tetramer whose staggered ends can overlap with other tetramers to form an extended filament. Humans possess more than fifty IF genes which are differentially expressed in different tissues. The different IF proteins may have distinctive molecular characteristics, affecting such features as solubility. Whilst in general IF proteins constitute approximately 1% of the total cell protein, in specialised cell types such as keratinocytes this figure may be as high as 85% (Fuchs & Cleveland, 1998). Whilst originally IFs were believed to form a relatively stabile lattice there is increasing evidence that they are dynamic structures that can reversibly connect the plasma membrane to intracellular compartments, exerting a modulatory influence upon cell shape. Neurofilaments (NFs) are the predominant type of IF in most adult neurons and there is evidence to suggest that they regulate the increases in axonal diameter that occur following synapse formation, a key determinant of conduction velocity, and that they are involved in driving neuronal outgrowth (Houseweart & Cleveland, 1998). Abnormal accumulation of NFs is a feature of the

pathology of various human neurodegenerative disorders.

Evidence that IFs can cross-link microtubules and actin filaments has come from the observation that the injection of peptides corresponding to a conserved region present in IF proteins into fibroblasts can disrupt all three filamentous networks (Goldman et al, 1996). Studies of plectin, a 400 kDa protein that interacts with IFs, have revealed that it can link actin and IF filament bundles and form cross-bridges between IFs and microtubules (Svitkina et al, 1996). That this protein plays an essential role in the integration of the different components of the cytoskeleton is illustrated by the effect of its null mutation in mice: the skin disorder Epidermolysis Bullosa Simlex (EBS), muscular dystrophy and death within two-three days of birth (Andra et al, 1997). The pathological consequences of the mutation of IFs or associated proteins has been reviewed by Fuchs & Cleveland (1998).

IF.4 The Cytoskeleton and Cellular Adhesion

Adhesion structures such as desmosomes, tight junctions and adherens junctions all contain linkages to cytoskeletal proteins and it is believed that the multi-molecular complexes of proteins that form at these sites of cytoskeletal/extracellular interface can act as both the source of and target for signalling pathways. To permit morphological changes and cell motility sites of adhesion must necessarily be dynamic. Cell-substrate adhesions are generally based on integrin-type receptors whereas cell-cell adhesions such as adherens junctions contain cadherin (reviewed by Yamada & Geiger, 1997). One well-studied example of the latter is the anchorage of keratin IFs at desmosomes. This enables integration of IFs across cell boundaries and provide a structural basis for the mechanical strength of epidermal tissues (reviewed by Chou et al, 1997). Focal adhesions, the contacts made between cultured cells and their extracellular matrix, have been used as an *in vivo* model system for the analysis of integrin-based adhesive structures. The assembly of focal adhesions is associated with tyrosine phosphorylation and the recruitment of proteins that can specifically interact with actin, e.g. α-actinin, to the cell membrane. Focal adhesion assembly can be induced in response to activation of the Rho family GTPases (reviewed by Hall, 1994) and also to integrin clustering and activation (reviewed by Yamada & Miyamoto, 1995).

1G Non-Catalytic Signalling Modules

Conserved protein modules, present in diverse signalling molecules, act as the basis for the organisation of specific protein-protein and protein-phospholipid interactions. The presence of a number of these domains within a single molecule permits the establishment of a versatile network of multi-molecular interactions that may then mediate a co-ordinated response to extracellular stimuli. Over the last ten years many such domains have been identified and several of these are described below.

1G.1 Src Homology 2 Domains

Src Homology 2 (SH2) domains are conserved protein modules of approximately 100 amino acid residues that bind tyrosine-phosphorylated peptides (as reviewed by Pawson, 1995 and Cohen et al, 1995). They are present in mammalian cells and a single, divergent SH2 domain sequence has been reported in S.cerevisiae (in the nuclear protein SPT6, Maclennan & Shaw, 1993) but are not believed to be present in plants or bacteria. The affinity of the interaction between the SH2 domain and its optimal target peptide is in the range 10-100nM. SH2 domains are found in a variety of protein contexts and can be associated with catalytic domains, for example with kinase domains in the src family of non-receptor tyrosine kinases, and also within non-catalytic molecules known as adaptor proteins such as grb 2. Tandem SH2 domains are a feature of several signalling molecules, including the p85 subunit of PI-3-K, SHP-2, ZAP-70 and PL-Cγ1. It has been proposed that the simultaneous interaction between two tandem SH2 domains and bi-phosphorylated protein motifs confers a higher level of specificity than that seen typically for individual SH2 domains (Ottinger et al, 1998).

The primary sequence carboxyl-terminal to the phosphotyrosine residue of the SH2 target peptide is critical in determining the specificity of the interaction (Cantley & Zhou, 1994). This has been studied extensively by Songyang et al (1993) who used recombinant SH2 domains to select target peptides from a degenerate phosphopeptide library. One group of SH2 domains (including src, abl and crk) preferentially selected sequences with the general motif pTyr-hydrophilic-hydrophilic-Ile/Pro whilst a second (including the p85 and phospholipase Cγ SH2 domains) selected pTyr-hydrophobic-X-hydrophobic and this difference is reflected in the distinct tertiary structure of the phosphopeptide binding site in these two classes of SH2 domain. The structure and target binding mechanism of SH2

domains will be described elsewhere in greater depth.

SH2 domain function mey be regulated by direct phosphorylation of the SH2-domain containing protein. Whilst a primary role of SH2 domains is to mediate interactions between different proteins, intramolecular interactions can serve as mechanisms for the regulation of enzyme activity. This is the case for the src family kinases which are held in a catalytically-inactive state by an intramolecular interaction between the SH2 domain and a carboxyl terminal phosphotyrosine, Tyr 527 (Xu et al, 1997). Alternatively, the effect of phosporylation may be to regulate the interaction with target proteins. Phosphorylation of a tyrosine in the carboxyl terminal SH2 domain of p85 correlates with reduction in the binding of a subset of target proteins without detectable change in the enzyme activity of PI-3K (von Willebrand et al, 1998).

Interaction between an SH2 domain and its tyrosine phosphorylated target may also has a regulatory effects function, for example, the facilitation of further phosphorylation of the substrate. The catalytic activity of Hck, a Src family kinase, is increased in the presence of a high affinity phosphopeptide target (Pellicena et al, 1998). The interaction between p190-RhoGAP and the tandem SH2 domains of p120-RasGAP induces a conformational change in p120 that increases the accessibility of its SH3 domain to ligands (Hu & Settleman, 1997)

1G.2 Phosphotyrosine Binding Domains

A phosphotyrosine-binding (PTB) domain distinct from an SH2 domain was first identified in the Shc adaptor protein. Others were then identified on the basis of sequence similarity (Bork and Margolis, 1995). The average length of these regions is around 160 amino acid residues but there is considerable variability. PTB domains recognise phosphopeptide motifs in which the phosphotyrosine is preceded by residues that form a β-turn (Kavanaugh et al, 1995), usually with the consensus NPXY, and specificity is conferred by hydrophobic amino acid residues 5-8 residues amino-terminal to the phosphotyrosine. These domains are predominantly found in docking proteins that recruit other proteins to activated receptors and, in some cases, can interact with unphosphorylated peptides (e.g. Li et al, 1997). Interactions between PTB domains and phospholipids can also occur; the Shc PTB domain can bind acidic phosphoinositides. This activity may reflect the similarity in the tertiary

49

structures of the PTB and PH domains (Zhou & Fesik, 1995). Other proteins in which PTB domains have been identified include the insulin receptor substrates, IRS-1 and IRS-2 (Yenush et al, 1996) and the proto-oncogene cbl (Lupher et al, 1997).

1G.3 Src Homology 3

Sequence comparison between src and other molecules led to the identification of a second module that could mediate protein-protein interactions, the SH3 domain. These are approximately 60 amino acid residues in size and bind proline-rich peptide sequences of approximately 10 amino acid residues with a core PXXP motif that forms a left-handed polyproline type II helix (Feng et al, 1994). The affinity of the interaction between an SH3 domain and the polyproline region of its ligand are estimated to be within the 5-100μM range (Pawson, 1995). The affinity of the interaction between the SH3 domain and a larger sequence form the ligand may be considerably higher: the Grb2 binds to the carboxyl terminal two hundred amino acid residues of Sos with a K_d of around 2nM (Sastry et al, 1995). However, the interaction between the amino terminal SH3 domain of the PIX exchange factor and a 22 amino acid residue peptide containing a proline-rich region from Pak has a K_d of approximately 20nM, suggesting that in some cases structural integrity of the protein sequences surrounding the polyproline region do not influence the binding affinity (Manser et al, 1998). The ligand peptides are pseudo-symmetrical and can potentially bind in either an amino- to carboxyl terminal orientation (Class 1) or a carboxyl- to amino orientation (Class 2, Lim et al, 1994). Phosphorylation of the ligand in the regions adjacent to the polyproline-rich sequence may down-regulate SH3 domain interactions (Chen et al, 1996).

SH3 domains are frequently found in conjunction with SH2 domains, as in the src kinase family and in adaptor molecules such as nck and grb2, but are also found independently, for example in the neutrophil oxidase component p67[phox] and proteins may have multiple SH3 domains, as found in the Rac1-binding protein POSH (Tapon et al, 1998). They are present in many cytoskeletal or cytoskeletally-associated proteins, for example the actin binding protein α-spectrin and non-muscle myosin 1b, and also in proteins that can regulate the cytoskeleton, as in the PLC-γ SH3 domain which targets this enzyme to the actin cytoskeleton (Bar-Sagi et al, 1993). Unlike SH2 domains they are present in

yeast.

1G.4 Pleckstrin Homology Domains

The pleckstrin protein is the major protein kinase C substrate in platelet cells and has two protein modules of approximately 120 amino acid residues which have come to be described as pleckstrin homology or PH domains (Haslam et al, 1993). The protein sequence homology between PH domains is generally low but, in those cases studied, the similarity between tertiary protein structures was found to be high (reviewed in Lemmon et al 1996) indicating the PH domain is a discrete functional unit. These domains bind the charged headgroups of specific phosphoinositides and may thereby target proteins to specific regions of the plasma membrane. PIP_2 was identified as a high affinity ligand of the PH domain of PLC-δ_1 with a dissociation constant in the micromolar range, but the majority of the reported interactions of other PH domains with this lipid are rather weak. However, other high affinity phospholipid-PH domain interactions have been described, for example the binding of the RasGap1 PH domain to IP_4 (Fukuda & Mikoshiba, 1996), an interaction that may directly link receptor-mediated PI-3 Kinase activation to the Ras signalling pathway.

PH domains are present in a wide variety of proteins and can be linked with other modules including SH2, SH3 and PTB domains with which they may synergise in activating specific signalling proteins. Interestingly, all known Dbl-homology domains (with predicted or proven guanine nucleotide exchange activity towards p21 GTPases) are followed by a PH domain. There are also a number of examples of PH domain-containing kinases that bind Rho family GTPases such as the ROK family, which binds RhoA, and MRCK, which binds Cdc42 (Leung et al, 1996 and 1998 respectively). Other than their interactions with phosphoinositides, binding to the $\beta\gamma$ subunits of heterotrimeric G proteins and to PK-C has been reported (reviewed by Shaw, 1996) suggesting a potentially diverse range of physiological ligands.

1G.5 Other Protein Modules

A large number of other specialised protein modules have been identified (Table 1.3). It is noteworthy that there is some overlap in the ligand specificity of some groups of these domains, for example both WW and SH3 domains bind proline-rich motifs. This suggests

that different domain families may mediate similar functions in the cell but potentially adds a further layer of complexity that may permit greater specificity.

Some modules interact with very specific sets of molecules. These include the p21 GTPase binding domain which may be found in proteins that interact with Rac1 and Cdc42Hs such as the family of p21-activated kinases (Paks, reviewed by Sells & Chernoff, 1997). The function of other types of protein module may be to mediate homo- or hetero-dimerisation. An example would be the DEATH domain (Feinstein et al, 1995) which is present in several proteins essential for the transduction of cytotoxic signals. The activity of some proteins, such as PK-C and chimaerin, may be regulated by phospholipid binding to a cysteine rich domains.

1H The GTPase Superfamily

GTP-binding proteins are involved, either directly or indirectly, in regulating diverse activities that include vesicle trafficking, actin polymerisation and cell proliferation. At least three major classes of these proteins have been identified: elongation and initiation factors that direct ribosomal protein synthesis; heterotrimeric G proteins that mediate transmembrane signalling and the Ras family of low molecular mass GTPases. The crucial regulatory feature of these different classes of proteins is their intrinsic GTPase activity and consequent ability to cycle between GTP- and GDP-bound states, effectively acting as molecular 'on/off' switches (reviewed by Bourne et al, 1990). The binding and hydrolysis of GTP triggers the transition between different conformational states. The differential abilities of the GTP and GDP-bound states to interact with other proteins is the basis of the molecular switch illustrated in Figure 1.4; the GTPase is considered 'on' when GTP-bound and 'off' when GDP-bound. Cycling between these two states is regulated by proteins that catalyse GTP/GDP exchange or up-regulate the intrinsic GTPase activity. The structure and function of heterotrimeric G proteins will be described briefly and the cellular functions of the low molecular mass GTPases discussed in greater depth.

1H.1 Heterotrimeric G Proteins

Heterotrimeric G proteins bind transmembrane receptors for sensory, hormonal or

Domain	Proteins in Which Domain is Present	Ligands or Function
Band 4.1	Ezrin, Radixin, Moesin	Membranes
Cysteine Rich Domain, CRD	PK-C, Chimaerin, Raf	Diacyl Glycerol, Phospholipids
Ca^{2+}-dependent Lipid-Binding, CalB	PK-C, PL-C, PL-D	Ca^{2+}, Phospholipid
Calponin Homology, CH	Dystrophin, PIX	Actin
FH1/FH2	Formin/ MDia	Profilin
LIM	Paxillin	Zn^{2+}, tyrosine-containing peptides
PDZ	PTP-1H, LIM Kinase	Carboxyl-terminal peptides
PTB	Shc, Cbl, IRS-1	Phosphotyrosine
PH	Spectrin, PL-Cγ1 Proteins with Dbl-homology domains	Phosphoinositides, $G_{\beta\gamma}$ complexes
Sterile Alpha Motif, SAM	Eph Receptor Tyrosine Kinases	Dimerisation
SH2	Src, Abl, PL-Cγ	Phosphotyrosine
SH3	Src, PL-Cγ, Spectrin	Poly-proline motifs
WD40	G_β subunits	Protein Binding
WW	Dystrophin	Poly-proline motifs

Table 1.3: Examples of Protein Signalling Domains

The names of different protein modules, examples of the proteins in which they are found and their ligands or functions are described. The abbreviations used include: PL, phospholipase; PTP, protein tyrosine phosphatase; G_β/ $G_{\beta\gamma}$, subunits of heterotrimeric G proteins.

neurotransmitter signals and transduce their signals (reviewed Emala et al, 1994). They are formed from three separate polypeptides: the α subunit, which binds and hydrolyses GTP, and the β and γ ($\beta\gamma$) subunits which act as a functional monomer and anchor the complex to the cytoplasmic face of the plasma membrane. The heterotrimeric complex is considered inactive and the α subunit is in a GDP-bound state. Upon activation of a heptahelical transmembrane receptor by ligand binding, the α subunit of the heterotrimeric complex binds the cytoplasmic region of the receptor. A conformational change is induced reducing the affinity for GDP which consequently dissociates, allowing GTP to bind. This results in dissociation of the α subunit from the heterotrimeric complex, freeing it to interact with and activate an effector protein, for example adenylate cyclase. Its activity persists until the bound GTP is hydrolysed whereupon it returns to its original conformation and the inactive heterotrimeric G protein complex is re-established (reviewed by Neer, 1994).

The existence of multiple isoforms of each G protein subunit gives rise to many different heterotrimeric complexes. Four sub-types of α subunit have been identified on the basis of amino acid residue similarities: αs, αi, αq and α12 (Hamm & Gilchrist, 1996) and also their different sub-types of the β and γ subunits. The combination of specific subunits will produce a G protein with unique pharmacological and physiological characteristics and hence the coupling of a G protein to an activated receptor can lead to specific and selective downstream effects.

The model of signal transduction as outlined above is believed to be generally applicable but does not indicate the potential complexity of the signalling output. Not only may a single receptor signal through different G proteins but a particular G protein may activate different effector systems. While the α subunit was conventionally held to regulate effector activation, more recent evidence indicates that the $\beta\gamma$ complex also participates in many G protein functions, interacting with proteins including potassium channels (Doupnik et al, 1997), adenyl cyclase (Weng et al, 1996), PI-3 Kinase (Lopez-Ilasaca et al, 1998) and members of the Rho family of low molecular mass GTPases (Harhammer et al, 1996). Synergistic cross-talk interactions occur between G proteins specific for different neurotransmitters, so that the activation of one signalling pathway amplifies signalling within a separate pathway (reviewed by Selbie & Hill, 1998). Furthermore, G protein-mediated signalling may also affect pathways downstream of receptor tyrosine signals; for example,

certain G protein-linked receptors contain tyrosine residues which, if phosphorylated, would be putative binding sites for SH2 domain-containing proteins.

1H.2 The Ras Superfamily of Low Molecular Mass GTPases

The low molecular mass GTPase Ras proteins were first identified as the transforming agents of the Harvey and Kirsten stain of rat sarcoma viruses (Barbacid, 1987). Four 'true' Ras proteins are expressed in mammalian cells: H-Ras, K-Ras, K-Ras-B and N-Ras. Each of these can function as an oncogene upon mutational activation and such mutations contribute to some 30% of human malignancies (Bos, 1989). These proteins regulate cellular process that include proliferation, differentiation (Bourne et al, 1991) and apoptosis (Downward, 1998b). A fifth protein, R-Ras, is 55% identical to H-Ras but cannot itself transform cells although it does interact with Bcl2, a regulator of apoptosis (Fernandez-Sarabia & Bischoff, 1993). The true Ras proteins are strongly related to members of the Rap family and Rap1A can reverse K-Ras-induced cell transformation; this may be due to competition for Ras effectors. To date, over fifty Ras-related GTPases have been identified and these have been classified into sub-families on the basis of protein sequence similarities; the identity between sub-families is approximately 30% and 40% within a single sub-family (Kahn et al, 1992).

The gene products of the Ras superfamily are characterised by their low molecular mass (20-35 kDa) and their GTP-binding domain; they are commonly referred to as p21s. The p21s can be activated in response to a variety of stimuli, including the activation of receptor tyrosine kinases, integrin clustering and the activation of heterotrimeric G proteins. Members are present in the most simple eukaryotes (e.g. Lohia & Samuelson, 1996), lower and higher eukaryotes (e.g. Didsbury et al, 1989 and Bourne et al, 1991 respectively) and plants (e.g. Dallery et al, 1996). Their *in vitro* rates of GTP hydrolysis and GDP dissociation are approximately 0.008 and 0.02/min respectively (Bourne et al, 1990). The *in vivo* activity is finely controlled by different families of regulatory proteins. GTPase activating proteins (GAPs) down-regulate p21 activity by increasing the intrinsic GTPase activity of the p21, so promoting the conversion of GTP to GDP and consequently returning the p21 to its inactive conformation. Guanine-nucleotide exchange factors (GEFs) mediate activation by catalysing the exchange of GTP for GDP; guanine nucleotide dissociation inhibitors (GDIs)

inhibit this nucleotide exchange (Boguski & McCormick, 1993). These regulatory proteins will be discussed in more depth at a later point.

Specific functions have been ascribed to some of the p21 subfamilies, such as the roles of the Arf sub-family in vesicle trafficking, the regulation of transport across the nuclear membrane by the Ran family, the involvement of Rab proteins in endocytosis and exocytosis. Proteins of the Rho sub-family play major roles in regulating cytoskeletal rearrangements in response to extracellular signals (Hall, 1994), the activation of the neutrophil NADPH oxidase complex, transcriptional activation, endocytosis and cell cycle control. Members of different sub-families may also mediate similar activities; for example both Ras and Rho-type proteins can activate transcription via MAP kinase cascades (Pulverer et al, 1991 and Coso et al, 1995).

Hierarchies of different Ras-related GTPases may regulate cellular activities, acting co-operatively or antagonistically. Ras can induce membrane ruffling in fibroblasts in a Rac-dependent manner (Ridley and Hall, 1992) whereas constitutively-activated Cdc42 can oppose Rho-mediated effects on remodelling of the actin cytoskeleton (Kozma et al, 1995) There are further examples of the involvement of multiple GTPases in the production of a particular phenotype, such as a requirement for Cdc42 in the Ras-mediated transformation of Rat1 cells (Qiu et al, 1997). Each p21 can form its own set of regulatory and effector interactions and hence the connection between different p21s may be complex.

1H.3 Lipid Modification of p21-GTPases

p21 proteins are targeted to membranes by a post-translational modification, the covalent attachment of isoprenoid lipids via a thioester linkage (Clarke, 1992). Ras family members with carboxyl terminal CAAX sequences (where A represents an aliphatic residue and X usually a serine or methionine) are prenylated by farnesyltransferase. Type I geranylgeranyltransferase also recognises a CAAX motif although the preferred 'X' residue is a leucine, typical of the Rho family proteins, whereas the substrate of the Type II enzyme is the carboxyl terminal CC or CXC motif typical of the Rab sub-family proteins. Subsequent methylation can increase the affinity of the lipid group for the membrane by a factor of approximately ten (Bhatnagar & Gordon, 1995).The geranylgeranyl modification has a fiftyfold greater affinity for the membrane than the farnesyl modification and the different

specificities of the modifying enzymes implies that there will therefore be differences in the relative abilities of the p21 sub-families to interact with the membrane. There is evidence to suggest that the process by which lipid-modified proteins are directed to specific regions of the cell is regulated by protein-protein interactions (Seykora et al, 1996).

1H.4 The Three-Dimensional Structure of p21s

The crystal structures of several different wild-type and mutant Ras proteins complexed with both diphosphate and triphosphate guanine nucleotides have been resolved (Bourne et al, 1990) and more recently structures of two members of the rho sub-family, Rac 1 (Ihara et al, 1998) and RhoA (Hirshberg et al, 1997), complexed with non-hydrolysable GTP analogues, have become available. The overall topology of the GTPase fold is well conserved not only between the different p21s but even with elongation factor Tu: the polypeptide backbones are almost superimposeable despite having only around 30% primary sequence identity.

Ras folds into a series of six β-sheets which comprise the hydrophobic core of the protein and are connected by hydrophilic loops and α-helical regions. Five specific regions, all located within the loops on one side of the protein are critical for nucleotide exchange, GTP hydrolysis and GTP-induced conformational change. Specific sequence motifs in these regions are well-conserved between different p21s. Comparison of the GTP and the GDP bound structure indicated that the conformational changes induced by γ-phosphate are predominantly localised within two specific regions: residues 30-38 (Switch 1) in the second loop and residues 60-76 (Switch 2), which are found in the fourth loop and the short α-helical region that immediately follows it (Milburn et al, 1990). Both these regions are highly exposed and form a continuous strip on the surface of the molecule. Analysis of point mutations within and around the Switch 1 region has defined key residues required for effector interactions; this region is conserved in all p21s, indicating that specificity determinants must lie elsewhere in the protein. It has been proposed that the highly motile Switch 2 region may contribute to the selectivity of effector binding (Moodie et al, 1995).

The Rho sub-family proteins contain a thirteen amino acid residue sequence (the insertion region) that in not found in Ras proteins and has been identified as a novel effector domain (e.g. McCallum et al, 1996); excluding this region the crystal structures of RhoA and

Rac1 superimpose well on that of Ras. In both cases this sequence folds into two helices and a loop structure. Outside this domain, the most prominent differences between these and the Ras structure are found in the Switch regions. The structures of two other small GTPases, Arf and Ran, also showed significant differences in these regions (Amor et al, 1994 and Scheffzek et al, 1995 respectively); these structures also differed from those of Rac1 and RhoA.

Interpretation of p21 structural data allows rationalisation of the effect of certain point mutations that result in oncogenic transformation. Activating mutations fall into two classes: those which have reduced intrinsic GTPase activity and are unresponsive to GAPs (mutations at residues 12, 13 61 and 63 in Ras) and those which have an overall lowered affinity for guanine nucleotide and are predicted to preferentially exist in a GTP-bound form in the cell (mutations at residues 28, 116-119, 144 and 146). There are also dominant negative mutants, such as S17N-Ras, that not only cause loss of function but cause dominant inhibition of endogenous cellular Ras protein. This is believed to be due to competition with the wild-type protein for the upstream activators of Ras proteins, exchange factors (Polakis & McCormick, 1993). Mutation of equivalent sites in other p21s produces proteins with the same dominant positive and dominant negative characteristics and these have proved valuable research tools. Point mutations in the effector domain of Ras (residues 32-40) have been used to study its cellular functions. Such substitutions do not perturb nucleotide binding or GTP hydrolysis but lead to a wide range of dysfunctions with the most drastic effects caused by mutation of Aspartate 38. It is believed that specific effector domain mutations can uncouple interactions with specific effectors; for example, POSH, binds F37ARac but not Y40CRac (Tapon et al, 1998).

1H.5 Regulation of Ras Family Proteins
1H.5A GTPase Activating Proteins

p21GTPases have relatively low rates of GTP hydrolysis and their *in vivo* inactivation is dependent upon GTPase activating proteins, GAPs, which therefore have a critical role in maintaining control of the binary switching mechanism of p21s. The first such protein to be identified was p120-RasGAP (Trahay & McCormick, 1987), a GAP for H-Ras, K-Ras, N-Ras and R-Ras, and since that time GAPs have been identified for all other p21subfamilies

(reviewed by Boguski &McCormick, 1993). Sequence analysis of p120-GAP indicates that, in addition to the approximately 250 residues of the carboxyl-terminal GAP domain, it has an SH3 domain, two SH2 domains, a PH domain and a PL-A$_2$-homology region implicated in the binding of Ca^{2+} to a C2 domain; complex multi-domain structure is a typical feature of GAP proteins. The SH2 domains mediate the interaction of p120 with two phosphoproteins, p62 and p190 Rho GAP (Settleman et al, 1992). This last interaction suggests the potential for cross-talk between Rho and Ras-mediated signalling pathways. Another protein identified with GAP activity towards Ras is Neurofibromin. Dysfunction in the neurofibromin gene result in the heritable condition Type 1 Neurofibromatosis, characterised by the abnormal growth of Schwann cells and increased risk of malignant tumours (Bollag et al, 1993). The GAP activity of this protein can be inhibited *in vitro* by interaction with tubulin but the physiological relevance of this observation is not known.

IQGAP-1 (Weissbach et al, 1994) and IQGAP-2 (Brill et al, 1996) are a novel family of proteins containing a RasGAP-like domain. They can interact with the Rho-related p21s, Rac1 and Cdc42 (Kuroda et al, 1996). IQGAPs can also bind and cross-link actin filaments and hence may directly connect the activities of two different families of p21 and the cytoskeleton (Bashour et al, 1997). RasGAPs, including IRA1 and IRA2 have been identified in yeast (Bollag G & McCormick F, 1991). A C.elegans GAP, CeGAP, that has activity towards Ras, Rab and the Rho family proteins has also been identified (Chen et al, 1994); such broad specificity is unusual.

1H.5A.1 GAPs for Rho Family Proteins

The first GAP activity described for a member of the Rho subfamily of GTPases was a 29 kDa protein that stimulated the GTPase activity of Rho but not Ras (Garret et al, 1989 and 1991). This was subsequently found to be a carboxyl-terminal truncation product from a 50kDa protein (Lancaster et al, 1994). p50 RhoGAP is a ubiquitously-expressed protein that can stimulate the GTPase activity of Rho, Rac and Cdc42 *in vitro* (with a preference for Cdc42). Unlike RasGAP which has a hundred-fold greater affinity for the GTP-bound form of the substrate, p50 interacts equally well with GTP and GDP-bound Rho proteins (Schaber et al, 1989). This protein also has a proline-rich region which can interact *in vitro* with the SH3 domains of Src kinase and the p85α regulatory subunit of PI-3 Kinase (Barfod et al,

1993).

Diekmann et al (1991) showed that other proteins with *in vitro* GAP activity for Rho family proteins were related in sequence. Peptide sequence from the GAP domain of p50 was found to have homology to Bcr, the product of the breakpoint cluster gene. It is a cytosolic protein which, in addition to the GAP domain, has serine/threonine kinase activity, a Dbl homology domain (see next section) and a PH domain. The breakpoint cluster region is involved in the Philadelphia Chromosome translocation (Heisterkamp et al, 1985) that is a characteristic of some human leukaemias. A fusion between a truncated Bcr gene product lacking the GAP domain and the Abl cytosolic tyrosine kinase (p185Bcr-Abl) is present in the cells of patients suffering from chronic myelogenous leukaemia. A closely related protein, Abr, is similar in domain structure and substrate specificity to Bcr but lacks its kinase activity (Tan et al, 1993). Several of the proteins that regulate Rho family proteins are indicated in Figure 1.4 which illustrates the p21 GTPase cycle.

Other proteins containing a Bcr homology domain include p190RhoGAP (Settleman et al, 1992) and the chimaerin family of proteins; the latter have distinct patterns of expression within the nervous system, possibly implying unique regulatory roles in cellular regulation or development. Like other GAPS described, p190 is a multidomain protein, active preferentially towards Rho *in vitro* but with activity for Rac and Cdc42 as well. It was isolated on the basis of its interaction with p120RasGAP. There is evidence to suggest that this interaction up-regulates the GAP activity of p190 (McGlade et al, 1993): when the amino teminus of p120 is stably expressed in fibroblasts it is constitutively associated with p190 and when serum levels are reduced to 0.5%, disruption of the actin cytoskeleton occurs, akin to that seen in Swiss 3T3 cells when Rho activity is down-regulated. There are domains with similarity to the Bcr GAP sequence in the p85α and β regulatory subunits of PI-3 Kinase although these have not been demonstrated to have any GAP activity *in vitro*.

There is evidence supporting an association between loss of RhoGAP activity with tumourogenesis and developmental disorders in human. A gene encoding a putative RhoGAP, DLC-1, is often deleted in liver cancers, suggesting a potential role in tumour suppression (Yuan et al, 1998). Mutations in the Oligophrenin-1 gene which would be predicted to cause loss of function of the protein product are associated with X chromosome-linked mental retardation; this protein contains a Bcr-homology domain

GTPase Cycle
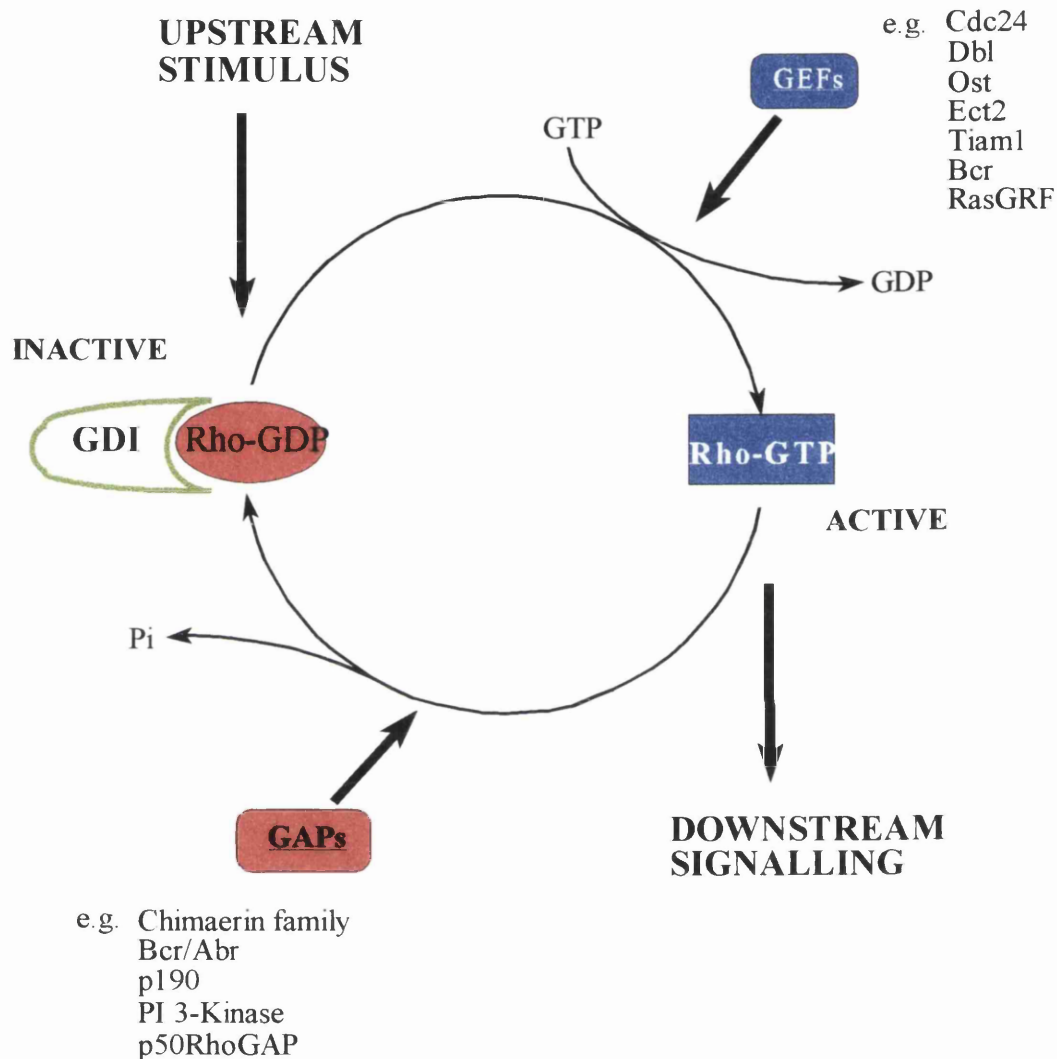
Figure 1.4: The p21 GTPase Molecular Switch

Low molecular weight GTPases (p21s) cycle between GDP- and GTP-bound, inactive and active states. This cycling is regulated by different classes of proteins, nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), postive and negative regulators of p21 function, respectively. Examples of GEFs and GAPs for the Rho family of p21s are listed. Guanine nucleotide dissociation inhibitor (GDI) sequesters the p21, preventing its activation.

(Billuart et al, 1998).

The Bcr-homology Rho GAP domain is approximately 140 amino acid residues in size and commonly found at the carboxyl terminal of large, multifunctional proteins. . Residues conserved between RhoGAPs are largely confined to one face of this bundle and it has been speculated that this may be the site of interactions with target proteins. The Bcr homology domain has no significant sequence homology with the RasGAP domain but it has recently been proposed that similarities in overall topography suggest a relationship between the two GAP domains (Bax, 1998). Whilst the overall identity between different RhoGAP domains is relatively low (20-40%), there are three major blocks of sequence conservation. A mutational analysis of the $\alpha 1$ chimaerin GAP domain (Ahmed et al, 1994) focused on these conserved sequences and demonstrated that the GAP activity could be isolated from the ability to interact with p21s. This implies that those proteins with putative RhoGAP domains that have not been shown to be enzymatically active may still bind Rho family proteins. RhoGAPs have also been identified in non-mammalian organisms, including S.cerevisiae (Zheng et al, 1994), D.discoidium (Ludbrook et al, 1997) and C.elegans (Chen et al, 1994).

1H.5A.2 The Three-Dimensional Structure of the Bcr-Homology Domain

The crystal structures of the Bcr homology domains of p85α and p50RhoGAP have been described (Musacchio et al, 1996 and Barrett et al, 1997 respectively); these domains fold into a series of seven and nine α-helices respectively with a four-helix bundle at the core. The interactions of these four helices, αA, αB, αE, αF, are roughly parallel. Residues conserved between Bcr homology domains are largely confined to one face of this bundle which may be a site for interactions with p21s. It has been proposed that the conserved arginine 85 and asparagine 194 amino acid residues of the p50RhoGAP domain, located in the A-αA loop and the αF helix respectively, may be involved in p21-binding and GTPase activation. Interestingly, in p85, which has not been shown to have GAP activity, a valine is found at the position equivalent to that of asparagine 194 in p50RhoGAP, suggesting that his amino acid residue may be essential for enzyme activity. A ribbon diagram indicating the tertiary structure of the p85 Bcr homology domain is shown in Appendix 3. An alignment of the protein sequences of several such domains highlighting the blocks of sequence

conservation is also shown.

1H.5B Nucleotide Exchange Factors

Activation of p21s is mediated by exchange factors that catalyse the dissociation of GDP, the guanine nucleotide exchange factors (GEFs). In yeast model systems, genetic loss of GEF function has similar effects to the loss of Ras itself. GEFs bind the GDP-bound p21, stimulating nucleotide dissociation. The cellular concentration of GTP is higher than that of GDP and, once free to do so, the p21 will bind GTP immediately and hence become activated. The conformational change induced upon activation reduces the affinity for the GEF which can then dissociate (Quilliam et al, 1995). In effect, GEFs act towards p21s in a manner analogous to that of the action of ligand-activated seven transmembrane receptors in the activation of heterotrimeric G proteins. Dominant loss-of-function p21 mutations, e.g. N17, have reduced affinity for GTP and are defective in the final stage of this process so that GDP and the GEF are more likely to be retained, sequestering the p21 into an inactive complex (Lai et al, 1993).

1H.5B.1 GEFs for Ras Proteins

Ras GEFs share a domain of approximately 200 amino acid residues in which three conserved blocks are separated by variable regions. In common with GAPs, the GEF domain is present in a complex protein environment, associated with other protein signalling modules. A well-established example of Ras activation mediated by GEF activity is that which occurs in response to activation of the EGFR. The adaptor protein Grb2 is complexed to the exchange factor Sos in the cytosol and recruited to the membrane upon intracellular tyrosine phosphorylation of the receptor. This also brings Sos to the membrane where it then activates Ras. The Grb2/Sos pairing in mammals is functionally equivalent to Drk/Sos in D.melanogaster and a Grb2 homologue has been identified in C.elegans, Sem5 (Kayne & Sternberg, 1995); this evidence suggests that this mechanism of p21 activation is very well conserved. Scd25 and Cdc25, two RasGEFs found in S.cerevisiae can interact with human Ras (Bollag & McCormick, 1991), implying that the p21/GEF interaction is mediated by highly conserved residues.

1H.5B.2 GEFs for Rho Family Proteins; the Dbl Homology Domain

The product of the Dbl oncogene, a cytosolic phosphoprotein, is the prototype for a large class of GEFs specific for Rho family proteins (Cerione & Zheng, 1996). A Dbl homology (DH) domain has been identified as a common feature of all known RhoGEFs. Interestingly, all known DH domains are associated with a carboxyl-terminal PH domain, although this is dispensable for GEF activity (Zheng et al, 1996). The DH domain was identified as a region of approximately 180 amino acid residues (Hart et al, 1994) with significant sequence similarities to Cdc24, the physiological activator of Cdc42 in S.cerevisiae (Zheng et al, 1994a), and to Bcr, which also has a Rac GAP domain. Other proteins containing Dbl homology domains include FGD1, whose gene is responsible for the human developmental disorder faciogenital dysplasia (Olson et al, 1996), the T cell tumour invasive gene product, Tiam 1 (Habets et al, 1994), and Vav, which can induce cellular transformation and is necessary for normal development. The PIX family of exchange factors contain a DH domain; these proteins interact with Pak and so may provide a point of potential cross-talk between Cdc42 and Rac-mediated signalling pathways (Manser et al, 1998).

The specificity of each of these GEFs may be restricted to one or possibly two members of the Rho family: for example, Dbl activates exchange only on RhoA and Cdc42, FGD1 on Cdc42, Tiam 1 on Cdc42 and Rac1. Trio, a serine/threonine kinase that interacts with the transmembrane tyrosine phosphatase LAR1, has two DH domains one of which is specific for Rac1 and the other for RhoA; this was found to be the case both *in vivo* and *in vitro* (Bellanger et al, 1998) and suggests a potential mechanism by which Rac1 and RhoA-mediated signalling pathways may interact. The exchange factor RasGEF2, which is primarily expressed in neurons has a DH domain in addition to its RasGAP domain can bind and activate both Ras and Rac and co-ordinate the activation of both the ERK and JNK kinases (Fan et al, 1998).

Regulation of *in vivo* GEF activity may, in some cases, be directed by tyrosine phosphorylation. Tyrosine phosphorylation of vav upregulates its exchange activity towards Rac1 *in vivo* (Crespo et al, 1997) but since there was no effect on Dbl activity it is unclear whether this is a general mechanism for control of exchange factor activity. Mutational analysis of the interactions of RhoA and Cdc42 with their respective GEFs, Lbc and Cdc24, indicated that multiple residues in the p21 were involved in the formation of a functional

64

interaction but that in each case there was an absolute requirement for a tyrosine (Tyr32) in the effector region (Li & Zheng, 1997). Other critical residues could be required either for interaction or catalysis. These differed in the two cases examined, raising the possibility that activation of a Rho family protein by a specific GEF may involve a unique mechanism.

GEFs specific for other p21 sub-families, including Ral, Ran, Rab and Rap, have also been identified. These appear to be unrelated in sequence to the other families of GEFs.

1H.5C Guanine Nucleotide Dissociation Inhibitors

The guanine nucleotide dissociation inhibitors (GDIs) sequester p21s in the cytosol in a 1:1 complex, preventing GTP/GDP exchange and their consequent translocation to the membrane. Rho GDI interacts with the GTP-bound form of Rho, Rac and Cdc42 proteins (Nobes & Hall, 1994) and inhibits both the intrinsic and GAP-stimulated rates of GTP hydrolysis. Its interaction with Cdc42 is mediated by the insert region (Wu et al, 1997). In the absence of GDI, it is predicted that Rho proteins would be firmly associated with the membrane since the geranylgeranyl modification at their carboxyl termini attaches to membranes far more securely than, for example, the farnesyl groups of Ras proteins (Boguski & McCormick, 1993). When introduced into cells, Rho-GDI can disrupt both Rac1/Cdc42 and RhoA-mediated reorganisation of the cytoskeleton (Coso et al, 1995) but its role is not clear-cut; it is also found the Rac1-GDI complexes can still activate the neutrophil NADPH oxidase (Segal & Abo, 1993). GDI is not a requirement in this system, however; the complex can be replaced functionally by recombinant Rac1-GTP.

Unlike GAPs and GEFs of which there are many, there appear to be a very limited number of different GDIs. RhoGDI is ubiquitously expressed whereas D4-GDI is primarily restricted to haemopoietic cells and is believed to interact specifically with haemopoietic Rho-type GTPases (Gorvel et al, 1998). RhoGDI-3 interacts specifically with RhoB and RhoG but not with RhoA or Rac (Zalcman et al, 1996). RhoGDIγ is preferentially expressed in the brain and pancreaas and binds Cdc42 and RhoA. Thus, there is a family of RhoGDIs that differ in their p21-binding preferences, tissue expression and binding affinities.

1H.6 p21 Effector Proteins

Downstream effects of p21s are mediated by effector proteins which interact preferentially

with the p21 in its GTP-bound form. The GTPase effector domain described previously is critical to these interactions and, when conformational change is induced by GTP binding, it becomes exposed and available to interact with target proteins (Wittinghofer & Nassar, 1996). The diversity of cellular processes regulated by p21s suggests that there are multiple mechanisms by which these proteins may induce specific effects: parallel effector pathways may be activated at the same time; different cellular contexts may influence the interpretation of the p21 signal or specific and co-ordinated downstream effects may be regulated by variations in the level and duration of the p21 signal.

1H.6A Effectors of Ras

Two well-studied targets of Ras activation are the serine/threonine kinase Raf 1 and PI-3-kinase. Interaction with Raf1 was first demonstrated using the yeast two hybrid system. This leads to Raf activation and subsequently the activation of the MAP kinase pathway (Daum et al, 1994) upon which processes including C.elegans vulval development and the differentiation of D.melanogaster photoreceptor cells depend. There is also evidence that this effector pathway may influence apoptosis, either positively or negatively. In neuronal cells the activation of the MAP kinase pathway protects cells from the cell death that would normally result form the withdrawal of neurotrophic factors, an effect opposed by activation of the JNK pathway (Xia et al, 1995). Conversely, high level activation of the MAP kinase pathway in Swiss 3T3 cells actively induces cell death (Fukasawa et al, 1995).A model has been suggested in which the interaction of Ras with Raf is regulated by the protein 14-3-3. In unstimulated cells Raf is held in the cytosol in an inactive conformation by 14-3-3, which is then displaced when Raf interacts with Ras-GTP and the effector is recruited to the membrane where it may activate its target (Rommel & Hafen, 1998). Ras-GTP interacts directly with the p110 catalytic subunit of PI-3-kinase and activates it; inactivation of either PI-3-K or Raf can inhibit Ras-mediated cellular transformation (Rodriguez-Viciana P et al, 1997). When activated by Ras, PI-3-kinase binds protein kinase B; this interaction has been reported to mediate an anti-apoptotic signalling pathway (Marte & Downward, 1997). The use of Ras effector domain mutants has indicated that the Raf and PI-3-K effector pathways can be separated biochemically but these mutants are not sufficient for cellular transformation indicating the fundamental importance of multiple effector pathways

stemming from a single activated p21.

1H.6B   Effectors of Rho Family Proteins

Rho family proteins have a functionally diverse set of potential effector proteins including protein kinases, phosphatases, adaptor proteins and lipid kinases, although physiological significance has not been ascribed to all of these interactions.

1H.6B.1   Rac- and Cdc42-Binding Kinases

Paks, serine/threonine kinases that bind to and are activated by Rac1 and Cdc42, have been purified on the basis of their interaction with Rac1(Manser et al, 1994). αPak (PAK1) was isolated from rat brain and was found to be closely related to the yeast Ste20p kinase, a component of the pheromone signalling pathway. Interaction with the GTP-bound forms of Rac1 or Cdc42 through a conserved p21-binding motif results in activation of the kinase. Mammalian tissues contain at least three Paks, αPak, a 68 kDa protein enriched in brain, muscle and spleen (Manser et al, 1994), βPak (PAK3), a 65 kDa protein enriched in brain but with a cell-type expression pattern distinct from that of αPak (Manser et al, 1995) and γPak (PAK2, Teo et al, 1995), a ubiquitously expressed 62 kDa protein (Lim et al, 1996). The species in which PAK proteins have been identified include C.elegans (Chen et al, 1996) and Drosophila (Harden et al, 1996). Other related kinase families have been identified; the GCK or germinal centre kinases and the PH domain-containing Paks.

Further information regarding the potential role of Paks in signalling pathways has been obtained by the identification of proteins that interact with Pak, such as the adaptor protein Nck whose second SH3 domain mediates an interaction with a proline rich region at the amino-terminus of Pak (Galisteo et al, 1996). The PIX family of exchange factors catalyse nucleotide exchange on Rho, Rac1 and Cdc42 in ascending order of efficiency *in vitro* although their *in vivo* activity appears to be restricted to Rac (Manser et al, 1998). Recent evidence suggests that Pak may act both upstream, via PIX, and downstream, as an effector, of Rac1 (Obermeier et al, 1998). Rac1 has been shown to bind p35, the regulator of the neuron-specific cyclin dependent kinase, cdk 5 (Nikolic et al, 1998). This complex co-localises with α-Pak at neuronal peripheries and causes it to become hyperphosphorylated, inhibiting its kinase activity. This is a potential mechanism for regulating Pak activity at

specific sites within the cell. The localisation of the p35/cdk 5 complex suggests that it may be of importance in the establishment of neuronal morphology.

Other kinases that bind to Rac/Cdc42 have been identified. These include p120-ack, a cellular tyrosine kinase that binds Cdc42 (Manser et al, 1993), pp70^{S6K}, a serine/threonine kinase involved in cell cycle progression that is activated upon interaction with either Rac1 or Cdc42 (Chou & Blenis, 1996), and the related Prk-2 serine/threonine kinase (Quilliam et al, 1996). Another MDK-related kinase, MRCK, has been identified as a specific target of Cdc42-GTP (Leung et al, 1998). Two isoforms, α and β, have been identified: the mRNA of the former is enriched in brain and lung, the latter in lung and kidney. Microinjection of MRCKα induces cytoskeletal rearrangement, identifying this protein as a possible downstream effector of Cdc42.

1H.6B.2 RhoA-Binding Kinases

A set of serine/threonine kinases that interact with Rho have been identified. ROKα, a larger isoform ROKβ (Leung et al, 1995 and 1996 respectively). The latter is identical to the independently-isolated ROCK (Ishizaki et al, 1996). ROKs also interact slightly with Rac. These proteins contain a kinase domain with high sequence homology to that of the myotonic dystrophy kinase (MDK) although this protein is not itself a Rho target. In the presence of activated RhoA, ROK is translocated to the membrane. Rho actually binds both the kinase and its substrate: the myosin binding subunit (MBS) of myosin light chain (MLC) phosphatase. Phosphorylation of the MBS by ROK down-regulates MLC phosphatase activity and it has been shown that activation of Rho leads to increased phosphorylation of MLC (Kimura et al, 1996). Phosphorylation of MLC promotes its interaction with actin and hence ROK provides a connection between the activation of Rho and acto-myosin based contractility.

1H.6B.3 Lipid Kinases

The Rho family p21s can interact with and regulate the activity of two lipid kinases. Rho and Rac bind to and activate Type I PIP-5-kinase in a GTP-dependent manner. The Rho effect was demonstrated using murine fibroblasts (Chong et al, 1994) and could not be reproduced in Rat1 cells and liver tissue lysates from which Rac and PIP-5-kinase were co-

immunoprecipitated (Tolias et al, 1995). This may reflect variations between different cell-types. The stimulation of PIP-5-kinase by recombinant Rho resulted in increased levels of PIP_2 and consequently uncapping and polymerisation of actin filaments (Hartwig et al, 1995). Cdc42 and Rac1 can bind to and activate PI-3-K *in vitro* in a GTP-dependent fashion (Zheng et al, 1994b and Bokoch et al, 1996 respectively). PI-3-K catalyses the D3 phosphorylation of phosphatidyl inositol lipids to produce 3-PIP, $3,4$-PIP_2 and $3,4,5$-PIP_3, all of which act as regulators of cellular processes including rearrangements of the actin cytoskeleton and the activation of PK-C (reviewed by Toker & Cantley, 1997). Since PI-3 K is also a Ras effector, it may act as a molecular connection between Ras and Rho family signalling events.

1H.6B.4 Non-Catalytic Targets of Rho Family Proteins

There are also a number of putative Rho family effectors that have no known catalytic function. These include p67[phox], whose interaction with Rac is a step in the activation of the neutrophil NADPH oxidase (Diekmann et al, 1994), and the Rho binding proteins Rhotekin and Rhophilin (Reid et al, 1996 and Watanabe et al, 1996 respectively). The Wiskott-Aldrich Syndrome Protein (WASP), a specific target of Cdc42-GTP, has been implicated as a downstream target of Cdc42 in the regulation of the actin cytoskeleton (Symons et al, 1996). WASP directly interacts with a cytoskeletally-associated protein, PSTPIP, which is required for the actin-bundling induction by WASP and the interaction between the two is dependent on tyrosine phosphorylation of the SH3 domain of PSTPIP (Wu et al, 1998). Interestingly, WASP can also interact with one of the Nck SH3 domains (Rivero-Lezcano et al, 1995) and also with Pak (Bagrodia et al, 1995a).

1H.6B.5 The Diversity of Rho Family Protein Effectors

p21s regulate a complex network of downstream signalling events and the evidence indicates that these are mediated by an equally diverse set of effector proteins. Different p21s may utilise the same effector, possibly leading to alternative signalling outputs. Alternatively, the activated p21 may mediate signalling via a number of different effectors at any one time. There is little evidence linking single potential effector proteins with a single, specific downstream effect. In a complex cellular environment there may be expected to be exquisite

control of the activation of p21s and this would translate into a precise balance of the use of the different potential effectors. Furthermore co-operation between different families of p21 has been observed. For example, in NIH 3T3 cells, Ras-mediated stimulation of proliferation required Rho signalling to suppress Ras-induced expression of the cell-cycle inhibitor p21-Waf1/Cip1 (Olson et al, 1998). In another case it was found that Rac-dependent ruffling could be induced by Ras and that this process was sensitive to the PI-3K inhibitor wortmannin (Hawkins et al, 1995). Therefore it may be that the relationship between an input signal and the output effect is mediated through complex interactions involving cross-talk at both the p21 level and the effector level.

1H.7 <u>GTPase Activating Proteins As Potential p21 Effectors</u>

A general feature common to both p21 effector proteins and GAPs is their specific interaction with the GTP-bound p21. This suggests that it may be appropriate to consider GAPs themselves as potential effectors, particularly as the RhoGAP domain is conserved in many diverse multidomain proteins. At the present time the evidence of this is limited. The SH2-SH3 domain region at the amino-terminus of p120-RasGAP can reproduce the effects of activated Ras in the inhibition of muscarinic atrial potassium channel currents (Martin et al, 1992) and antibodies raised to the SH3 domain of p120 can block Ras-activated maturation in Xenopus oocytes (Duchesne et al, 1993), arguing in favour of an effector role for the amino terminus of the protein. The induction of stress fibre formation, a Rho-type phenotype, that is seen in fibroblasts upon the injection of p120 is also dependent on the integrity of the SH3 domain (Leblanc et al, 1998). It has also been suggested that the RacGAP n-Chimaerin may act as an effector for Rac: microinjection of the isolated GAP domain inhibits the formation of Rac-induced actin structures which is consistent with its anticipated role in the downregulation of Rac activity whereas when the full length protein is injected into either fibroblasts or neuroblastoma cells there is an increase in Rac and Cdc42-dependent actin structures (Kozma et al, 1996).

GAPSs for p21s are generally complex molecules composed of different cellular signalling modules; beyond their interaction with their target p21 and their catalytic activity they have the potential to mediate further protein-protein interactions in their own right and may thus act as effectors as well as regulators. p122 has been identified as a GAP for RhoA

that can also bind to PL-Cδ1, stimulating its lipase activity (Homma & Emori, 1995). An interaction between a novel RhoGAP (whose preferred *in vitro* substrate is Cdc42) and a PDZ domain of the tyrosine phosphatase PTPL1, a potential negative regulator of RTK-mediated signalling, has also been demonstrated (Saras et al, 1997). A model in which interaction with a GAP led to activation of a downstream signalling pathway and the simultaneous down-regulation of the original signal may offers a simple mechanism for effectively 'switching off' the p21, allowing restricted signalling 'bursts'.

1H.8 Functions of the Rho Family

The first Rho protein was cloned serendipitously from an Aplysia library (Madaule & Axel, 1985) and over 35 homologous proteins have since been identified from different organisms. This group of p21s includes the various Rho, Rac and Cdc42 proteins. With the exception of RhoE (Foster et al, 1996) and TTF (Dallery et al, 1995) which are GTPase-deficient, the Rho family proteins are believed to act as binary switches in signalling pathways in a manner analogous to the Ras paradigm.

1H.8A Transcriptional Activation by Rho Family GTPases

Initial studies of the roles of Rho family proteins focused on their involvement in the regulation of the actin cytoskeleton but, since that time, they have also been shown to be potent activators of the c-Jun N-terminal kinase, JNK, and p38, MAP kinases which, when activated translocate to the nucleus and phosphorylate transcription factors, promoting transcriptional activation. An association between the activation of Rac1 and Cdc42 and up-regulation of these kinases has been demonstrated in several different cell types (e.g. Coso et al, 1995); activation of JNK has also been detected in response to Dbl, the Cdc42 activator (Minden et al, 1995). The known substrates of JNK include the amino-terminus of c-jun, Elk and ATF2; these last two transcription factors are also targets for p38. In contrast to the ERK signalling cascade that can be activated by Ras, the p38 and JNK pathways are poorly activated by mitogens and respond well to inflammatory cytokines and cellular stresses. Few of these stimuli have been directly linked to activation of a specific p21. There is some evidence supporting a role for Pak acting downstream of Rac in the activation of JNK: constitutively active Pak can stimulate JNK activity (Bagrodia et al,

1995b), Pak is implicated as a mediator of angiotensin-stimulated JNK activation (Schmitz et al, 1998) and Pak signalling is required for the JNK activity that is induced by Fas in an apoptotic signalling pathway (Rudel et al, 1998). Pak has also been reported to mediate co-operation between the ERK kinase cascade activation via Raf1 and the Rho family proteins Cdc42 and Rac1, phosphorylating and activating MEK (Frost et al, 1997). Conversely, Tapon et al (1998) reported that Pak was not the Rac target for JNK activation in COS-1 cells, indicating that there may be cell-type differences in the mechanisms for the induction of transcription by Rac and Cdc42.

Rho can act as a mediator of transcriptional action via the serum response element (SRE), a regulatory element to which the serum response factor (SRF) binds. This sequence is present in the promoters of many genes regulated by growth factor. Stimulation by LPA, serum, or heterotrimeric G proteins can induce SRF-dependent signalling to the SRE (Hill et al, 1995). Rac1 and Cdc42 can mediate SRF-dependent transcription in a RhoA-independent fashion, but activation does not correlate with activation of JNK or p38. These results suggest that there are at least two pathways regulated by Rho family proteins that can lead to SRF-mediated transcriptional activation.

Rho, Rac and Cdc42 have all been implicated in activation of the nuclear transcription factor NF-κB (Sulciner et al, 1996 and Perona et al, 1997). NF-κB exists in an inactive cytosolic complex with the inhibitory IκB subunit. Activation is believed to depend on the phosphorylation-induced degradation of the inhibitory subunit. This frees the transcription factor to translocate to the nucleus (Verma et al, 1995). It has been reported that the Rho family protein mediate activation of NF-κB by promoting the phosphorylation of IκB, although the mechanism by which this occurs is unclear. There is data to suggest that it may be via an increase in the cellular levels of reactive oxygen species (ROS). An increase in ROS levels can stimulate NF-κB which can itself cause an increase in cellular ROS (Lo & Cruz, 1995). Expression of activated Rac1 or Ras also elevates ROS (Sulciner et al, 1996). Since in neutrophils Rac is also known to play an essential role in the generation of superoxide radicals by the neutrophil NADPH oxidase (Bokoch, 1995), it might be speculated that the mediation of Rac signalling processes by ROS is a more commonly used mechanism that had previously been realised.

1H.8B Rho Family Proteins and the Actin Cytoskeleton

Microinjection studies in fibroblasts led to the association of Rho, Rac and Cdc42 with an ability to regulate actin polymerisation and the formation of distinct actin structures: Rho induces stress fibre formation (Ridley et al, 1992), Rac the production of ruffling and lamellipodia (Ridley & Hall, 1992) and Cdc42 causes the formation of filopodia (Kozma et al, 1996).

Rho is activated downstream of extracellular factors, for example lysophosphatidic acid (LPA) treatment of serum-starved Swiss 3T3 fibroblasts induces the formation of stress fibres (Ridley & Hall, 1992). Exposure of the cells to serum has the same effect but no such response is seen when the cells are pre-treated with bacterial C3 toxin, an agent which specifically inhibits Rho by ADP-ribosylation. Stress fibre formation is associated with the clustering of proteins at the cell membrane, producing large complexes termed focal adhesions which contain proteins including vinculin, talin, paxillin and focal-adhesion kinase. These structures mediate integrin clustering, act as sites of cytoskeletal adhesion, interact with cell signalling molecules and are associated with high levels of phosphotyrosine (Craig & Johnson, 1996).

The injection of activated Rac1 into serum-starved Swiss 3T3 cells leads to a rapid accumulation of actin at the cell periphery, an effect that can be abolished by dominant negative N17Rac1 (Ridley et al, 1992). This is visualised by the formation of lamellipodial structures, broad extensions of the cell periphery that are implicated in cell motility (Ridley & Hall, 1992). A variety of different stimuli can induce Rac-dependent actin polymerisation, including activation of Cdc42, activation of the PDGF receptor and Ras activation. Cdc42 can be activated in 3T3 fibroblasts by treatment with bradykinin and it mediates the formation of filopodia at the cell periphery (Kozma et al, 1995), fine projections thought to be involved in the recognition of the extracellular environment. (Chang et al, 1995). Filopodia formation was followed by the production of Rac-type lamellipodial structures. Both Rac and Cdc42 induce the assembly of multimolecular integrin-based focal complexes at the plasma membrane of fibroblasts (Nobes & Hall, 1995). These complexes are morphologically distinct from Rho-induced focal adhesions being notably smaller but have the same set of constituent proteins.

73

1H.8C Rho Family Proteins Regulate Similar Morphological Events in Different Cell Types

Whilst many of the earlier studies of the cellular effects of Rho-family GTPases were performed in fibroblast cells, more recent evidence suggests that they may have similar activities in the regulation of actin remodelling in other cell-types. The actin rearrangements seen in macrophages upon injection of activated RhoA, Rac1 and Cdc42 reflect those seen in fibroblasts and neuroblastomas, i.e, stress fibre, lamellipodia and filopodia formation respectively (Allen et al, 1997 and Kozma et al, 1997).The Drosophila Rac homologue, Drac1 has been shown to be required for actin assembly at adherens junctions of the wing disc epithelium (Eaton et al, 1995). Cdc42 and other Rho family homolgues are responsible for budding and the regulation of polarised cell growth in S.cerevisiae (reviewed by Tanaka & Takai, 1998). Over-expression of Cdc42 affects the regulation of cytokinesis in fibroblasts, inducing the accumulation of multinucleate cells (Qui et al, 1997); its effects are mediated via Rac1. Thus the Rho family of GTPases appear to regulate actin polymerisation in different cells types through biochemically similar processes although the outcomes may manifest themselves in a cell-type specific manner.

1H.8D p21 GTPases May Act in a Heirarchy

When Cdc42 was injected into fibroblasts there was a rapid formation of filopodia; at a later timepoint ruffles and lamellipodia were observed (Kozma et al, 1995). However, co-injection of dominant negative Rac with Cdc42 abolished the latter effect. Similarly, bradykinin treatment did not induce subsequent ruffling when the cells were pretreated with dominant negative Cdc42. These results suggest a GTPase hierarchy operates in control of the actin cytoskeleton: activated Cdc42 induces the activation of Rac1. There is also evidence that Rac1 can activate Rho: in Swiss 3T3 cells the microinjection of Rac1 was shown to induce ruffling initially and subsequently stress fibres (Ridley et al, 1992). Whether the Cdc42/Rac and Rac1/Rho activation events are linked to form a GTPase cascade is a point of some debate.

1H.8E Rho Family Proteins Regulate Actin Rearrangement in Neuronal Cells

The complex morphological structures of different neuronal cell types, many of which

contain specialised actin-rich structures, suggests a potential role for the Rho family p21s in neuronal development. Activation of Rac or Cdc42 in NIE115 neuroblastomas can promote lamellipodia and filopodia respectively (Kozma et al, 1997). The formation of lamellipodia and filopodia in response to an acetyl choline gradient can be blocked by the introduction of the dominant negative Rac1 and Cdc42, respectively. This differs from results obtained using fibroblasts; in these cells Rac-dependent actin structures did not form in the presence of dominant negative Cdc42 (Kozma et al, 1995), suggesting that the integration of Rho family proteins may differ in different cell types. The response to activated Cdc42 in these cells could be competed by co-injection with RhoA, and acetyl choline inhibited LPA-mediated neurite retraction. Similarly, NIE115 cells overexpressing Tiam-1, a GEF for Rac1, do not respond to LPA, an effect that can be overcome by co-expression of activated RhoA (Leeuwen et al, 1997). Thus it appears that Rac1/Cdc42 and RhoA-mediated signalling can act antagonistically in neuroblastoma cells, implying that a balance between these two pathways may determine neuronal morphology.

1H.8F The Roles of the Rho Family p21s in Neuronal Development

Correct directional growth of axons is thought to require contractile actin-myosin filaments and demands a constant interpretation of the extracellular environment and appropriate responses to development cues. The evidence suggests that these responses take the form of a selective stabilisation or destabilisation of actin polymerisation in filopodia and lamellipodia which results in directional outgrowth (Bentley & O'Connor, 1994). If growth cones are deprived of filopodia by treatment with cytochalasin B, an agent that inhibits formation of actin filaments, their pathfinding becomes disorientated (Bentley & Toroian-Raymond, 1986).

The effect of expression of dominant negative and constitutively active Rac1 and Cdc42 has been studied in the sensory neurons of Drosophila (Luo et al, 1994). Drac1 and Dcdc42 are highly expressed in the developing nervous system. Expression of either the activated or the dominant negative Drac1 resulted in defects in axonal outgrowth which appeared to arise from disorganisation of the actin cytoskeleton but dendrite formation was unaffected. A more generalised set of neuronal abnormalities was obtained when the Dcdc42 proteins were expressed. Results consistent with a preferential effect of Rac in axons rather

than dendrites were also obtained when Purkinje cells were examined in transgenic mice expressing a constitutively active form of human Rac1 (Luo et al, 1996). There was an extensive loss of presynaptic terminals and dendrite formation was also affected: dendritic spines were reduced in size but increased in number.

Studies indicate that RhoA promotes growth cone collapse and neurite retraction in cultured and primary neuronal cells. Using the NIE115 neuroblastoma cell line Kozma et al (1997) showed that neurite outgrowth could be induced by the injection of C3 toxin; such outgrowth could be blocked by co-injection with dominant negative Rac1 or Cdc42. These mutants could also block the neurite outgrowth that would be the usual cellular response to serum starvation. Injection of constitutively activated RhoA led to growth cone collapse. C3 toxin stimulated the outgrowth of chick dorsal root ganglion (DRG) neurons (Jin & Strittmatter, 1997). These results indicated that RhoA could block neurite outgrowth whilst Cdc42 and Rac1 promoted it, furthermore implying competition between the two pathways. Injection of the catalytic domain of ROKα has been shown to act induce neurite retraction in PC12 cells (Katoh et al, 1998). This activity was not sensitive to C3 toxin, indicating that this kinase is acting as a downstream effector of RhoA. The role of Rho family p21s in the dendritic development of cortical neurons has also been studied (Threadgill et al, 1997). It was found that expression of dominant negative Cdc42 or Rac1 mutants, p190RhoGAP led to reduction in the number of dendrites. Interestingly, injection of C3 toxin gave same result, suggesting that the functions of RhoA in neuronal development may differ not either between cells types or within different parts of the same cell.

Members of the collapsin/semaphorin family also play an important role in axonal guidance, the creation of the complex pattern of neural connections. Inhibition of growth cone collapse is regulated by a family of collapsin response mediator proteins (CRMPs, Wang & Strittmatter, 1996) which are differentially expressed in the nervous system. Jin & Strittmatter (1997) demonstrated that collapsin's action on growth cones were mediated by Rac1 but not Cdc42 or RhoA in DRGs.

Thus, Rho, Rac and Cdc42 appear to mediate morphologically different effects in neuronal cells and are required for correct neuronal development. Analysis of the mRNA expression patterns of RhoA, RhoB, Rac1 and Cdc42 in adult rat brain revealed that all four were present at high levels in hippocampal neurons, the cerebellum and Purkinje and

granular cells. Strong expression was also observed in the brainstem, thalamus and neocortex (Olenik et al, 1997).This widespread distribution would be consistent with a general role in the regulation of cellular processes in the brain. However, the activities of the Rho family p21s could be modulated by brain specific regulators and effectors. It is tempting to speculate that it is the role of Rho family proteins in the development of complex cellular morphologies such as are found in the nervous system that demands the number and diversity of GAPs and GEFs that have been identified to date.

1I The Chimaerin Family of Neuronal RacGAPs

There are four known chimaerin RhoGAPs, two splice variants from each of the α and β chimaerin genes: $\alpha 1$ (n-chimaerin) and $\alpha 2$ chimaerin and $\beta 1$ and $\beta 2$ chimaerin. The proteins diverge at their amino-termini but they share a homology to the C1 regulatory region of PK-C (Parker et al, 1986) and, at the carboxyl terminal, a RhoGAP domain. The GAP activity of chimaerin *in vitro* is restricted to Rac and Cdc42 although the concentration of chimaerin required to stimulate equivalent levels of GTP hydrolysis is approximately two hundred fold greater in the case of Cdc42 (Manser et al, 1992). The combination of a RhoGAP domain and a cysteine-rich region is found in other proteins, including the unconventional myosin heavy chain, myosin IXb, and mgcRacGAP, human GAP which is enriched in the testis (Wirth et al, 1996 and Toure et al, 1998).

1I.1 α and β Chimaerins

$\alpha 1$ chimaerin, was isolated from human hippocampal and retinal cDNA libraries (Hall et al, 1990). The cDNA hybridises to a 2.2 kb species in human mRNA and a 2.3kb species in rat brain mRNA but is not detected in rat kidney, heart, spleen, muscle or testis. $\alpha 1$ chimaerin mRNA is present at the highest levels in the cerebral cortex, hippocampus pyrimidal layers, the dentate gyrus and pyriform cortex. In the cerebellum its expression is restricted to the Purkinje cells. The $\alpha 1$ chimaerin mRNA is also developmentally regulated, with detectable levels in embryonic rat brain from day 15 which increase post-natally from birth to 20 days (Lim et al, 1992). The mRNA encodes a protein with a molecular mass of 38kDa. It is predicted that the unique amino-terminal of $\alpha 1$-chimaerin, 35 residues which are not present in $\alpha 2$ chimaerin, fold into an amphipathic helix that could potentiate membrane-binding.

	Chimaerin	Expression
AH CRD GAP	α1, 38 kDa	Brain
SH2 CRD GAP	α2, 43 kDa	Brain, Testis, Intestine
CRD GAP	β1, 30 kDa	Testis
SH2 CRD GAP	β2, 46 kDa	Brain
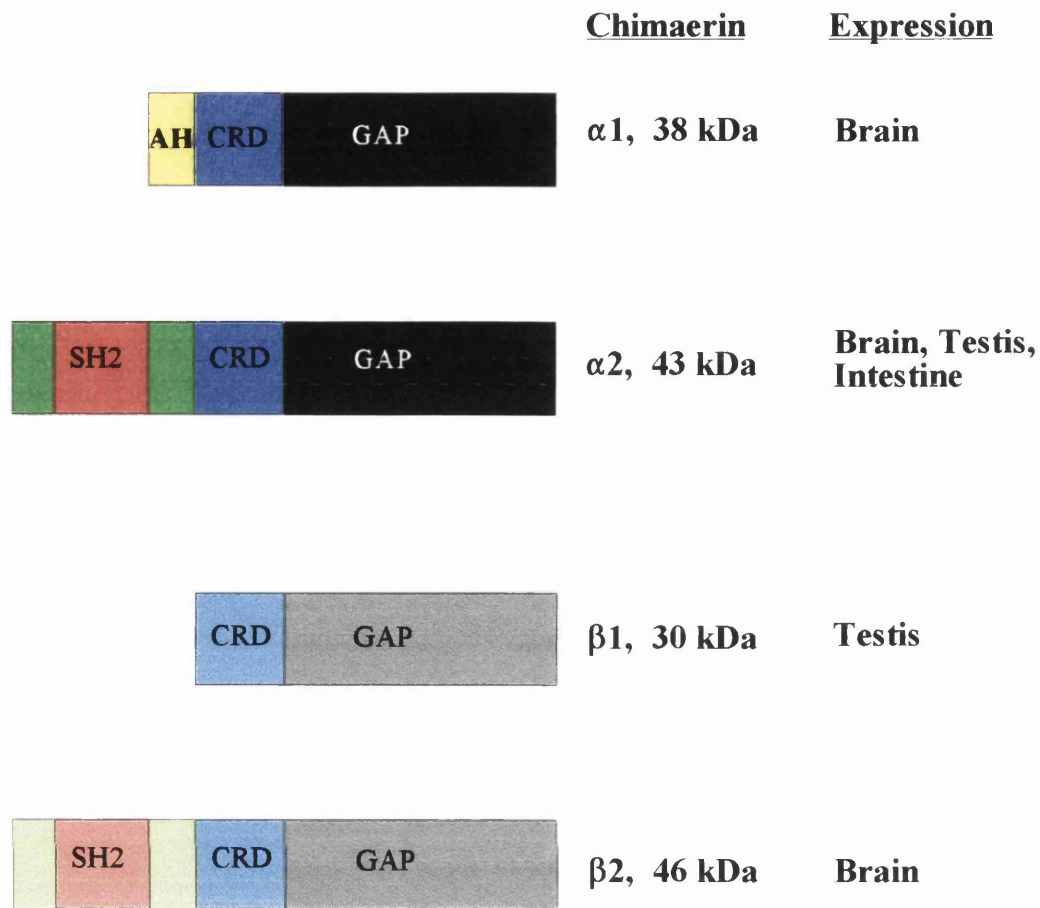
Figure 1.5: A Schematic Representation of the Protein Domain Structure of the Chimaerin Family of Rho GAPs

This figure indicates the protein domain structure of the four members of the chimaerin family of RacGAPs. The abbreviations used are: SH2, Src Homology 2 domain; GAP, GTPase activating protein domain; CRD, cysteine rich/phorbol ester-binding domain; AH, region that is predicted to fold into an amphipathic helix. The approximate molecular mass of each protein is given in kDa and the regions in which they are expressed is described.

The isolation of variant chimaerin cDNAs from human retinal and hippocampal libraries led to the identification of a splice variant with an alternate amino terminal containing an SH2 domain, α2 chimaerin (Hall et al, 1993). This has 38% identity with the Abl and Src SH2 domains. A human cDNA probe hybridises to a 2.2kb message in rat brain and testis and to a slightly smaller mRNA in intestine. Like the α1 chimaerin mRNA, the α2 chimaerin message is selectively expressed in neurons in the adult rat brain, the highest levels being found in the cortex, hippocampus and piriform cortex; the message is detectable in embryonic rat brain and gradually decreases after birth. The α2 chimaerin message is also expressed in testis. The highest levels are present in early pachytene spermatocytes and no expression is detected in spermatids. Analysis of the exon/intron organisation of the α chimaerin gene, which is localised to chromosome 2, revealed that α1 and α2 chimaerin are alternate splice variants of the same gene. Interestingly, splice junctions of the chimaerin gene are conserved with those of the related PK-C and Bcr genes.

α1 and α2 chimaerin are also differentially expressed in cultured cells (Dong et al, 1994). The expression levels of both are lower in cell lines than *in vivo*. In the cell lines examined, α1 chimaerin was detected only in neuronal cells whereas α2 chimaerin was also present in the non-neuronal HepG2 and HeLa cell lines. The expression of both chimaerin transcripts is up-regulated when SK-N-SH cells undergo neuronal-type differentiation in serum-free medium but only α1 chimaerin mRNA expression is increased in response to KCl-induced membrane depolarisation of these cells, indicating that the two transcripts of the α chimaerin gene are under different transcriptional regulation (Dong & Lim, 1996)

A cDNA highly related to α1-chimaerin was isolated from a rat testis library (Leung et al, 1993). It encodes a 30kDa RacGAP, β1-chimaerin, with 77% identity to the α1-chimaerin and a PK-C homology region with 93% identity. Its expression is restricted to the testis at the acrosomal assembly stage of spermatid development, later than α2 chimaerin. β1-chimaerin lacks an SH2 domain and has a short amino-terminus with low similarity to that of α1-chimaerin. Antibodies raised against β1-chimaerin also detected an abundant 46 kDa species in rat cerebellar granule cells whose expression is increased post-natally (Leung et al, 1994). The corresponding cDNA, β2-chimaerin, encodes a protein with cysteine-rich and GAP domains identical to those found in β1-chimaerin, with an additional amino-terminal SH2 domain, highly related to that of the α2 chimaerin SH2 domain and sharing

its most anomalous feature: an amino-terminal glutamate residue rather than the conventional tryptophan. This possibly defines a distinct sub-class of SH2 domain. The identical cDNA sequences encoding the CRD and GAP domains of β1 and β2 chimaerin indicates that they are also splice products of a single gene. Decreased expression of β2-chimaerin has been associated with malignant gliomas (Yuan et al, 1995).

Thus, the chimaerins form a novel family of RhoGAPS. There is a high level of sequence identity between the α and β proteins which are differentially expressed, predominantly in the brain and testis. The chimaerins diverge at their amino-termini and it can be speculated that these sequences may be important in determining the cellular localisation or the potential effector functions of the different proteins. The protein domain structure of these proteins is indicated schematically in Figure 1.5.

1I.2 The PK-C Homology Domain of Chimaerin

This domain has approximately 48% homology to the cysteine-rich sequences found in the C1 region of PK-C. The $CX_2CX_{13}C$ portion of this $CX_2CX_{13}CX_2CX_7CX_7C$ motif has the potential to form a 'Zinc finger' structure (Freedman et al, 1988). Related domains are found in several molecules, including Raf1 and the Cdc42-binding kinase, MRCK (Hu et al, 1995 and Leung et al, 1998). Both the C1a and C1b motifs present in PK-C act as phospholipid-dependent phorbol ester receptors (Ono et al, 1989); phorbol esters are analogues of the naturally-occurring lipids that are potent tumour-promoting agents (Diamond, 1984). The chimaerin cysteine rich domain (CRD) also interacts with phorbol esters and the characteristics of binding are similar to those observed for PK-C (Ahmed et al, 1992 and Areces et al, 1994): the in vitro binding affinities are similar for both, being in the nanomolar range, the interaction is inhibited by competition with diacylglycerol and the binding is zinc-dependent (Ahmed et al, 1991). Site-directed mutagenesis of certain conserved cysteine residues in the CRD can abolish or reduce phorbol ester binding (Kazanietz et al, 1995). α2-chimaerin also binds phorbol ester and, recently, the interaction of phorbol ester with β2-chimaerin has been shown to alter its sub-cellular distribution in vivo, suggesting that the CRD may play a role in directing these proteins to their intracellular targets (Caloca et al, 1997).

1I.3 Lipid Modulation of Chimaerin GAP Activity

The isolated GAP domain of α1-chimaerin has a higher GAP activity than the full-length protein, implying that the CRD may have a regulatory function (Ahmed et al, 1993). Phospholidids, including phosphatidylserine and phosphatidic acid, stimulate GAP activity and phorbol esters can act synergistically to further up-regulate α1-chimaerin activity. The activation of GAP activity by phospholipids requires the presence of the CRD. These findings mirror those obtained in studies investigating the regulation of PK-C activity by phospholipid/phorbol ester. A further set of lipids down-regulate the GAP activity of α1-chimaerin: lysophosphatidic acid (LPA), PI, PIP, PIP_2 and arachidonic acid (AA). With the exception of AA, the effects mediated by these lipids are dependent on the presence of the CRD. AA also inhibits the GAP activity of p120-RasGAP (Bollag & McCormick, 1991). Interestingly, there is evidence that Rac1 promotes AA release (Peppelenbosch et al, 1995), possibly suggesting a Rac1-mediated positive feedback mechanism which could lead to the extension of Rac1 activation.

The GAP activity of α2-chimaerin can be stimulated by phosphatidylserine and, whilst there is no stimulation by phorbol esters alone, some synergy is observed in the presence of limiting amounts of phospholipid (Hall et al, 1993). However, treatment with inositol phospholipids or arachidonic acid up-regulates the GAP activity of α2-chimaerin (Teo, Ph.D. Thesis, 1994). A further difference between the two chimaerin isoforms is that, in the absence of phospholipid, the level of α2 chimaerin GAP activity is similar to that of the isolated GAP domain. In the absence of modulators, recombinant α2 chimaerin is a more potent GAP than α1 chimaerin. The only molecular difference between these two proteins lies in their amino-terminal region and the implication is that this is the basis for the disparity in their respective GAP activities although the *in vitro* studies used recombinant proteins and might not reflect the *in vivo* situation.

1J Src Homology 2 Domains: Structure and Function

The structures of several different SH2 domains, complexed with high affinity phosphopeptide ligands, have been determined by NMR and X-ray crystallography (reviewed by Schaffhausen, 1995). This allows rationalisation of the mechanism of the interaction with the specific phosphopeptide ligand and defines two separate classes of SH2

domain.

1J.1   <u>The Three-Dimensional Structure of the SH2 Domain</u>

SH2 domains are compact, globular structures with a large central antiparallel β-sheet flanked by two α-helices. Similarities in the secondary structures of those SH2 domains studied led to the adoption of a nomenclature in which the β-strands are labelled βA-βG, the helices αA and αB and the loops are named by the letters of the secondary structures that they join. This facilitates comparison of different sequences (Eck et al, 1993), as illustrated by the alignment of the primary sequences of several SH2 domains shown in Figure 1.7. The most variable regions of the sequences, in composition and size, are the loops between the secondary structures; whether this reflects important functional differences between different SH2 domains is not known. The apposition of the amino and carboxyl termini of the SH2 domain on the face opposite the phosphotyrosine-binding site allows the domain to be inserted into a protein with minimal structural alteration. The crystal structure of a large fragment of the src kinase, comprising the SH2, SH3 and kinase domains and the carboxyl-terminal tail demonstrates that this is the case (Xu et al, 1997) and that the SH2 domain conformations when isolated and within a protein context are the same.

1J.2   <u>The Structure of the Phosphotyrosine-Binding Site and Mechanism of Ligand Binding</u>

The side-chain of the phosphotyrosine ligand projects into a deep pocket formed from well-conserved residues. At the base of this pocket the phosphate group of the ligand makes two hydrogen bonds to the positively-charged side-chain of the βB5 arginine of the highly conserved FLVRES motif (Waksman et al, 1992). This residue is invariant in all known SH2 domains and even conservative mutations can abolish the interaction with phosphotyrosine (Bibbins et al, 1993). The fundamental role of this residue suggests that the basis of the SH2 domain preference for phosphotyrosine rather than phosphoserine/threonine is that the longer sidechain of phosphotyrosine can extend sufficiently into the pocket to form this vital contact (Lee at al 1994). The precise contacts made with the ligand within the binding pocket vary somewhat in different SH2 domains but, in src SH2 domains and others, an extensive network of hydrogen bonds is made with the phosphate group. These contacts

include that made with the well-conserved αA2 arginine and there are also several hydrophobic interactions with the phenol group of the phosphotyrosine, one provided by βD6 lysine. This residue forms one part of the mouth of the binding pocket, another being formed by the βD4 histidine. This last residue is conserved in src family kinases and is important in establishing the binding site by providing structural support for the phenol ring of the target phosphotyrosine and maintaining active site geometry.

The affinity of the interaction between an SH2 and its specific target can be as much as three orders of magnitude greater than that of its interaction with a random phosphotyrosine-containing peptide or with isolated phosphotyrosine. That this is due to additional contacts made when the SH2 domain interacts with a phosphopeptide of optimal sequence is illustrated by the difference in the crystal structure when an SH2 domain is complexed with a peptide target of high rather than low affinity. Low affinity ligands interact with the SH2 domain at a single site, the phosphotyrosine binding pocket (Waksman et al, 1992), whereas high affinity ligands interact both at the phosphotyrosine binding pocket and at a second site (Waksman et al, 1993 and Eck et al, 1993). This second set of contacts are predominantly with the amino acid residues immediately carboxyl-terminal to the phosphotyrosine of the peptide ligand and SH2 domains can be divided into two classes on the basis of differences in the nature of these interactions. Class I SH2 domains which include the src family kinases make contacts with three amino acid residues (termed +1 to +3). Analysis of the phosphopeptides selected from a degenerate library indicate a preference for hydrophilic amino acid residues at the +1 and +2 positions and a hydrophobic residue at the +3 position (Songyang et al, 1993) which is accommodated by a small binding pocket on the surface of the domain (Waksman et al 1993). Accordingly, the src target peptide has the sequence pYEEI. The second class of SH2 domain is typified by that found in the SHP-2 tyrosine phosphatase which forms contacts with a series of at least five, predominantly hydrophobic amino acid residues immediately carboxyl-terminal to the phosphotyrosine. These residues fit into an extended groove across the binding surface of the SH2 domain (Lee et al, 1994).

One factor contributing to the different binding sites is the identity of the βD5 residue: in Class I SH2 domains this is an amino acid residue with a bulky sidechain (Songyang et al, 1994) which separates the phosphotyrosine-binding pocket from the

Figure 1.6: 3D Images of an SH2 Domain Interacting with a Phosphopeptide Target

Three-dimensional images of the human Lck tyrosine kinase (p56-Lck, EC 2.7.1.112), a member of the Src family were obtained from the SWISS-3DIMAGE database (Acession number P06239) which can be accessed through the ExPASy WWW server at http://expasy.hcuge.ch/pub/Graphics/. The entire SH2 domain is shown in the upper panel; helical regions are indicated by cylinders and β-strands are indicated by broad ribbons. The SH2 domain is complexed with a phosphopeptide target. The phosphotyrosine (Tyr-P) is shown in red/purple and the peptide at its carboxyl terminal is indicated in green. The lower panel shows an enlargement of the phosphotyrosine-binding pocket. The phosphate group of the ligand is inserted into the binding pocket, co-ordinated by two arginines, whilst the carboxyl terminal residues can form contacts with the module outside the binding pocket. This is a Class I SH2 domain.

P56-LCK SH2 domain: Interaction with Phosphotyrosine
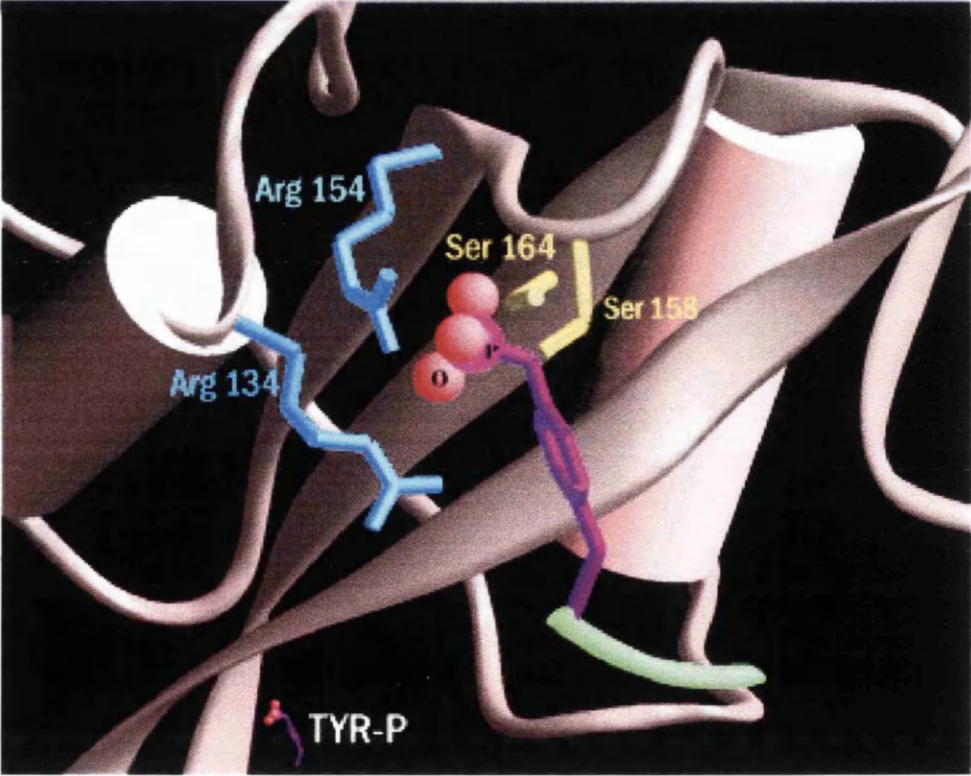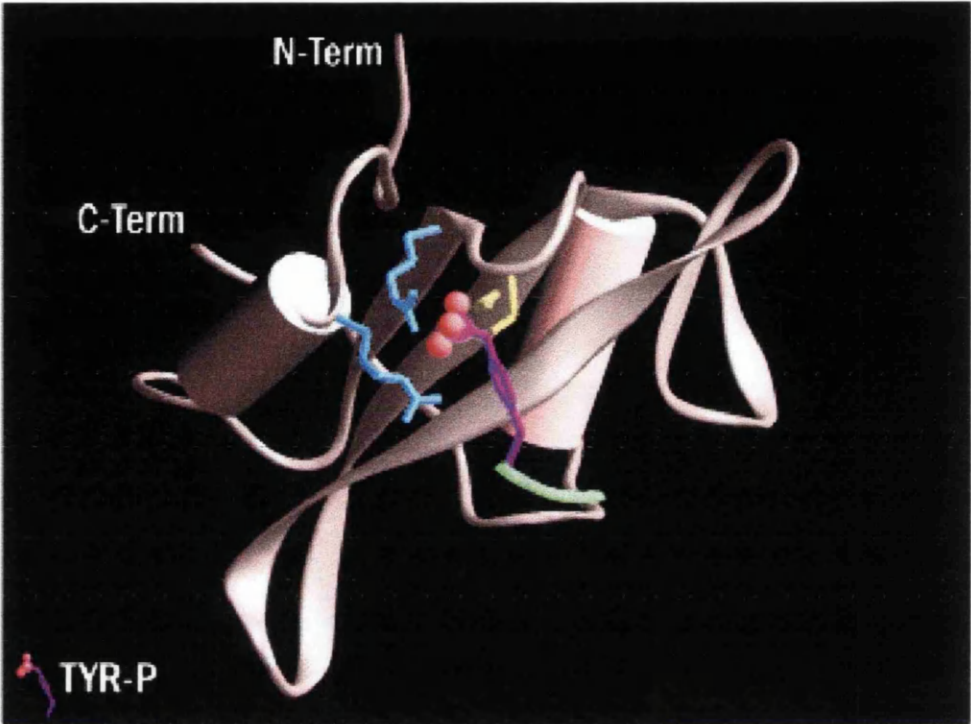
hydrophobic pocket at the +3 position whereas the Class II proteins have a smaller, non-aromatic sidechain at this position. The effect of this variation is to produce a slender, hydrophobic channel between the pockets rather than a bipartite binding site with two distinct pockets. The predicted βD5 amino acid residue in α2 chimaerin is phenylalanine and in β2 chimaerin is a tyrosine, the same as that in the src SH2 domain, suggesting that the chimaerin SH2 domains may be of the Class I type.

That the specificity of the interaction between an SH2 domain and its target is governed by the precise contacts made between the amino acid residues immediately carboxyl-terminal to the phosphotyrosine of the target and SH2 domain residues outside the phosphotyrosine-binding pocket was illustrated by experiments performed by Marengere at al (1994). A single threonine residue in the +3 src SH2 binding pocket was altered to the tryptophan found in the Sem5/Grb2 SH2 domain at this position and it was found that the mutated protein preferentially selected the pYENP phosphopeptide that is the optimal target of the Sem5 SH2 domain.

The three-dimensional structure of the Class I SH2 domain of the human p56-lck cytosolic tyrosine kinase complexed with a high affinity phosphopeptide ligand is shown in Figure 1.6. The projection of the phosphate into the binding pocket is evident. The αA1 and βB5 arginine residues that co-ordinate the phosphate group are indicated.

Further investigation of the original 'two-pronged plug in a two-hole socket' model for phosphopeptide/SH2 domain interactions that is outlined above have provided evidence that it may be an oversimplification. Thermodynamic studies of the interaction of the Src SH2 domain with phosphotyrosyl peptides suggested that high affinity binding was only partially determined by the interactions between the +3 residue of the binding pocket and the +3 binding pocket of this Class I SH2 domain (Bradshaw et al, 1998). The data obtained was however consistent with a binding mechanism that did not involve substantial conformational changes which is in agreement with the original model.

There are relatively few contacts made between the SH2 domain and amino acid residues amino-terminal to the phosphotyrosine; these residues turn away from the structure. In general, interactions that do occur are with the peptide backbone rather than the sidechains and would so be unlikely to play a role in target selection. One exception to this is the SH2 domain of the SHP-2 phosphatase whose αA2 residue interacts with the

phosphotyrosine-2 residue of the target peptide. This requirement for target recognition can be abolished by point mutation of the αA2 residue (Huyer & Ramachandran, 1998).

1J.3 Mutational Analysis of SH2 Domain-Target Interactions

Analysis of the binding of Src SH2 domain containing single amino acid residue substitutions to a peptide containing the regulatory phosphotyrosine 527 from the Src carboxyl-terminal has allowed further understanding of individual SH2 domain residues in target recognition and binding (Bibbins et al, 1993). As predicted, mutation of the invariant βB5 arginine abolished binding; the same result was obtained when the βA1 tryptophan was mutated to glutamate, although the more conservative tryptophan to tyrosine mutation had no effect. Interestingly, the effects of the tryptophan to glutamate mutation could be partially reversed if the SH3 domain were also present, suggesting that sequences outside the SH2 domain may act to stabilise the interactions of the SH2 domain. An arginine to alanine substitution at the αA2 position attenuated the interaction with the phosphopeptide by some 20% indicating that the contacts made with the phosphate of the target by the wild-type arginine are not critical determinants of phosphotyrosine binding. Changes in the conserved βD4 histidine (which does not contact the phosphotyrosine) can also adversely affect target binding: the more conservative the substitution, the less pronounced the effect.

Point mutation of the Grb10 SH2 domain has shown that phosphotyrosine-dependent binding to the EGFR can be separated from phosphotyrosine-independent binding to the kinases MEK-1 and Raf1. However, an arginine to leucine substitution revealed that the βB5 arginine was essential for interaction with both sets of targets (Nantel et al, 1998). The use of specific point substitutions in the known binding site of the STAT 2 SH2 domain on the activated IFNα receptor indicated that such mutations could affect downstream signalling processes (Krishnan et al, 1998).

1J.4 Atypical SH2 Domains

Other than the βB5 arginine, invariant in all known SH2 domaind, the most highly conserved residue is that at the βA1 position, the first in the SH2 domain sequence. Only four SH2 domains are known in which the amino acid residue at this position is not tryptophan, those found in α2 and β2 chimaerin (glutamate), EAT-2 (tyrosine) and ZAP70

```
        βA   AA    αA        AB        βB       BC     βC        CD       βD

Src     WYF  GKIT  RRESERLL  LNPENPRG  TFLVRES  ETTKG  AYCLSVSD  FDNAKGL  NVKHYKI
α2 Chim EFH  GMIS  REAADQLL  I   V  AEG SYLIRES QRQPG  TYTLALRF  GSQ      TRNFRL
β2 Chim EFH  GIIS  REQADELL  G  G  VEG AYILRE   QRQPG  CYTLALRF  GNQ      TLNYRL
ZAP-70  FFY  GSIS  RAEAEEHL  KLAGMADG  LFLLRQC  LRSLG  GYVLSLVH  D   V    RFHHFPI
EAT-2   YYH  GCLT  KRECEALL  LKGGVD  G NFLIRDS  ESVPG  ALCLCVS   FKK      LVYSYRI


        βD'  DE   βE    EF        βF    FB  αB            BG         βG

Src     RKL  DSG  GFYI  TSR       TQF   S   SLQQLVAYYSKH  ADGLCHRLT  NVC  PTS
α2 Chim Y  Y DG   KHFV  GE        KRF   E   SIHDLV        TDG        LI T L YIETKA
β2 Chim F  H DG   KHFV  GE        KRF   E   SIHDLV        TDG        LI T L YIETKA
ZAP-70  ERQ  LNG  TYAI  AGG       KAH   C   GPAELCEFYSRD  PDGLPCNLR  KPC
EAT-2   FRE  KHG  YYRI  ETDAHTPR  TIF   P   NLQELVSKYGDP  GQGLVVHLS  NPI
```

Figure 1.7: Alignment of the Sequences of Four Atypical SH2 Domains with the Src SH2 Domain Sequence

The protein sequences of four SH2 domains, those of the RacGAPs α2 and β2 chimaerin (marked α2 and β2 chim), the amino-terminal SH2 domain of the ZAP-70 tyrosine kinase and the SH2 domain of Eat-2, a protein that is up-regulated in Ewing's sarcoma tumour cell lines, are aligned with the Src SH2 domain sequence. These four SH2 domains are atypical in that their first residue is not tryptophan. Different β strands are labelled βA-βG and helices αA and αB and indicated in green. Loops are identifed by the helices that they connect. Helical regions are boxed in blue and β-strand regions in red.

88

(phenylalanine), (Hall et al, 1993, Leung et al, 1994, Thompson et al, 1996 and Hatada et al, 1995, respectively). These, and most notably those in the chimaerins, are all more hydrophilic than usual. The sequences of these atypical SH2 domain are aligned in Figure 1.7.

ZAP 70 has two tandem SH2 domains and it is the more amino-terminal domain whose sequence is anomalous. The structure of these modules, in combination with a doubly-phosphorylated target peptide, has been resolved and a novel phosphotyrosine-binding mechanism revealed: sequences from the more carboxyl-terminal SH2 domain are required to complete the phosphotyrosine binding pocket of the other, again suggesting that the interaction between an SH2 domain and its ligand may be modulated by sequences outside the SH2 domain (Hatada et al, 1995).The chimaerin and EAT-2 domains can both bind phosphotyrosine immobilised on a column (shown later in this thesis and by Thompson et al, 1996 respectively) but, by comparison with the ZAP 70 SH2 domain whose phosphotyrosine-binding ability is drastically modified by quite a conservative substitution, may do so in an unconventional manner.

1J.5 Phosphotyrosine-Independent SH2 Domain Interactions

A number of examples of phosphotyrosine-independent SH2 domain interactions have been reported, including that of the src family kinase, p56-lck, with a 62kDa ligand, p62 (Park et al, 1995), which associates with RasGAP and also with the lambda and iota isoforms of PK-C (Sanchez et al, 1998). The SH2 domain of the adaptor protein Grb 10 has been shown to interact constitutively with Raf 1 kinase in the yeast-two hybrid system and also with MEK1 kinase upon stimulation of the cells with insulin (Nantel et al, 1998). These interactions could occur in the absence of tyrosine phosphorylation. The p56-lck/p62 interaction is dependent on the phosphorylation of the p62 Ser 59 residue but the evidence suggests that this is in a regulatory context. A general requirement for phosphoserine/threonine was demonstrated for the binding of Bcr by the Abl SH2 domain (Pendergast et al, 1991) and the Raf 1/Fyn interaction was dependent on the serine phosphorylation of Raf 1 (Cleghon & Morrison, 1994). However, it is unclear whether the interactions themselves are directly mediated by phosphorylation. The estimated affinities of these types of interaction were approximately two orders of magnitude lower than those observed for high affinity

phosphotyrosine-dependent interactions. To date no precise SH2 domain binding sites have been mapped on these phosphotyrosine-independent ligands, there is no structural information available and there is little understanding of the role that they may play in signalling processes.

PIP$_3$, one of the possible products of PI-3K activity, can interact specifically with the SH2 domains of the p85 regulatory subunit of PI-3K and effectively compete with tyrosine-phosphorylated target proteins (Rameh et al, 1995). The p85 subunit inhibits the p110 PI-3-K catalytic subunit (Yu et al, 1998a) and this activity requires its amino-terminal SH2 domain (Yu et al, 1998b). This suggests that competition of the peptide ligand with the lipid ligand could provide a mechanism for the regulation of PI-3-K activity.

Non-phosphorylated ligands of the Grb2 SH2 domain have been reported (Oligino et al, 1997). An interaction between this SH2 domain and an eleven amino acid residue cyclic petide has been demonstrated *in vitro*; this peptide can compete with a phosphotyrosine-containing peptide ligand for the SH2 domain. Analysis of the effect of amino acid residue substitution in the SH2 domain suggested that the cyclic peptide was binding to the phosphotyrosine binding pocket. The novel peptide ligand contained a YXN motif similar to that found in the natural phosphopeptide ligand and its ability to compete with such a ligand was increased upon phosphorylation of the tyrosine in this motif. It can be speculated that a greater range of potential ligands could expand the signalling role of SH2 domains.

1K Analysis of Protein-Protein Interactions

Protein-protein interactions are central to all cellular processes and signal transduction pathways can be defined in terms of the specific series of such interactions connecting an extracellular signal to an intracellular effect. The identification of specific binding partners is essential to the understanding of this process. Large, relatively stable multi-subunit complexes may co-purify and then the various interactions between different polypeptides can be studied once the components have been identified. However, many protein-protein interactions are transient and hence not very amenable to investigation. In addition, any particular interaction may dependent on a prior interaction: the transient association between a kinase and its substrate leading to phosphorylation of that substrate may be essential if the

substrate protein is subsequently to bind a downstream target. The concentrations of signalling molecules at successive stages of a transduction pathway may be low and furthermore may be sequestered by interaction with a binding partner. Thus to identify a specific binding partner it is necessary to use a technique that will permit the isolation of a single, potentially labile target that may be expressed only transiently and at low levels, under circumstances that will promote or enable any modification upon which the interaction may be absolutely dependent. Different methods that may be used to isolate and identify interacting proteins are discussed briefly below. General techniques for the identification of interacting proteins reviewed comprehensively in Phizicky & Fields, 1995.

1K.1 Protein Purification Techniques

Classical methods of protein purification rely on a series of chromatographic separations of a complex mixture of potential target proteins, enriching for the desired protein at each stage by identifying the fractions in which it is present at the highest levels by biochemical assay. Such an approach is appropriate if a protein with known biochemical activity is to be purified. This activity can then be assayed at different stages of purification. The ability to interact with p21s whilst immobilised on a filter was exploited to monitor the purification of the p21-activated kinase α-pak at each stage (Manser et al, 1994). Native $\alpha 1$ and $\alpha 2$ chimaerin were purified from rat brain extracts on their immunoreactivity towards specific antibodies (M.Teo, Ph.D Thesis 1994). The selection of an appropriate source of the protein to be purified is also a factor that must be considered in the development of a purification strategy. The development of a novel GTPase overlay assay technique allowed tissue extracts to be screened for the presence of GAPs for Rho family proteins (Manser et al, 1992). If a suitable assay system is not available then other approaches must be used.

1K.2 The Use of the Yeast-Two Hybrid System

A variety of methods have been developed to screen large libraries for genes or fragments of genes whose products may interact with a protein of interest. The chief advantages of this approach are that a very large number of potential targets can be screened relatively quickly and, once identified, a clone of the target is immediately available. Much information has also been obtained using the powerful techniques available for the genetic analysis of certain

organisms, for example D.melanogaster and S.cerevisiae.

The yeast-two hybrid system relies upon the modular nature of many site-specific transcriptional activators that consist of separate DNA-binding and transcriptional activation domains which do not need to be covalently attached to permit the initiation of transcription. A protein of interest, the 'bait' protein, is fused to one domain of a DNA-binding domain/ transcriptional activator pair and co-expressed in a cell with another protein fused to the other domain of the pair. When the DNA binding domain binds to the promoter of the of a suitable reporter gene, the transactivator is brought into close proximity by the interaction of the bait and its target protein thus promoting transcription from the reporter gene, commonly the E.coli lacZ gene; the level of transcriptional activity is used as a measure of protein-protein interaction. Two commonly used systems utilise the yeast GAL4 (Chien et al, 1991) or the E.coli LexA (Zervos et al, 1993) DNA-binding domains. This system can be used to screen a library of activation domain hybrids to identify potential targets and it was in this way that the interaction between Ras and Raf 1 was confirmed (Van Aelst et al, 1993).

An inducible mammalian tyrosine kinase can be co-expressed with the activation and DNA-binding domain fusions to create a system in which the phosphorylation of potential target proteins may reflect more closely their physiological patterns. If Src is used a mutated form of the kinase is required because the expression of the wild-type protein is lethal in yeast (Boschelli et al, 1993). This may be of especial relevance when a tyrosine-dependent SH2 domain interaction is being sought although there are examples of the successful use of the yeast two hybrid system in such cases, including the determination of the interaction between the Grb 10 SH2 domain and the activated insulin receptor (Hansen et al, 1996).

1K.3 The Use of Affinity Chromatography

Affinity chromatography is the isolation of a target on the basis of its affinity for a ligand that has been immobilised on agarose resin. An advantage of this technique is its wide applicability: all that is required in principle is a sufficient quantity of 'bait' protein and a suitable source of potential target proteins. There is no absolute requirement for a biochemical assay that can estimate the interaction between two proteins or an enzyme activity that can be followed through the purification. It is also an extremely sensitive

technique, one that can be made more so by the use of radioactively- labelled target proteins, and one that tests all potential target proteins in the mixture equally in the sense that any protein bound will have competed successfully against all the other proteins present in the population. Examples of proteins isolated using this method include the pak-interacting PIX nucleotide exchange factors (Manser et al, 1998) and the myotonic dystrophy kinase-related Cdc42-binding kinases, MRCKs (Leung et al, 1998).

Bacterially-expressed recombinant proteins are commonly used to prepare affinity columns and they may either be covalently coupled to an activated agarose matrix such as Affigel or may be tethered non-covalently by a high affinity binding interaction with a suitable resin; for example a GST fusion protein can be bound to a glutathione agarose column. Isolated SH2 domains have been bacterially expressed that can bind with high affinity to specific phosphopeptide targets (Mayer & Baltimore, 1994) indicating that they can be both functional and stable entities when excised from their original protein context. This suggests that they may be able to bind their physiological ligands whilst immobilised on a column. The source of target proteins may be extracts prepared from tissues or cultured cell in which the 'bait' protein is known to be expressed, or alternatively a source known to be enriched in the target of interest. Control columns can be run in parallel to establish that proteins are bound to the affinity column specifically. Binding proteins can be analysed by electrophoresis and possibly peptide sequence information be obtained. This may enable immediate identification of the isolated protein if database searches reveal identical peptides or alternatively degenerate primers can be designed to enable the isolation of a cDNA encoding the purified protein by PCR. This approach has been used succesfully to isolate a target of an SH2 domain: the neuronal protein synaptojanin was purified from rat brain extract on the basis of its affinity for the carboxyl terminal SH2 domain of PL-Cγ1 (Ahn et al, 1998).

1K.4 The Identification of Targets of the α2 Chimaerin SH2 Domain

The yeast two hybrid system has been used by a member of our group to screen for target proteins of the α2 chimaerin SH2 domain. A positive clone was sequenced and identified as the B13 subunit of the inner mitochondrial membrane NADH ubiquinone oxidoreductase, the first and largest enzyme of the mitochondrial respiratory chain (Walker et al, 1992) has

been shown to bind the α2 chimaerin using the yeast two hybrid system. Introduction of a non-lethal but kinase-active Src into the system did not affect the interaction of this protein with α2 chimaerin (C.Monfries, Personal Communication).

A second putative target of the α2 chimaerin SH2 domain with an apparent molecular mass of approximately 65 kDa has been identified in rat brain extracts. The native protein was partly purified using a conventional chromatographic techniques: the enrichment of this protein was monitored during purification using an overlay binding assay (M.Teo, Ph.D Thesis, 1994). Peptide sequence information was obtained and the protein identified subsequently as TOAD-64 (for Turned On After Division), a neuronally expressed phosphoprotein of approximately 64 kDa whose expression is developmentally regulated (Minturn et al, 1995a). Its expression levels are highest in the late embryonic/early post-natal rat brain cortex, decreasing to lower levels in the adult. This correlates well with the expression of α2 chimaerin mRNA (Hall et al, 1993) and also the timescale of neuron maturation. TOAD-64 is related to Unc-33, a C.elegans gene involved in axonal outgrowth and is found in lamellipodia and filopodia in growth cones (Minturn et al, 1995b). Collapsin is a member of the semaphorin family of molecules and is implicated in axonal pathfinding as a repulsive guidance cue (reviewed by Muller et al, 1996). Growth cone collapse in response to collapsin in dorsal root ganglion neurons is mediated by a TOAD-64/Unc-33-related protein, named the collapsin response mediator protein (CRMP, Goshima et al, 1995). Growth cone collapse could be blocked by the injection of anti-CRMP antibodies. A family of four CRMP genes have since been identified and they are differentially expressed in the nervous system (Wang & Strittmater, 1996). Of the four, TOAD-64 is mostly closely related to CRMP-2. CRMP protein share sequence similarity with dihydropyrimidinases (DHPases) which hydrolyse pyrimidine rings, although no enzymatic activity has been detected by *in vitro* studies (Wang and Strittmater, 1997). Recently human CRMP-2 has been shown to be incorporated into the neurofibrillary tangles that are a feature of Alzheimers Disease, suggesting that this protein may have a pathological role in the formation of the tangle-bearing neuron, so contributing to disease progression (Yoshida et al, 1998).

CHAPTER TWO

MATERIALS & METHODS

Material and Methods

2A Materials

General laboratory chemicals were from Sigma or BDH (Analar Grade). Purified water was produced in the laboratory using an Elga Option 4 Purification System (water was deionised, purified by reverse osmosis, filtered and sterilised by ultraviolet light). PBS tablets were from Oxoid. Absolute alcohol was from Haymans Laboratories. Microbiological reagents were from Difco. Radionucleotides were from Amersham with the exception of γ^{32}P-GTP which was from NEN. Other reagents were obtained from the sources detailed below or in Section 2B.

2A.1 Reagents for DNA Work

Antibiotics were obtained from Sigma. E.coli strains XL-1Blue and BL21 were obtained from Stratagene. Restriction enzymes were from Gibco-BRL where available or alternatively from New England Biolabs. pGEX vectors were from Pharmacia and Plasmid Purification and Clean-Up kits were from Promega. DNA modifying enzymes were obtained variously from Amersham, Pharmacia or Promega). The Transformer Site-Directed Mutagenesis Kit was from Clonetech. Mutagenic primers (GCF2, 3, 4, 5, 6) and the sequencing primers CH17 and CH18 were synthesised by the Molecular Medicine Unit at King's College London. dNTPs were from Pharmacia. The Sequenase Version 2.0 kit for DNA sequencing was from United States Biochemical. The Random Primed DNA Labelling Kit was form Boehringer-Mannheim. Hybond-N filters were from Amersham and other filter papers were from Whatman. Autoradiography film was from Kodak.

2A.2 Reagents for Protein Work

Glutathione agarose was from obtained from Sigma and Affigel-10 resin from Biorad. Coomassie Brilliant Blue-R protein stain was from Sigma and Silver Staining Kits were from Biorad. Thrombin was obtained from Sigma and Benzamidine Sepharose from Pharmacia. Dialysis membranes were from Medicell and protein concentrators from Amicon. The Protein Assay Kit from Biorad was used to estimate protein concentrations. Acrylamide/bis acrylamide solutions were obtained from Scotlab. Nitrocellulose membranes were from

Schleicher & Schuell, polyvinyl difluoride (PVDF) membranes from ICN. Anti-Glutathione-S-Transferase antibody was from Santa Cruz, anti-actin antibody from Amersham and anti-β tubulin antibody from Sigma. RC20 anti-phosphotyrosine antibody was from Transduction Laboratories. S Sepharose, Q Sepharose and Sephadex G-50 resins were from Pharmacia. Electrochemiluminescent (ECL) reagents were from Amerham, as was ECL-Hyperfilm. Titermax adjuvant was from Sigma. Protein Kinase A (catalytic subunit) was from Sigma. ATP and GTP were from Pharmacia and Sigma respectively.

2A.3 Other Reagents

Clones of α2 chimaerin sequences in Bluescript KII+ (921.2) and the pGEX-2T expression vector were provided by W.C.Sin and in pGEX-2TK by C.Monfries. These constructs are described in Appendix I. pGEX-p13 and pGEX-p64 were obtained from C.Monfries. Anti-α2 chimaerin antibody was produced by G.Michaels and M.Teo.

2B Methods

2B.1 Preparation of Glassware and Reagents.

All glassware, pipette tips and eppendorf tubes were sterilised by heating to 120°C for a period of 6 hours in a dry oven. Other consumables were guaranteed sterile by the manufacturer. Reagents were either autoclaved at 121°C for 20 minutes at 20 lbs/inch^2 or passed through 0.2μm filters (Flow Laboratories) to sterilise, as necessary.

2B.2 Production of Competent E.coli

A glycerol stock of the required bacterial strain (e.g. XL-1Blue, BL21) was streaked out onto an LB/agar plate (LB: 1% w/v tryptone/ 1% w/v NaCl/ 0.5% w/v yeast extract; 15% w/v agar added to prepare plates) and incubated overnight at 37°C. A single colony was picked and used to inoculate 5ml Luria-Bertani medium, supplemented with 50μg/ml tetracycline. The culture was grown overnight at 37°C then diluted 100-fold into fresh LB medium, again supplemented with 50μg/ml tetracycline, and incubated at 37°C with shaking until the absorbance at 600nm was between 0.8 and 1.0. The cells were pelleted in sterile 50ml Falcon tubes (15 minutes at 3000g in a Beckman J6-HC, 4°C) and the pellets resuspended in a volume of ice-cold 100mM $CaCl_2$ equivalent to half that of the original

culture (250ml). The cells were spun down (15 minutes at 3000g, 4°C) and the pellets resuspended in 50 ml of ice-cold 100mM $CaCl_2$. Sterile glycerol was added to a final concentration of 10%, the cells aliquoted into eppendorfs and snap frozen before storage at -70°C. All consumables used in this procedure were cooled to 4°C before use.

2B.3 Transformation of Competent E.coli

Circularised plasmid molecules, either intact or with single-stranded nicks are transformed into competent E.coli more than a hundred-fold more efficiently than linearised molecules. Approximately 100ng of purified plasmid DNA, containing a specific antibiotic resistance gene, was incubated with 200μl competent E.coli for 30 minutes in pre-chilled eppendorf tubes. The cells were heat-shocked for at 42°C for 90 seconds in a water bath and then allowed to incubate on ice for a further 5 minutes. At this point 400μl of SOC medium (2% w/v tryptone/ 0.5% w/v yeast extract/ 10mM NaCl/ 2.5mM KCl/ 10mM $MgCl_2$/ 10mM $MgSO_4$) was added and the tubes transferred to a shaker-incubator set at 37°C for 1 hour. The transformed cell mixture was plated on LB/agar plates containing the appropriate antibiotic to select for cells containing the plasmid. The plates were incubated at 37°C for 16 hours to allow the formation of bacterial colonies. They could then be stored at 4°C until required.

The choice of bacterial strain depended on the application: XL-1Blue were routinely used for DNA manipulations such as sub-cloning and BL-21 for the expression of recombinant proteins

2B.4 Purification of Plasmid DNA

2B.4A Small Scale Purifications (Minipreps)

2B.4A.1 Obtaining Bacterial Cultures for Plasmid Purifications

A sterile toothpick was used to pick a single colony from an LB-agar-antibiotic plate on which a plasmid transformation mixture had been plated. This colony was used to inoculate 1-5ml of LB medium, supplemented with ampicillin at 100μg/ml, which was then incubated overnight at 37°C with agitation. If a high-copy number plasmid, e.g. Bluescipt-SK II, were to be purified, approximately 1ml of culture was used. If the plasmid in question were of a lower copy number, e.g. pGEX-2TK, 5ml of culture would be used for the miniprep.

2B.4A.2 Production of a Cleared Bacterial Lysate

The Wizard Minipreps DNA Purification System (Promega) was used to isolate plasmid DNA from bacterial cultures according to the manufacturers instructions. Briefly, 1 or 5 ml of bacteria were pelleted by centrifugation (5 minutes at 3000rpm in a Beckman J6-Hc, 4°C). The cell pellet was resuspended in 200µl Cell Resuspension Solution (50mM Tris-HCl pH7.5/10mM EDTA/100 µg/ml RNase A). 200µl Cell Lysis Solution (0.2M NaOH/1% SDS) was added and mixed gently by inversion. When cell lysis was complete, as determined by a clearing of the solution, 200µl of Neutralisation solution (1.32M Potassium acetate pH 4.2) was added and gently mixed by inversion. A floccular precipitate containing genomic DNA and cell debris formed at this stage and was pelleted by centrifugation at top speed in a microfuge for 10 minutes at 4°C. The supernatant was decanted and the plasmid DNA extracted.

2B.4A.3 Purification of Plasmid from a Cleared Bacterial Lysate

1ml of Wizard Minipreps DNA Purification Resin was added to the cleared bacterial lysate and mixed by inversion. The resin and bound plasmid DNA was transferred to the barrel of a 2ml syringe, pushed into a mini-column and washed with 2ml of Promega Column Wash Solution (125ml of 200mM NaCl/ 20mMTris-HCl pH 7.5/ 5mM EDTA diluted with 170ml Ethanol). The mini-column was spun at top speed in a microfuge for 20 seconds at room temperature to dry the resin and the plasmid was eluted by application of 50µl TE (10 mM Tris-HCl pH7.5/ 1mM EDTA) to the top of the column. After a 1 minute incubation the plasmid DNA could be recovered by centrifugation for 20 seconds at top speed in a microfuge at room temperature. A typical 'prep' would yield approximately 5µg pure plasmid DNA.

2B.4B Large scale Purifications (Maxipreps)

For larger scale plasmid purifications the Promega Wizard Maxipreps DNA purification system was used. 500ml bacterial cultures were prepared by inoculating 5ml of LB/ampicillin with a single colony and growing for 6-8 hours at 37°C then diluting this culture 100-fold into fresh LB/ampicillin and incubating overnight at 37°C with agitation. The cells were then pelleted (centrifugation at 5000g for 10 minutes at room temperature in a Beckman J6-HC)

99

and resuspended, lysed and neutralised as described in Section 2B.4A.2, all volumes being scaled up appropriately. The floccular precipitate/cell lysate was centrifuged (14,000g for 15 minutes at room temperature in a Beckman J2-21) and the supernatant filtered through Whatman #1 Paper to remove residual cell debris. DNA was precipitated from the filtrate by addition of 0.5 volumes of room-temperature isopropanol. The DNA was pelleted by centrifugation (14,000g for 15 minutes at room temperature) and resuspended in 2ml of TE. 10 ml of Wizard Maxiprep DNA Purification Resin was added to the DNA solution, the slurry mixed by inversion and transferred to a maxi-column whereupon a vacuum was applied to draw liquid through the column. Two 12.5 ml aliquots of Promega Column Wash Solution were drawn through the column under vacuum to wash the resin and then 5ml of 80% ethanol applied to further rinse it. The vacuum was maintained for a further minute and residual wash solution removed by centrifugation at 1300g for 5 minutes in a swing-out rotor. A vacuum was applied to the column for 5 minutes to ensure that it was completely dry and the DNA eluted by applying 1.5ml of TE buffer, preheated to 70°C, to the top of the column. After incubating for 1 minute the plasmid DNA solution was spun out from the column (centrifugation at 1300g for 5 minutes in a swing-out rotor). Typical DNA yields varied between 100µg and 500µg, depending on the plasmid copy number.

2B.5 Quantification of DNA in Solution

The concentration of DNA in solution was estimated by measuring the absorbance of a sample diluted in TE buffer at 260nm. At this wavelength a 50µg/ml solution of double-stranded DNA has an absorbance of 1.0. Levels of contamination can be estimated by measuring the absorbance at 280nm (resonance of tryptophan) and calculating the ratio of the two values (A_{260}/A_{280}). A pure preparation of DNA should give a value of 1.8.

2B.6 Digestion of DNA by Restriction Enzymes

For routine analysis of plasmid samples, 100ng DNA was digested in a 10µl reaction volume using the manufacturers recommended buffer. The incubation temperature was as directed by the enzyme manufacturer, routinely 37°C. Digestion reactions were incubated for between 1 and 16 hours and a ten-fold excess of enzyme was used. In no case was the amount of glycerol present allowed to exceed 10% v/v lest the restriction enzyme should

exhibit star activity. For larger quantities of DNA, the reactions were carried out in larger volumes. DNA could then be ethanol-precipitated from the reaction solution and resuspended in a smaller volume if necessary (see Section 2B.7). When restriction digests were performed with two enzymes (e.g. to excise a fragment from the a plasmid) whose buffering requirements were incompatible, the reactions were carried out sequentially, the DNA being ethanol-precipitated after the first reaction and then resuspended in the appropriate buffer for the second enzyme.

2B.7 Ethanol Precipitation of DNA

DNA-containing solutions were diluted to 500µl with TE buffer and 1ml of ethanol (2 volumes) and 50µl 3M sodium acetate pH 5.2 (0.1 volumes) added. Samples were incubated at -20°C for between 1 and 16 hours. Under these conditions DNA is not soluble and precipitated DNA was pelleted by centrifuging for 20 minutes at 20,000g (4°C) in a microfuge. The supernatant was decanted and the pellet washed with 1ml of 70% ethanol to remove contaminating salts. The pellet was dried under vacuum in a Savant Speed-Vac and resuspended in the desired buffer.

2B.8 Inactivation / Removal of Restriction Enzymes

After digestion it may be necessary to inactivate the restriction enzyme lest its presence should adversely affect subsequent manipulations. Some enzymes could be inactivated by incubation at 65°C for 20 minutes; such information is provided routinely by the manufacturer. In other cases, the Promega Wizard 'clean-up' system was used. The sample was diluted such that the volume was between 50µl and 500µl, 1ml of 'clean-up' resin was added and the sample mixed gently. The slurry was transferred to a mini-column using a syringe and the flow-through discarded. The resin and associated DNA (plasmid or fragment) were washed with 2ml of 80% isopropanol. The column was spun at top speed in a microfuge at room temperature for 2 minutes to remove isopropanol from the column and then left at room temperature for 15 minutes to allow evaporation of residual isopropanol. DNA was eluted by applying 50 µl of TE, preheated to 70°C, to the top of the column and incubating for 1 minute. The 'clean' DNA was then recovered by centrifugation at top-speed in a microfuge for 2 minutes. These 'clean-up' columns were also used to

remove low molecular mass DNA molecules and free nucleotides from samples.

2B.9 Separation of DNA Fragments by Electrophoresis

Sufficient agarose was added to 0.5x TBE buffer (10xTBE stock: 900mM Tris/ 900mM boric acid/ 20mM EDTA) to give a concentration of between 0.8% and 1.2% w/v. The exact concentration used was dependent upon the expected size of the DNA fragments to be separated but 1%w/v was appropriate for routine use. The agarose was dissolved in the buffer by boiling for 5 minutes and then allowed to cool to approximately 50°C before pouring into a horizontal gel tray (Biorad). A comb was positioned in the tray to produce sample wells. Once set, the gel was submerged in 0.5x TBE buffer in a 'DNA Sub-Cell' (Biorad) and samples loaded. The samples had been mixed with 6x Loading Buffer (0.25% w/v Bromophenol Blue, 0.25% w/v Xylene Cyanol FF, 30% v/v Glycerol) to weight them down in the wells and allow visualisation of the separation of the fragments by movement of the dye fronts (bromophenol blue runs with linearised, double stranded fragments of 300 base pairs whilst xylene cyanol runs with fragments of about 4000 base pairs). Samples were electrophoresed at 150 Volts for approximately 50 minutes and then soaked in solution of 0.5µg/ml Ethidium Bromide/ 0.5xTBE for 20-30 minutes. Ethidium Bromide binds DNA and allows it to be visualised under ultraviolet light. Permanent records of gels were obtained by Polaroid photography. To allow estimation of the size of the fragments, HaeIII-digested Φx174 and HindIII-digested λ bacteriophage (obtained from Gibco-BRL) were run in parallel with the samples as markers.

2B.10 Purification of DNA Fragments from Agarose Gels

To purify fragments of DNA derived, the vector (1-5µg) was digested and the whole digest loaded into a single, wide well of an agarose gel. After electrophoresis, the fragments were stained with Ethidium Bromide for a maximum of 5 minutes and then visualised under long wavelength UV light. Agarose containing the fragment of the appropriate size was then excised from the gel and dissolved in 1ml of Promega DNA Clean-Up Resin. The 6M Guanidinium Chloride present in the slurry of resin completely disrupts the agarose within a few minutes, releasing the DNA fragment into solution. TAE/agarose gels are more sensitive to guanidinium-induced solubilisation than TBE/agarose gels and so 1x TAE

(0.04M Tris-Acetate/ 1mM EDTA) was used rather than 0.5x TBE for this specific procedure. Once dissolved, the fragment was recovered using the Promega Clean-Up column protocol described in Section 2B.8.

2B.11 Enzymatic Modification of DNA Molecules

2B.11A Blunt-ending Fragments

When sub-cloning a fragment into a vector it was sometimes necessary to remove the single-stranded overhangs produced by the enzymes used to excise the fragment from its parent vector. Either the 5' or the 3' strand may be left overhanging depending on the enzyme used.

2B.11A.1 Filling in 5' Overhangs

0.2-1µg of DNA was treated with 2 units of the Klenow fragment of DNA Polymerase 1 (Amersham) in the presence of 5mM $MgCl_2$ and 0.1mM dNTPS. The reaction was incubated at room temperature for 15 minutes and then the Klenow heat-inactivated by incubation at 65°C for 20 minutes. Klenow is active in TE buffer and virtually all restriction enzyme buffers and so this reaction could be performed directly after DNA digestion.

2B.11A.2 Cutting Back 3' Overhangs.

0.2-1µg of DNA was treated with 2 units of T4 DNA Polymerase for 15 minutes at 12°c in the presence of 0.1mM dNTPs and 5mM $MgCl_2$. The enzyme was then inactivated by heating to 65°C for 20 minutes. As with the 5' filling-in reaction, the enzyme and required reagents could be added directly to the buffer used for the digestion reaction.

2B.11B 5' Phosphorylation of Single Stranded DNA

Oligonucleotides were phosphorylated at their 5' end using T4 DNA Polynucleotide Kinase (PNK, Pharmacia). Approximately 2µg of DNA was phosphorylated in a 20µl reaction volume containing 2µl of 10x OnePhorAll Buffer Plus (1x concentration: 10mM Tris-Acetate pH 7.5/ 10mM Magnesium Acetate/ 50mM Potassium Acetate, Pharmacia) and 1mM ATP. The reaction was incubated at 37°C for 60 minutes until terminated by the addition of 1µl of 0.5M EDTA. The phosphorylated oligonucleotide could then be annealed to another single stranded DNA molecule.

2B.11C 5' Dephosphorylation of DNA

When a plasmid vector was digested with a single enzyme or with two enzymes that produced compatible overhangs, religation of the parent vector during attempts to construct a hybrid fragment/vector molecule would give rise to a circularised product that could be efficiently transformed into competent E.coli so causing high background levels of colonies containing the plasmid alone rather than the hybrid plasmid/insert molecule. To reduce the ability of the parent vector to religate under these conditions, the digested plasmid was treated with Shrimp Alkaline Phosphatase (USB/Amersham) to dephosphorylate the 5' ends of the molecule so preventing religation. This enzyme is active in the majority of restriction enzyme buffers and so could be added directly to the tube in which the plasmid had been digested. 2 units of shrimp alkaline phosphatase were added per microgram of DNA and the reaction incubated at 37°C for 2 hours. The enzyme was then heat-inactivated by incubation at 65°C for 20 minutes. The dephosphorylated vector could be used directly in a ligation reaction.

2B.12 Ligation of DNA Fragments into Plasmid Vectors

Fragments of DNA could be inserted into appropriately-digested plasmid expression vectors by incubating the two in the presence of T4 DNA Ligase (Promega). Approximately 100ng of digested plasmid was incubated with an equimolar quantity of insert fragment in the presence of 1 unit of T4 DNA Ligase for 1-16 hours at 16°C. The reaction was performed in the ligation buffer supplied by the manufacturer (10x Ligase Buffer: 300mM Tris-HCl pH 7.8/ 100mM $MgCl_2$/ 100mM DTT/ 10mM ATP) in a total volume of 10μl. In general, if DNA ends with complementary overhangs were to be ligated, 1 hour was a sufficient reaction time. If blunt DNA ends, produced either by digestion with a restriction enzyme such as Sma1 which does not give rise to overhangs or by filling in/cutting back of existing overhangs, were to be ligated the reaction was incubated overnight. After ligation, 5μl of the reaction mix was transformed into competent XL-1 Blue cells. Cohesive or 'sticky-end' ligations are more efficient than blunt-end ligations and best results were obtained when the 5' and 3' ends of the fragments had been prepared by digestion with two enzymes which produce non-complementary overhangs, allowing only one possible orientation of insertion (directional cloning). In other cases, the orientation of the insert was determined by digestion

with an enzyme that would cut towards the end of the insert so that the predicted size of the digestion products would depend on the orientation.

2B.13 Identification of Clones Containing Inserted DNA Fragments.

If a high efficiency of correct insertion of fragment into plasmid was anticipated, positive clones could be identified by simply picking a number of colonies (up to 18) with sterile toothpicks, inoculating 5ml overnights and preparing the plasmid DNA. Digestion with restriction enzymes that excise the insert fragment followed by agarose gel analysis enabled positive clones to be identified. In practice, such an approach was only practical for directionally-cloned fragments which are ligated into plasmid with high efficiency. When blunt-ended fragments were inserted, the background of colonies containing only religated plasmid was such that it was more appropriate to screen a large number of clones, immobilised on a membrane, with radioactively-labelled insert fragment.

2B.13A Preparation of Radioactively Labelled DNA Fragments Using the Random Prime Labelling Technique

All components for the labelling reactions were obtained from the Random Primed DNA Labelling Kit produced by Boehringer Mannheim with the exception of α ^{32}P-dCTP (3000Ci/mmol) which was obtained from Amersham. This method relies upon the use of a mixture of all possible hexanucleotides which will contain sequences which can hybridise to any fragment that is to be labelled. Klenow enzyme can then extend the complementary strand from the 3' hydroxyl terminus of the hexanucleotide, incorporating radiolabelled dCTP that has been included in a reaction mix containing the other three nucleotides. The DNA fragment acts solely as a template for synthesis of new, radioactively-labelled DNA which may then be used to detect clones containing that DNA sequence.

2B.13A.1 Preparation of the Probe

Between 50 and 200ng of DNA was used as a template for the labelling reaction. Fragments that had been blunt-ended were never used in these reactions because free nucleotides would be present in solution; these would reduce the specific activity of the radiolabelled product. The volume of the sample was made up to 10μl with deionised water and boiled in a water bath for 3 minutes to denature the DNA. The DNA solution was spun down, cooled on ice

and the following reagents added: 5μl of Random Prime Labelling Buffer (containing the hexanucleotide mix, reaction buffer, and dATP/dGTP/dTTP at a concentration of 0.1mM in this mix), 4μl of α^{32}P-dCTP, 1μl of 1 unit/μl Klenow. The reaction was incubated at 37°C for 1 hour before heat-inactivating the Klenow. The probe was diluted into 180μl of TE and either purified (see below) or used directly.

2B.13A.2 Purification of the Probe by Gel Filtration

The probe could be separated from free nucleotides by gel filtration chromatography on Sephadex G-50. This resin retains low molecular mass species, so retarding their progress through a column and separating them from larger species such as labelled DNA molecules. The resin was swollen in deionised water and equilibrated in TE pH 7.5. A slurry of the resin/buffer could be stored at 4°C if sodium azide was added to a concentration of 0.2% w/v to inhibit fungal growth. A 1ml syringe was plugged with glass wool to approximately the 0.5ml mark and a G-50 slurry added. The syringe was placed within a 15ml glass tube and the gel bed settled by centrifugation at 2000rpm for 5 minutes; these steps were repeated until the resin was at the 1ml mark. The labelled DNA solution was pipetted onto the top of the column, the column centrifuged at 1000rpm for 10 minutes at room temperature and the flow through collected into an eppendorf. The purified probe could be stored at -20°C prior to use if necessary.

2B.13B Preparation of Filters for Hybridisation

Colonies were picked from a master plate with a toothpick and gridded out onto fresh LB/agar/ampicillin plates. These were incubated overnight at 37°C and the colonies lifted onto a round nylon-based filter (Hybond-N from Amersham) which could be treated to expose and attach the DNA. The plate was then incubated at 37°C for 2-6 hours to allow regrowth of the colonies. The filter was marked with biro so that the top of the grid of colonies could be identified and then rested on the surface of the agar such that no air bubbles were trapped. Once the filter had soaked through it was removed in one swift movement and placed colony-side up on a piece of Whatman 3MM paper pre-wet in Denaturing Solution (0.5M NaOH, 1.5M NaCl) for 1 minute to lyse the cells. It was then transferred to 3MM paper pre-soaked in Neutralising Solution (1.5M NaCl, 0.5M Tris-HCl pH 8.0, 1mM EDTA) for 5 minutes before a final 5 minute incubation on 3MM pre-wet in

2xSSC (0.15M Sodium Chloride, 15mM Sodium Citrate). The filters were covered in Saran wrap and the DNA cross-linked to the filters by exposing the filter, DNA side down, to short-range wavelength ultraviolet light for 5 minutes. Bacterial debris could be removed at this stage by wiping with a tissue. The filters were air-dried and either used immediately or stored at room temperature until required. In some cases relatively few colonies were produced on transformation of a ligation reaction and it was more appropriate to use the transformation plate directly for the colony lift rather than preparing a gridded plate. If this was so, the filter and plate were aligned by marking both with India Ink whilst the filter was resting on the plate.

2B.13C Hybridisation of the Probe to the Filter.

Filters were pre-wet in 2xSSC and transferred to Hybaid hybridisation tubes and any excess solution drained away. 10ml of hybridisation buffer (7%w/v SDS/ 0.49M Na_3PO_4 pH 6.8) was added and the tube rotated in a Hybaid hybridisation oven until the temperature reached 65°C. At this stage the probe was denatured by boiling for 5 minutes and then was diluted into hybridisation buffer. The filters were incubated with this hybridisation solution for 4-16 hours at 65°C before removing the filters and washing to remove non-specifically associated probe. The first wash was for 20 minutes in 2xSSC/0.1% SDS at room temperature and the second, more stringent, wash was for 20 minutes in 0.2xSSC/0.1% SDS at 40°C. The filters were then dried on 3MM paper, Saran-wrapped and exposed to Kodak X-omat film at -70°C for 1-16 hours. Clones containing the desired insert sequence bind the radioactive probe and give rise to a autoradiographic signal. By aligning the original plate with the developed film, those colonies containing plasmid with inserted sequences can be identified. Overnight cultures could be grown, the plasmid purified and the presence of a correctly inserted DNA fragment confirmed by restriction digest.

2B.14 Site-Directed Mutagenesis of the α2-Chimaerin SH2 Domain

Point mutations were introduced in the α2 chimaerin SH2 domain at positions equivalent to those known to be important for the interaction of the Src SH2 domains with a phosphopeptide substrate (Bibbins et al,1993). Four separate mutations were made in the α2 chimaerin SH2 domain sequence of Bluescript KII+ clone 921.2 using the Transformer

Site-Directed Mutagenesis Kit (Clonetech). The protocol relies upon the simultaneous annealing of two oligonucleotide primers to one strand of a denatured double-stranded plasmid. A mutagenic primer introduces the desired mutation and a selection primer mutates a unique restriction enzyme site in the plasmid such that a new unique recognition site is created. A 200-fold molar excess of primers to plasmid was used during the annealing reaction to promote the incorporation of both the selection and the mutagenic primers and this enabled selection of clones containing the mutation on the basis of their altered susceptibility to restriction digest. By introducing the point mutations into the full length $\alpha 2$ chimaerin sequence it was possible subsequently to produce both the altered full-length and SH2 domain recombinant proteins following a single mutagenesis step. Further details of the mutagenesis procedure are given in Appendix 2.

2B.14A Oligonucleotide Primers for the Introduction of Point Mutations In the Chimaerin SH2 Domain

Primers were designed such that the mismatch bases were towards the middle of the sequence. These nucleotides are underlined and the nucleotides in the wild-type sequence shown directly below .

2B.14A.1 The Selection Primer

The selection oligo mutates the Bluescript SK II+ SacI site to an AatII site

  GCF2:   5' CACCGCGGTGGA<u>CG</u>TCCAGCTTTTGT 3'

                                   GC

2B.14A.2 The mutagenic primers

GCF3: 5' CATCGGATGAAAC<u>CA</u>TCTTCCATAATAC 3' E^{49} is mutated to W

GCF4: 5' GTCGGCTGCTTCT<u>AG</u>GGAGATCATGCC 3' R^{56} is mutated to L

GCF5: 5' CCGCTGGCTCTCC<u>A</u>GGATGAGGTAGCTCC 3' R^{73} is mutated to L

GCF6: 5' GTAGAGCCTGAAGT<u>G</u>TCTGGTTTGACTTCC 3' N^{94} is mutated to H

2B14.B Mutagenesis Protocol

Approximately 2 µg of each primer was 5' phosphorylated by T4 Polynucleotide Kinase using the method described in Section 2B.11B. The 5' phosphorylated primers and the

Bluescript clone 921.2 were diluted to a concentration of 0.05μg/μl and four separate annealing reactions set up, each containing the plasmid (2μl), the selection primer (2μl) and one of the four mutagenic primers (2μl) diluted to a total volume of 20μl in Annealing Buffer (1x Annealing Buffer: 20mM Tris-HCL pH 7.5/ 10mM MgCl2/, 50mM NaCl). Samples were boiled for three minutes to fully denature the double-stranded plasmid, chilled on ice for five minutes and collected by centrifugation. 1μl T4 DNA ligase, 1μl T4 DNA polymerase and 3μl 10x Synthesis Buffer (containing a mixture of the four deoxynucleotides at a concentration appropriate for elongation from the primer) was added to each annealed primer/plasmid mix and the total volume increased to 30μl with deionised water. The samples were incubated at 37°C for 2 hours to permit synthesis of the mutant strand then heated to 70°C for 5 minutes to inactivate the enzymes. The DNA was then ethanol-precipitated and digested with SacI to linearise plasmid molecules that did not contain the point mutation introduced by the Selection Primer. After digestion, 50% of each sample was transformed into mutS E.coli, a bacterial strain deficient in mismatch repair. The protocol for transformation is identical to that described in Section 2B.3 but rather than plating onto LB/agar plates, each mixture was diluted into 5ml LB medium with 100μg/ml ampicillin and incubated overnight at 37°C. This procedure amplified the mixed plasmid pool, which was subsequently isolated from the overnight culture using the Promega Wizard Miniprep Kit. A second selection digest was then performed, using approximately 100ng of the purified mixed plasmid pool. At this stage each sample was transformed into competent XL-1Blue and the entire of each transformation reaction plated onto LB/agar/ampicillin plates and incubated overnight at 37°C. Subsequently, colonies were picked, cultures grown and the plasmid isolated. These samples were then screened for the presence of the desired mutation.

2B.14C Identification of Correctly Mutated Clones

The initial stage in the identification of positive clones was to confirm that the purified plasmid could be linearised by AatII but would not be digested by SacI. Clones fulfilling these criteria were further analysed. For the mutations introduced by primers GCF 3, GCF 4 and GCF 5 there were concomitant changes in the enzyme recognition sites around the altered nucleotides that enabled confirmation of the point mutation by restriction analysis, although the positive clones identified in this way were also sequenced. The presence of the

mutation introduced by the GCF6 primer could only be confirmed by sequencing the appropriate region of the clone. The sense strands of the GCF3, GCF4 and GCF5 clones were sequenced from the CH18 primer:

CH18: 5' CTATCGATCGATGAATATAGACCTCCTTGG 3'

The antisense strand of the GCF 6 clone was sequenced from the CH17 primer:

CH17: 5' ATCTCGAGAGTCACCAGATCGTGGATCGAC 3'

Once clones had been identified with the desired point mutation, sequences encoding either the chimaerin SH2 domain region or the full-length protein were sub-cloned into a bacterial expression vector.

2B.14D Subcloning Point-Mutated α2 Chimaerin Sequences into a Bacterial Expression Vector

The SH2 domain-containing fragments (with additional α2 chimaerin sequences encoding 48 amino acid residues amino terminal to the SH2 domain and 31 carboxyl terminal amino acid residues; chimaerin SH2 domain Contruct B, see Appendix 1) were excised from the Bluescript clones by digestion with EcoRI and NdeI and gel-purified. Fragments containing the full-length coding sequence were obtained by digestion with EcoRI and DraI, blunted-ended and ligated into SmaI-digested pGEX-2TK. The inserted sequence was fused in frame 3' to the sequence encoding Glutathione-S-Transferase (GST); expression of this protein and the insert to which it is fused was driven from an IPTG-inducible *tac* promoter. The GST tag enabled the purification of the fusion protein by affinity chromatography. These constructs also have a PK-A phosphorylation site immediately N-terminal to the inserted sequence which facilitates radioactive labelling of the expressed fusion protein.

2B.15 Sequencing of Double-Stranded DNA

2B.15A Chain Termination Sequencing Reactions

The Sequenase Version 2.0 kit produced by United States Biochemical was used to sequence DNA by the dideoxy chain termination method. The Sequenase 2.0 enzyme has high processivity, high fidelity and low exonuclease activity.

5μg of high purity double-stranded DNA template was denatured by incubating in an equal volume of 0.4M NaOH/0.4mM EDTA for 30 minutes at 37°C to denature,

recovered by ethanol precipitation and resuspended in 7μl of deionised water. Approximately 1pmol of the appropriate primer and 2μl of 5x Sequenase Reaction Buffer (200mM Tris-HCl pH 7.5/ 100mM MgCl$_2$/ 250mM NaCl) was added and the primer annealed to the template by heating at 65°C for 2 minutes in a water bath followed by slow cooling to room temperature. 1μl of 0.1M DTT, 2μl of Labelling Mix (1.5μM dGTP/dCTP/dTTP), 0.5μl of ^{35}S-dATP and 2μl of Sequenase 2.0 (diluted 1:8 in 10mM Tris-HCl pH7.5/ 5mM DTT/ 0.5mg/ml BSA) was added and the reaction incubated at room temperature for 5 minutes during which time DNA polymerisation extended from the primer. 3.5μl of the reaction was transferred to four tubes, each of which was pre-warmed to 37°C and contained the components of a termination reaction for one of the four nucleotides (a mixture of each of the four dNTPS at 80μM and one dideoxynucleotide at 8μM in 50mM NaCl). The termination reactions were mixed and incubated at 37°C for 5 minutes before ending the reactions by addition of 4μl of 'Stop' solution (95% formamide/ 20mM EDTA/ 0.05% Bromophenol Blue/ 0.05% Xylene Cyanol FF).

2B.15B Denaturing Acrylamide Gel Electrophoresis

The LKB Macrophor System was used for the electrophoretic analysis of DNA sequencing reactions. The thermostatic plate onto which the gel mixture is poured was coated with Repel-Silane and the upper glass plate was coated with Bind-Silane (75μl diluted in 20ml ethanol/5ml glacial acetic acid) so that, after running, the gel could be lifted away from the thermostatic plate, remaining attached to the glass plate. 4% acrylamide (acrylamide/bis acrylamide in a ratio of 19/1) gradient gels containing 42% w/v urea were cast according to manufacturers directions. The thickness of the gel was graduated from 0.2mm at the top to 0.4mm at the bottom, a device intended to improve the range of fragment sizes that could be resolved by the gel. After polymerisation the gel was arranged vertically in the Macrophor unit, the upper and lower buffer reservoirs filled with 1x TBE, the thermostatic plate connected to a cooling unit and the gel pre-run at 1000V until the temperature of the thermostatic plate stabilised at 55°C. Immediately before loading onto a gel the samples were denatured by heating to 75°C for 2 minutes and 4μl of each termination reactions for each sample were loaded in the order of the nucleotides 'GATC' and the gel run at 1000V until the samples had run into the gel. The voltage was then increased to 1200V and the gel

run until the Bromophenol Blue dye front approached the bottom of the gel. The glass plate with the gel attached was lifted away from the thermostatic plate, soaked in 10% v/v glacial acetic acid for 1 hour to remove the urea and dried overnight at 80°C in a drying cabinet. The dried gel was subsequently exposed to Kodak X-omat film at room temperature for 1-3 days. The sequence of the template could then be read from the autoradiograph.

2B.16 Expression of Recombinant Proteins in Bacteria.

Protein expression can be driven from the isopropyl-β-thiogalactopyranoside (IPTG)-senstive promoters of bacterial expression vectors such as pGEX, allowing large-scale production of recombinant proteins. An overnight culture was prepared by inoculating LB/ampicillin medium with a single colony from a fresh transformation plate and incubating at 37°C for 16 hours. This was diluted between twenty-fold and one hundred-fold into prewarmed LB and incubated with agitation until the culture had reached a stage of vigourous growth (assessed as an absorbance of approximately 0.5 at 550nm). To ensure adequate aeration, flasks were never filled beyond a quarter of their maximum capacity. Protein expression was induced by addition of (IPTG) to a final concentration of 0.5mM. After further incubation, cells were harvested by centrifugation (3Krpm, 15 minutes, 4°C in a Beckman J6-HC), resuspended in 0.1 volumes of Phosphate Buffered Saline (PBS: 140mM NaCl/ 3mM KCl/ 4mM Na_2HPO_4/ 1mM KH_2PO_4 pH 7.4), transferred to 50 ml Falcon tubes (each tube containing the equivalent of 250ml of the original culture volume) and pelleted once more. Cell pellets were stored at -20°C prior to purification of the fusion protein. Routinely, the initial dilution was twentyfold, all stages of protein expression were performed at 37°C and the induction period was approximately two hours. However, when expressing the $\alpha2$ chimaerin SH2 domain as a recombinant protein, better results were obtained by diluting the overnight culture one hundred-fold, growing the cultures at a lower temperature (30°C) for a longer period (24 hours) prior to inducing for a shorter time (1 hour at 30°C). The protein yield varied widely; whilst up to 10mg of GST could be purified from one litre of culture, only around 100μg of GST-$\alpha2$ chimaerin SH2 domain would be obtained from a similar volume.

112

2B.17 Fusion Protein Purification

2B.17A Purification of GST (Glutathione-S-Transferase) Fusions

10 ml of ice-cold PBST (PBS/1% Triton-X-100) was added to each bacterial pellet and the cells lysed by sonication (20 second bursts with 5 second pauses). At this stage, although not subsequently, the buffer was supplemented with 1mM DTT, and a cocktail of protease inhibitors (all from Sigma): $1\mu g/ml$ pepstatin A; $1\mu g/ml$ aprotinin; $1\mu g/ml$ leupeptin and 1mM 4-(2-aminoethyl)benzenesuphonyl fluoride (AEBSF) or phenylmethylsuphonyl fluoride (PMSF). The cell lysate was cleared by centrifugation at 15,000g for 20 minutes at 4°C (Beckman J2-21), the supernatant decanted and incubated with Glutathione Agarose (swollen by incubation with 200ml distilled water/g resin for two hours at room temperature and equilibrated into PBST) for 1 to 2 hours at 4°C. The glutathione agarose beads were pelleted by centrifugation (2000Krpm, 5 minutes at 4°C in a Beckman J6-HC), the supernatant decanted and the beads resuspended in 10 column volumes of PBST in a 15ml Falcon tube. After pelleting the beads, this batchwise washing procedure was repeated once more with PBST and then, if the fusion protein was to be eluted with glutathione, a further time with 50 mM Tris pH 8.0. Alternatively, if the fusion protein to be cleaved from the GST moiety, Thrombin Cleavage Buffer (TCB: 150mM NaCl/ 3mM $CaCl_2$/ 50mM Tris-HCl pH 7.5). The full fusion protein was eluted by incubating the washed beads in ten column volumes of 10mM reduced glutathione/50mM Tris-HCl pH 8.0 for ten minutes at room temperature. The slurry was then transferred to a column and drained. The purified fusion protein was present in the flow through and which was then concentrated to a volume of less than 1ml using an Amicon Centriprep-10 (centrifuged for 3 x 10 minutes at 3000rpm and 4°C in a Beckman J6-HC, draining the filtrate after each spin). The elution step could be repeated a second time to maximise yield.

To cleave the target protein from its GST moiety, the beads were incubated overnight at with 5 units of thrombin (Sigma)/expected mg of fusion protein in 2 column volumes of TCB at 4°C. The slurry was then transferred to a column and the flow-through (containing the cleaved protein) incubated with 0.5 ml of Benzamidine Sepharose (prewashed into TCB) for 15 minutes at 4°C to remove the thrombin. The slurry was then drained and the protein solution concentrated. Purified proteins were then analysed by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2B.17B Purification of MBP (Maltose Binding Protein) Fusions

The protocol was similar to that for the purification of GST fusions with the following exceptions: the cell lysis buffer used was 20mM Tris-HCl pH 7.5/ 2mM EDTA/ 2mM EGTA/ 0.5% Triton-X-100; the fusion protein is purified by affinity chromatography on amylose agarose (New England Biolabs), washed with 50mM Tris pH 8.0/0.15M NaCl and eluted by competition with 10mM maltose in the same buffer.

2B.18 Dialysis of Proteins

To remove low molecular mass contaminants, e.g. glutathione or maltose, or to change the buffer in which a protein had been purified, samples were dialysed overnight at 4°C against 3 changes of buffer of the desired composition. Each change was maintained for a minimum of two hours, which should be sufficient to ensure that the relative buffer concentrations of the sample and the dialysis medium had reached equilibrium.

2B.19 Determination of Protein Concentration

The Biorad Protein Assay Kit was used to measure the concentrations of proteins in solution. Between 1µl and 10µl of a protein solution was diluted to 0.8ml with deionised water and mixed with 0.2ml Biorad Dye Reagent (Coomassie Brilliant Blue G-250 in phosphoric acid and methanol). After 5 minutes incubation at room temperature, the absorbance of the sample at 595nm over a 1cm path was measured relative to a 'no protein' control. Protein concentration could be estimated by comparison of the absorbance read with a standard curve derived from the absorbances of known quantities of Bovine Serum Albumin (BSA) treated in this fashion.

2B.20 Analysis of Proteins by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A vertical, discontinuous SDS/polyacrylamide system was used (Biorad Mini Protean IF). Resolving gels containing between 9% and 12% acrylamide (30% stock solution, an acrylamide/bisacrylamide ratio of 37.5:11)/ 0.375 M Tris-HCl pH 7.5/ 0.1% w/v SDS/ 0.05% w/v Ammonium Persulphate (APS)/ 0.05% v/v TEMED were incubated at room temperature for approximately 1 hour to allow polymerisation to occur. Gels were of either 0.75mm or 1.5mm thickness. Stacking gels (4% w/v acrylamide/ 0.125M Tris-HCl pH 6.8/

0.1% w/v SDS/ 0.1% APS/ 0.2% TEMED) were overlaid and Teflon combs used to form sample wells. Samples were diluted into an equal volume of 2x Protein Sample Buffer (0.125M Tris-HCl pH 6.8/ 4% w/v SDS/ 40% v/v glycerol/ 10% v/v β-mercaptoethanol, 0.0002% w/v Bromophenol Blue), boiled for 5 minutes to fully denture the protein and loaded into the wells of the stacking gel. Proteins were separated at 180V for approximately 40 minutes (until the dye front reached the base of the gel) in SDS-Running Buffer (0.025M Tris/ 0.2M Glycine/ 0.1% w/v SDS). Prestained protein molecular weight markers (Gibco-BRL) were run in parallel with the samples to allow estimation of the apparent molecular masses of the separated proteins. For long term storage, gels were dried under vacuum at 60°C between sheets of cellophane in a Biorad Slab Dryer

2B.21 Two Dimensional SDS-PAGE

This procedure involves an initial separation of proteins of the basis of their electrical charge (isoelectric focusing, IEF) followed by a separation in a different direction on the basis of molecular mass. The resolving power of the combination of the procedures is far greater than that of a conventional one-dimensional SDS-PAGE separation and hence this method is appropriate for the analysis of samples containing a mixture of proteins. Samples were diluted 1:1 in Lysis Buffer (9.5M urea/ 10mM lysine/ 2% v/v NP-40/ 5% v/v ampholytes (pH 3.6-10, Pharmacia)/ 5% β-mercaptoethanol) and loaded onto the top of tube gels (length 8cm, diameter 2mm) that had been pre-equilibrated at the loading end with Lysis buffer. The IEF gels were prepared from a degassed acrylamide solution (55% w/v urea/ 4% w/v acrylamide (28.38% acrylamide/1.62% bis acrylamide)/ 2% v/v NP-40/ 5% v/v ampholyte pH 3.5-10/ 0.01% APS/ 0.05% TEMED) and poured into glass tubes that had been cleaned by sequential washes in concentrated hydrochloric acid, chloroform and finally ethanol. The gels were arranged vertically in an electrophoresis unit (Gel Electrophoresis Apparatus GE-2/L LS, Pharmacia), the samples loaded and overlaid with 0.5x Lysis Buffer. The top end of the gels were submerged in the Cathode Running Buffer (20mM NaOH, degassed) and the lower end rested in the Anode Running Buffer (10mM H_3PO_4). Proteins were focused at a constant voltage of 300V for 16 hours followed by 1 hour at 1000V. The IEF gels were expelled from their tubes and equilibrated in a small volume of 2x Protein Sample Buffer for 2 hours at room temperature. They were then loaded horizontally into the

single well of the Stacking Gel of the second dimension SDS-PAGE gel (modified from that previously described to contain 3% w/v urea; the composition of the resolving gel was unchanged) such that the basic end of the IEF was at the left-hand side of the gel. Protein Molecular Weight Standards (Gibco-BRL) were diluted into an equal volume of boiling 1% agarose, the solution poured into an IEF tube and allowed to set. The gel was extruded and a small portion run to the right hand side of the IEF gel.

2B.22   Visualisation of Proteins
2B.22A   Coomassie Blue Staining

Coomassie Brilliant Blue G-250 (Sigma) was dissolved in Destain Solution (0.5g in 1 litre of 40% v/v methanol/ 10% glacial acetic acid/ 50% dd H_2O; this solution will effectively fix proteins within the gel matrix). The solution was filtered through Whatman #1 Paper and stored at room temperature. SDS-PAGE gels were incubated in sufficient stain to cover the gel for 1 hour at room temperature with agitation. The stain solution was decanted and the background of the gel destained by incubating in several changes of Destain Solution over approximately 24 hours. The limit of detection using Coomassie Blue stain is approximately 250ng protein.

2B.22B   Silver Staining

When a more sensitive detection method was required, gels were silver-stained using the Biorad Silver Stain Kit. This method is 10-50 times more sensitive than Coomassie staining but care must be taken to use water of a very high purity and to handle the gels as little as possible to keep background staining to a minimum. Proteins were fixed by incubating the gel firstly in 40% v/v methanol/10% glacial acetic acid for 1 hour and then twice in 10% v/v methanol/glacial acetic acid for 30 minutes. The Oxidiser Reagent was added for 10 minutes then excess removed by rinsing the gel for 3x 10 minutes in deionised water prior to a 30 minute incubation in the Silver Reagent. Proteins were visualised by incubating in three changes of Developer Solution and, when the desired level of staining had been achieved, the reaction was halted by replacement of the Developer with 5% v/v glacial acetic acid. All incubations were at room temperature on an orbital shaker.

2B.22C India Ink Staining of Western Blots

To visualise proteins immobilised on a nitrocellulose filter, India Ink was diluted 1000-fold into PBS/0.1% Tween 20 (PBS-T20) and the blot incubated in this solution for 3-16 hours on an orbital shaker at room temperature. Once an adequate level of staining had occurred, excess stain could be removed washing the blot with several changes of PBS-T20.

2B.23 Immobilisation of Proteins on Membranes

2B.23A Preparation of Dot Blots

Protein samples were diluted to a volume of 10 μl and carefully pipetted onto a 1cm square of gridded nitrocellulose. The filter was allowed to dry at room temperature.

2B.23B Transfer of Proteins from Gel to Filter (Semi-Dry Blotting)

Gels, nitrocellulose filters (0.45μM pore) cut to the size of the gel, and extra thick sheets of filter paper (Biorad) were equilibrated in Transfer Buffer (48mM Tris/ 39mM glycine/ 0.0375% w/v SDS/ 20% v/v methanol) for 5 minutes at 4°C. One sheet of filter paper was placed on the platinum anode of the Biorad Trans Dry-SD Semi-Dry Transfer Cell and the nitrocellulose filter placed directly onto this. The gel was then placed on the filter, all air bubbles displaced, the remaining sheet of filter paper laid on the top of the stack and the steel cathode of the blotter fixed in place. Protein transfer was carried out at a constant voltage of 12V for 1.2 hours at 4°C. Nitrocellulose filters were either used immediately or air-dried and stored at room temperature. The efficiency of the transfer could be assessed by Coomassie staining a gel after transfer and comparing the amount of protein remaining with that seen on a duplicate gel that had not been blotted. In certain cases proteins were transferred to PolyVinyl DiFluoride (PVDF) membranes rather than nitrocellulose. If this was so, it was necessary to pre-wet the membrane in methanol prior to equilibration in Transfer Buffer and to ensure that at no point after blotting and during use did it dry out.

2B.24 Immunological Detection of Proteins Immobilised on Filters (Western Blotting)

2B.24A Standard Protocol

Filters were incubated in a blocking solution of 5% w/v dried milk powder (Marvel)/PBS-T20 either overnight at 4°C or at room temperature for 2 hours to block unbound sites on

117

the membrane. The filter was rinsed briefly in PBS-T20 and then incubated with the primary antibody, diluted according to manufacturer's instructions or to a concentration determined effective by experimentation, in 1% w/v Marvel/PBS-T20 and incubated for 1-2 hours at room temperature. The antibody solution was decanted and the filters washed for 6x 5 minutes in PBS-T20. The filters were then incubated in a secondary antiserum of Horseradish Peroxidase (HRP)-conjugated anti-immunoglobulin (Dako) diluted 1/1000 in 1% w/v Marvel/PBS-T20 for a maximum of 1 hour at room temperature. The secondary antiserum used was that appropriate to the species in which the primary antiserum had been raised. The filters were washed 6x 5 minutes in PBS-T20 and then incubated in Electrochemiluminescent (ECL) Reagent (Amersham, a 1:1 mixture of ECL Reagents 1 and 2) for 1 minute. Any excess fluid was removed by resting the filter briefly on Whatman 3MM paper, the filter was Saran-wrapped and exposed to ECL-Hyperfilm (Amersham) at room temperature for between 10 seconds and 40 minutes to visualise antibody binding to proteins on the filter.

2B.24B Immunological Detection of Phosphotyrosine

The RC20 antibody (Transduction Laboratories) is a bacterially-expressed recombinant anti-phosphotyrosine antibody (PY-20) that has been modified to give higher affinity. The constant region of the parent IgG has been remove to give a lower non-specific background and the antibody has been directly conjugated to HRP so eliminating the requirement for a secondary antiserum. Proteins were transferred to PVDF and the filters blocked in 1% w/v BSA dissolved in Wash Buffer (10mM Tris-HCl pH 7.5/ 100mM NaCl/ 0.1% Tween-20) for 20 minutes at 37° C. RC20 was diluted 1/5000 in 1% w/v BSA/Wash Buffer and the filters incubated in the solution for 20 minutes at 37° C. After washing with 5 changes of Wash Buffer over 15 minutes at room temperature phosphotyrosine-containing proteins were visualised by ECL.

2B.25 Production of Polyclonal Antibodies

Antibodies were raised to two recombinant proteins, p13-SHIP and p64-TOAD, expressed in bacteria and cleaved from their GST tag. A water-in-oil emulsion of the purified protein (approximately 100µg p13-SHIP or 20µg p64-TOAD) and the Titermax Classic Adjuvant

(Sigma) was prepared. The protein solution was diluted with water such that the final volume was equivalent to the that of the adjuvant. The adjuvant was vortexed for 30 seconds to ensure full resuspension and, whilst continuing to vortex, the protein was added in two equal aliquots, vortexing at full speed for two minutes after each addition. The emulsion was collected by spinning in a microfuge (2000rpm, approximately 20 seconds) and transferred to a 1ml syringe using a blunt-tipped 18 gauge needle. The injections were sub-cutaneous and of a maximum volume of 0.2ml. Two rabbits (Sandy Full Lops) were inoculated with each antigen/adjuvant emulsion. After the initial inoculation, a booster was administered after 2 weeks. Five subsequent injections were given at approximately 6 week intervals. Prior to the first injection, and between 10 and 14 days after the later injections, 10 ml bleeds were taken from each rabbit. The bleeds were stored in glass vials and clotting was allowed to complete by incubation at 37°C for 30 minutes. The end of a Pasteur pipette was sealed by flaming and used to gently detach the clot from the sides of the vial, which was then incubated at 4°C overnight during which time the clot contracted, extruding the serum. The serum was removed from the vial using a syringe and 19 gauge needle and spun at 10,000g for 10 minutes at 4°C (Beckman J2-21) to clear. The supernatant was aliquoted and stored with 0.2% w/v Sodium Azide at -70°C. The serum was tested for the presence of antigen-specific antibodies by immunoblotting.

2B.26 Preparation of Tissue Extracts

Rat tissues (brain, testis or intestine) were removed from Sprague-Dawley or Wistar rats of known ages and rinsed briefly in Extraction Buffer I (25mM MES pH 6.5/ 0.5mM $MgCl_2$/ 0.05mM $ZnCl_2$/ 0.1mM EGTA/ 0.05% v/v Triton-X-100) or Extraction Buffer II (50mM HEPES pH 7.5/ 0.1M NaCl/ 1mM EGTA). The tissue collected was weighed, fresh buffer added and an homogenate prepared. The volume of buffer added was such as would give a 20% w/v homogenate. Insoluble material was pelleted by centrifugation at 100,000g for 1 hour at 4°C (Beckman L8 Ultracentrifuge). The supernatant was decanted and either used immediately or stored at -70°C with 5% w/v glycerol. A further extraction could be carried out to prepare a 1% Triton-X-100-soluble extract: the insoluble material from the first extraction was resuspended in 1% Triton-X-100/Extraction Buffer II and the extraction procedure repeated. At all stages the solutions used were ice-cold and contained protease

inhibitors (1mM PMSF or AEBSF, 1µg/ml Aprotinin, 1µg/ml Pepstatin A, 1µg/ml Leupeptin) and phosphatase inhibitors (1mM Na_2VO_3, 10mM NaF) and 1mM DTT.

2B.27 Ion-Exchange Chromatography of Rat Brain Extracts

Rat brain extracts were loaded onto an S Sepharose (cation exchange) or a Q Sepharose (anion exchange) column that had been pre-equilibrated into the appropriate Extraction Buffer. After loading, the column was washed with approximately 20 Column Volumes (CV) of Extraction Buffer. A peristaltic pump was used to regulate the flow through the column during the loading and washing stages. A series of elutions was performed using two column volumes of buffer and a stepwise gradient of NaCl (0.1M, 0.2M, 0.3M 0.4M, 0.5M). All stages of the procedure were performed 4°C. The eluants were dialysed against an appropriate buffer, aliquoted, 5% v/v glycerol added and stored at -70°C.

2B.28 Preparation of Covalently-Coupled Affinity Columns

The supernatant solvent was removed and Affigel-10 N-hydroxysuccinamide-activated agarose bead matrix (Biorad) washed twice in 10 column volumes of cold, deionised water and a third time in the buffer used for the coupling reaction, routinely 0.5x PBS. Washing was completed within 20 minutes. Sufficient protein solution was added to the resin to give a coupled protein concentration of 1-2 mg/ml resin and buffer added such that the Affigel bed volume/total slurry volume ratio was in the order of 1/3. If a 'blocked' Affigel column (one in which the active sites have been reacted with a neutral agent) was required as a control, this was prepared by adding 0.1ml 1M Ethanolamine/ml bed volume Affigel to the same quantity of washed Affigel matrix used for the experimental column. Coupling reactions were allowed to proceed overnight at 4°C after which any unreacted sites on the column were blocked by a further 1 hour incubation in the presence of 0.1ml 1M Ethanolamine/ ml Affigel. The slurry was then transferred to a 5ml glass scinter cup, the cup placed within a 15ml glass Corex tube and the flow-through collected. The efficiency of the coupling reaction could be estimated by measuring the protein content of this sample (Biorad Assay). The Affigel was resuspended in 3 ml of 0.5x PBS and then spun at 1200rpm for 4 minutes at 4°C. The flow through was decanted from the Corex tube and this wash repeated twice more. There were two more washes with 1M NaCl/ column buffer (eg.

Extraction Buffer) and a final wash in column buffer. The Affigel-protein matrix was resuspended in the column buffer and transferred to a 50 ml Falcon tube to which a source of potential targets of the protein coupled to the matrix was then added (e.g. tissue extract)

2B.29 Isolation of Interacting Proteins by Affinity Chromatography

2B.29A Preparative Scale Purifications

Preparative scale affinity chromatography was used to select potential targets of the α2-chimaerin SH2 domain from rat brain extract. In some cases the affinity column was produced by covalent coupling of the chimaerin SH2 domain to Affigel 10 whereas in others a non-covalent column was used, produced simply by leaving the full GST fusion protein on a glutathione agarose column rather than eluting it during the purification and covalently linking it to a different agarose matrix. Where appropriate, GST or other columns were run in parallel as controls. During affinity purifications protease and phosphatase inhibitors were added to all buffers and the entire procedure was performed at 4°C.

10-100 ml of rat tissue extract, whole or fractionated, was incubated with the column matrix (routinely 1mg of protein per ml of beads) for 2-4 hours on a Spiramix (Denley). The slurry was then loaded into a glass column (200mm in length, 5ml capacity, glass frit at base, from Omnifit) and the flow-through of unbound material collected. The column was washed in 50-100 column volumes of 50mM NaCl diluted into the buffer of the sample applied to the column (Extraction Buffer), the flow being maintained by a peristaltic pump. Bound proteins were eluted with 5 column volumes of NaCl either in a stepwise gradient (0.1M, 0.2M, 0.5M, 1.0M) or simply with 1M NaCl. The columns could be stripped with 0.5% SDS after use to remove the fusion protein used to prepare the column and any very tightly associated proteins. Samples were dialysed against 2.5 mM Tris-HCl pH 7.5 to remove NaCl/SDS and were concentrated by freeze-drying in a Savant Speed-Vac. Each sample was resuspended to a final volume of 200μl (the buffer concentration would be approximately 62.5 mM). Samples were aliquoted and stored at -70°C prior to further analysis which was by peptide sequencing, 1 or 2 dimensional SDS-PAGE or biochemical assay.

2B.29B Analytical Scale Purifications

Small-scale affinity chromatography procedures used approximately 25μl of fusion protein-

agarose column matrix which was incubated with a source of potential binding proteins at 4°C in a total volume of 100μl for 1-2 hours. The slurry was transferred to a mini-column (Promega) and the unbound material expelled by application of pressure with a syringe. The minicolumns were inverted and placed in a eppendorf and the beads spun out by centrifugation in a microfuge. They were then resuspended in PBS, agitated for 5 minutes at 4°C and recovered once more. This washing procedure was repeated several times and the protein on the column and any bound proteins eluted in 1x Protein Sample Buffer. Samples could then be analysed by SDS-PAGE.

2B.30 Identification of Potential Target Proteins of the α2-Chimaerin SH2 domain by Peptide Sequencing

Analysis of eluants from α2-chimaerin SH2 domain affinity columns by 1 and 2 dimensional SDS-PAGE allowed the identification of several putative target proteins. Coomassie Blue-stained bands or spots were excised from the gel and tryptic peptides derived from these proteins were sequenced by Robin Philp at the Institute of Molecular and Cell Biology in Singapore.

2B.31 Assaying for Kinase and Phosphatase Activity
2B.31A *In Vitro* Kinase Assays and Phosphatase Assays

Protein samples were diluted to 10μl in deionised water and mixed with 4μl 5x Kinase Buffer (1x concentration: 50mM HEPES pH 7.0/ 5mM $MnCl_2$/ 5mM $MgCl_2$/ 1mM DTT/ 0.05% v/v Triton-X-100), 10μM ATP (Sigma) and 5μCi (γ^{32}P)ATP (Amersham, 3000Ci/mmol). The final reaction volume was 20μl and could include 1μg potential kinase substrate such as Myelin Basic Protein (Sigma). Reactions were incubated at 30°C for 15 minutes and terminated by the addition of an equal volume of 2x Protein Sample Buffer. The samples were run out on acrylamide gels which were then stained, dried and exposed to film at -70°C. Signals obtained could reflect either autophosphorylation of a kinase or phosphorylation of a kinase substrate by a kinase present in a mixed protein sample.

Myelin Basic Protein (MBP) was phosphorylated by Src kinase or β-pak according to this protocol to provide a source of potential phosphatase substate. Approximately 100ng of radio-labelled (MBP) was mixed with an equal volume of a protein sample and incubated

at 30°C for 30 minutes. The reactions were ended by the addition of Protein Sample Buffer and analysed by SDS-PAGE followed by autoradiography. Samples containing phosphatase activity would show a loss of phosphorylation relative to controls.

2B.31B In-Gel Kinase Assays

Protein samples containing putative kinase activities were separated by SDS-PAGE (0.75mm thickness gels) that had been prepared with 10% v/v glycerol. If desired, a potential kinase substrate, e.g. Myelin Basic Protein, could be polymerised into the gel matrix at a concentration of 0.1-1mg/ml (the stacking gel was prepared without the protein substrate). After electrophoresis the SDS was removed from the gel by incubating in three changes of 20% v/v isopropanol/ 50mM Tris pH 8.0 over 1 hour. Unless otherwise stated all incubations were at room temperature. The gel was then washed in several changes of 5mM DTT/50mM Tris-HCl pH 8.0 over a one hour period, denatured for an hour in 6M guanidinium chloride/ 2mM EDTA, 50mM DTT/ 50 mM Tris-HCl pH 8.0 and washed 3x 10 minutes in PBS to remove all the guanidinium chloride. Gel-bound proteins were allowed to renature by overnight incubation in three changes of 1% w/v BSA/ 0.1M NaCl/ 2mM EDTA/ 2mM DTT/ 0.1% v/v Triton-X-100/ 50mM Tris pH 7.5 at 4°C. The gels were re-equilibrated into In-Gel Kinase Buffer (50mM HEPES pH 7.5/ 10mM $MgCl_2$/ 2mM $MnCl_2$/ 2mM DTT/ 0.05% Triton-X-100) with three buffer changes over 2 hours and the kinase assay performed by incubation of the gels with 5μCi γ^{32}P-ATP/ml In-Gel Kinase Buffer. The gels were rinsed in PBS for 3x 10 minutes and washed overnight in several changes of 5% w/v trichloroacetic acid/ 1% sodium pyrophosphate to. Finally, proteins were fixed in the gel by incubation in 10% v/v methanol/ 5% v/v glacial acetic acid for 2x 10 minutes followed by 2x 10 minutes in 40% v/v methanol/ 10% v/v glacial acetic acid. The gels were dried and exposed to film at -70°C. Signals on the autoradiograph indicate the presence of an autophosphorylating kinase of a specific molecular mass. If a protein substrate was included in the gel, signals could indicate that a non-autophosphorylating kinase of a specific molecular mass is present if no corresponding signal is produced when the substrate is omitted.

2B.32 Assaying for Activity of GTPase-Activating Proteins

2B.32A Loading the p21-GTPase with γ^{32}-P GTP

Approximately 2μg of purified GST-Rac1 was incubated with 10μCi (1μl) of high specific activity γ^{32}P-GTP (6000Ci/mmol, NEN) in 20μl 20mM Tris-HCl pH 7.5/ 25mM NaCl, 0.1mM DTT/ 2μl of 0.5M EDTA for 10 minutes at room temperature. The reaction was terminated by the addition of $MgCl_2$, which inhibits nucleotide exchange, to a final concentration of 25mM. The GTP-loaded p21 could be stored on ice for a maximum of 1 hour before use.

2B.32B GAP Assays

4μl of the GTP-loaded Rac1 was stored on ice and diluted with 1μl of 30mM GTP, 3μl of 13 mg/ml BA, potential GAP proteins/GAP-interacting proteins, 0.1mM DTT, 20mMTris-HCl pH7.5 to a final volume of 30μl. The reaction was incubated in a water bath at 15°C and 5μl aliquots removed after 0, 4, 8 and 12 minutes. These were dotted onto 1cm squares of nitrocellulose and washed 6x 1 minute on ice in Wash Buffer (50mM Tris pH-HCl pH 7.5/50mM NaCl/5mM $MgCl_2$). The filters were dried and each square placed in a Beckman Snap-Cap scintillation vial. Beckman ReadySafe Scintillant was added and the counts measured in a Beckman LS 6000Sc Scintillation Counter. The counts were then plotted as a percentage of the counts at time zero, i.e. as a percentage of GTP remaining over time. The intrinsic GTPase activity of Rac1 was measured by performing one assay in parallel to the others with no added GAP.

2B.33 ^{32}P-Labelling of Fusion Proteins for Use as Probes.

Proteins expressed as GST fusions in the pGEX-2TK vector have a Protein Kinase A (PK-A) phosphorylation site immediately N-terminal to the insert sequence and C-terminal to the thrombin cleavage site of the fusion protein. This enables such proteins to be radioactively-labelled to a high specific activity. A 10 units/μl solution of the catalytic subunit of PK-A (Sigma, derived from bovine heart muscle) was prepared in 40mM DTT immediately prior to use.

2B.33A Labelling Proteins on a Glutathione Agarose Column

Approximately 10 μg of protein, associated with up to 100μl of glutathione agarose beads, was equilibrated in Heart Muscle Kinase Buffer (HMK, 20mM Tris-HCl pH 7.5, 100mM NaCl, 12mM MgCl$_2$) and resuspended in 7.5μl of 10x HMK Buffer, 2.5μl of PK-A solution, 2.5μl of γ^{32}P-ATP (25μCi) and 62.5μl of deionised water. The slurry was mixed and incubated for 30 minutes at 4°C on a Spiramix. The reaction was terminated by the addition of 5ml of 'Stop' Solution (10mM sodium phosphate pH 8.0/ 10mM sodium pyrophosphate/ 10mM EDTA/ 1 mg/ml BA) and the beads pelleted by centrifugation at 500g for 2 minutes. The supernatant was discarded and the beads resuspended in 10 ml PBS, incubated briefly at room temperature and spun down once more. The washing procedure was repeated four more times, the final wash being with 50 mM Tris-HCl pH 8.0. Radio-labelled fusion protein was recovered by eluting twice for 10 minutes at room temperature with 0.5ml 10mM glutathione/ 50mM Tris-HCl pH 8.0. These eluants were combined and the specific activity of the probe estimated by scintillation counting. The integrity of the probe was determined by SDS-PAGE analysis followed by autoradiography.

2B.33B Radioactive Labelling of Proteins in Solution

10μg of protein containing a site for PK-A phosphorylation was incubated with 5μl 10x HMK Buffer, 2.5μl of PK-A solution, 2.5μl of γ^{32}P-ATP (25μCi) in a volume of 50μl for 30 minutes at 4°C. The reaction was terminated by addition of 1ml 'Stop' Solution, sufficient Dextran Blue (Pharmacia) added to give a strong colouration and the protein solution applied to the top of a 1ml Sephadex G-50 column that had been pre-equilibrated in PBS. The columns were prepared in a 2ml syringe barrel blocked with two layers of Whatman GF-C filter cut to size with a cork borer. The Dextran blue acts as a marker for high molecular mass species and its movement through the column reflects that of proteins. The eluant was analysed by SDS-PAGE and the specific activity estimated indicated by scintillation counting.

2B.34 Detection of Proteins Immobilised on Nitrocellulose Filters with Radioactive Probes

2B.34A Denaturation/Renaturation Overlay Binding Assays

Proteins that had been transferred from gels to nitrocellulose filters were denatured by washing for 2x 5 minutes in 6M Guanidinium Chloride/ Hyb 75 (20mM HEPES pH 7.5/ 75mM KCl/ 2.5mM MgCl$_2$/ 0.1mM EDTA/ 1mM DTT/ 0.05% v/v NP-40) and allowed to renature slowly by washing in decreasing concentrations of guanidinium chloride (4x 10 minutes in 3M, 1.5M, 0.75M, 0.375M and 0.1875M Guanidinium Chloride. After transfer to fresh tubes, the filters were washed for 2x 30 minutes in Hyb75 prior to blocking non-specific sites by incubating firstly in 5% w/v Marvel/ Hyb 75 for 30 minutes and then in GST Blocking Buffer for a further 30 minutes. (GST was expressed in bacteria, the cells collected and resuspended in 0.05 volumes of Hyb 75 and a cleared lysate produced. This GST containing cell lysate was diluted five-fold into 5% w/v Marvel/Hyb 75 to give GST Blocking Buffer). An advantage of this particular method is that exposure of the filters to GST at this stage will reduce non-specific signals derived from the GST/bacterial contaminant moieties present in the probe. Probes were diluted into GST Blocking Buffer (modified to contain only 1% w/v Marvel) to give approximately 10^6 cpm/ml and incubated for 12-18 hours. The filters were washed for 3x 10 minutes in 0.1% w/v Marvel/ Hyb 75, covered in Saran Wrap and exposed to film. This entire procedure was performed at 4°C

2B.34B Conventional Overlay Binding Assays

Protein samples were either dot-blotted directly onto 1cm squares of nitrocellulose or transferred to filters after SDS-PAGE separation. The filters were blocked by incubation in 3% w/v BSA/Blocking Buffer (BB: 25mM MES pH 6.5/ 0.5 mM MgCl$_2$/ 0.05mM ZnCl$_2$/ 0.2 M NaCl/ 0.05% v/v Triton-X-100) for 2-16 hours at 4°C. The filters were washed in 0.1% w/v BSA/BB for 2 x 10 minutes at 4°C, prior to incubation with the probe, which had been diluted to 10^6 cpm/ml in 0.1% w/v BSA/BB, for 1 hour at room temperature. The probe was decanted, the filters transferred to fresh dishes and washed in BB for 6x 5minutes on ice. They were then covered in Saran Wrap and exposed to film.

CHAPTER THREE

RESULTS I

The Identification of Potential Targets of the α2 Chimaerin SH2 Domain.

Expression of a splice variant of chimaerin with an SH2 domain implicates this protein in tyrosine kinase signalling pathways. Identification of a target protein of the α2-chimaerin SH2 domain might allow the prediction of further interactions. The atypical features of the chimaerin SH2 domain suggest that it may interact with its target in a novel manner. α2-chimaerin is a neuronally expressed protein and, since brain is a highly abundant source of signalling proteins, brain extracts were used as the starting material for purifications of chimaerin target proteins.

Affinity chromatography was used to identify potential targets of the α2-chimaerin SH2 domain. The α2-chimaerin SH2 domain, expressed as a GST fusion protein, was coupled to an agarose matrix, either covalently or non-covalently, and used to select binding proteins from rat brain extracts. SDS-PAGE analysis enabled the identification of proteins that specifically bound the chimaerin affinity column which could than be isolated and peptide sequence information obtained. An alternative approach involved the use of radio-labelled α2-chimaerin SH2 domain to probe potential sources of target proteins that had been separated by SDS-PAGE and immobilised on nitrocellulose filters. A fundamental difference between these two techniques is that the results obtained by affinity chromatography may not be indicative of a direct chimaerin SH2 domain-target interaction, whereas binding assay studies using Western blots detect direct interactions *in vitro*.

3A The Use of Affigel Affinity Columns to Identify Potential Target Proteins

Two different types of affinity column were used: those in which the 'bait' protein had been covalently coupled to an agarose matrix (Affigel-10) and those in which a fusion protein was non-covalently associated with an affinity matrix (for example a GST fusion and glutathione agarose). One advantage of the former is that, once brain extract has been applied, the columns can be washed more stringently without the risk of dissociation of the fusion protein. The chimaerin SH2 domain construct used to produce protein for the preparation of affinity columns contained chimaerin protein sequence beyond the SH2 domain: ten amino acid residues at its amino terminal and thirty at its carboxyl terminal. This and other constructs used are described in Appendix 1.

3A.1 GST, GST-abl SH2 Domain and GST-α2 Chimaerin SH2 Domain Affinity Columns

If a GST-α2 chimaerin SH2 domain fusion protein is used to select binding proteins a GST column is an appropriate control. In initial experiments a GST-abl-SH2 domain column was also used; the abl SH2 domain has known target proteins and its interaction with phosphotyrosine has been established. These three fusion proteins were purified and analysed by SDS-PAGE to confirm that they were suitable for use in an affinity chromatography purification (Figure 3.1). The preparations did not contain any bacterial contaminants or fusion protein breakdown products that were detectable by Coomassie stain and these proteins were subsequently coupled covalently to an activated Affigel-10 matrix according to the protocol described in Section 2B.28. GST and GST abl SH2 domain columns contained approximately 2mg protein/ml bed volume whereas the concentration of the GST-α2 SH2 domain column was 1mg/ml. Rat brain extract (prepared in 0.05% Triton-X-100) was applied to each column, the columns washed and bound proteins eluted with a stepwise salt gradient. There were three further elutions, the first with glycine-HCl pH 3.6, the second with glycine-NaOH pH 10 and finally with 0.5% SDS. This last treatment appeared to remove a proportion of the fusion protein from the column, although the coupling procedure should have linked the protein to the matrix covalently. Any very tightly bound proteins were also eluted. 5% of the total protein in each sample was separated by SDS-PAGE and proteins visualised by silver staining (Figure 3.2).

A distinct set of rat brain proteins was selected by each column. Of the three columns tested, the abl SH2 domain bound the most protein and the GST the least (gels B and A respectively). The total quantity of protein binding to each column was very low but the specificity of the selection was shown by the differences in the proteins eluted from the chimaerin and the abl SH2 domain columns (gels C and B respectively). The quantity of protein eluted from the chimaerin column with SDS (C, lane 7) was high relative to the other elutions and it was found that a protein with an apparent molecular mass of around 55 kDa bound the chimaerin SH2 domain in an approximately equimolar fashion. At least three proteins were detected in the 25-35 kDa range in this sample as were a number of other proteins of higher molecular mass. A protein of around 55 kDa also bound the abl SH2
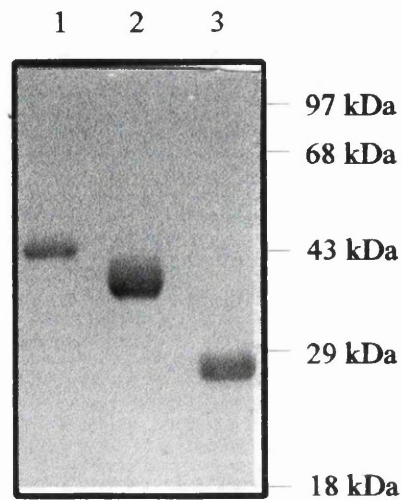
Figure 3.1: The Purification of GST-α2 Chimaerin SH2 Domain and Control Proteins for the Preparation of Affinity Columns

Samples of GST-α2 chimaerin SH2 domain (lane 1), GST-abl SH2 domain (lane 2) and GST (lane 3) protein preparations were separated by SDS-PAGE and proteins visualised with Coomassie Blue. These proteins were subsequently coupled to an activated agarose matrix (Affigel 10).
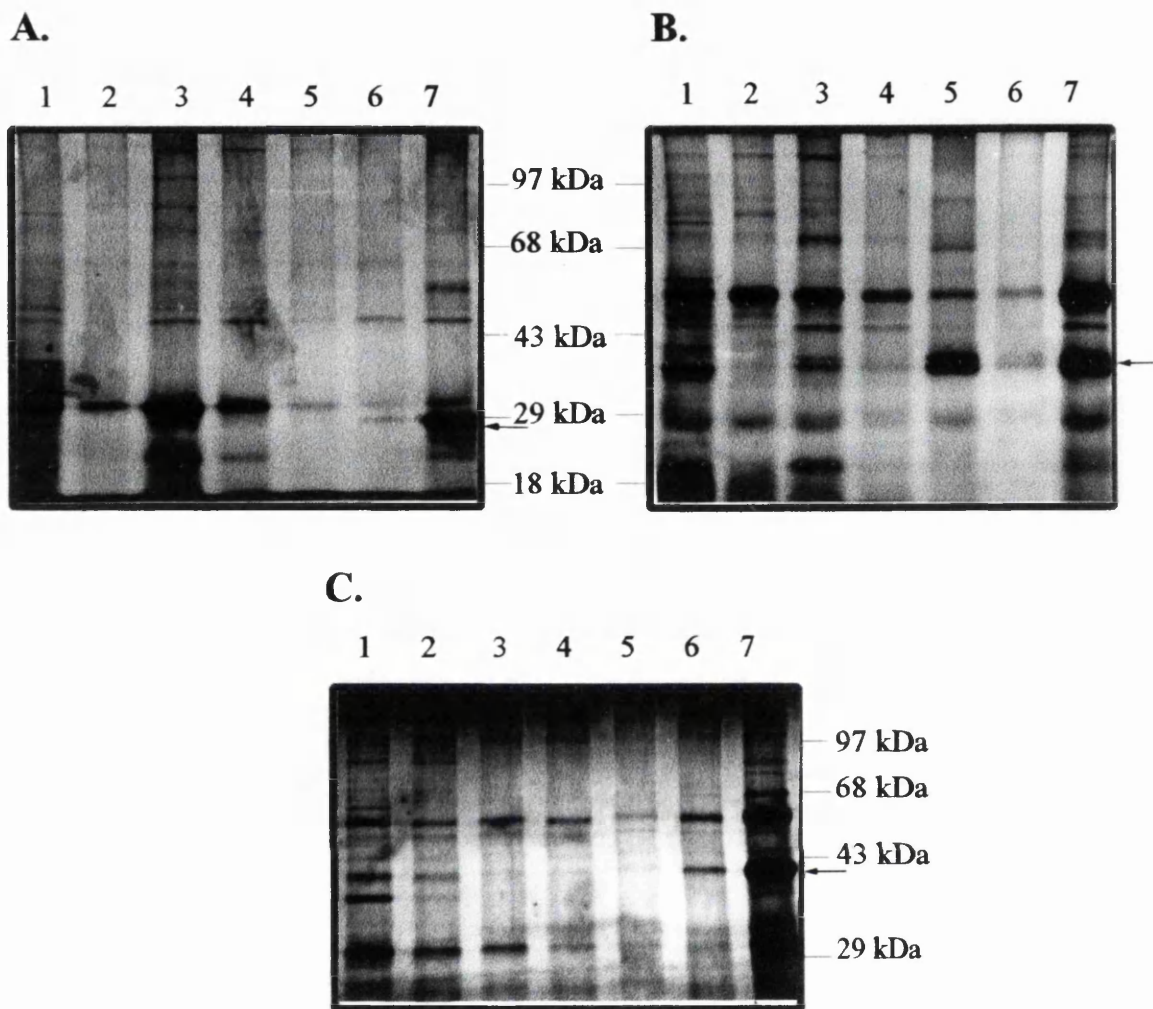
Figure 3.2: The Purification of Rat Brain Proteins by Affinity Chromatography

Approximately 30ml of rat brain extract (prepared in 0.05% Triton-X-100) was passed down a GST (A), GST-abl SH2 domain (B) or GST-α2 chimaerin SH2 domain (C) affinity column. The flow through was collected and the columns washed in Extraction Buffer. Proteins associated with the columns were eluted sequentially with 0.1M NaCl (lane 1), 0.2M NaCl (lane 2), 0.5M NaCl (lane 3), 1.0M NaCl (lane 4), pH 3.6 buffer (lane 5), pH 10 buffer (lane 6) and 0.05% SDS (lane 7) and analysed by SDS-PAGE. Proteins were visualised by silver staining. Arrows indicate the fusion protein present in the samples eluted with SDS (lanes 7, gels A, B, C).

domain column in relatively large amounts but otherwise the pattern of eluted proteins differed from that obtained using a chimaerin column, indicating that affinity chromatography can successfully be used to select specifically potential α2-chimaerin SH2 domain-binding proteins from rat brain extract. The fusion protein used to prepare the column was also released by treatment with SDS and the corresponding bands are indicated to the right of each gel in Figure 3.2.

3A.2 Phosphotyrosine-Containing Proteins Bind the Chimaerin SH2 Domain

SH2 domains bind phosphotyrosine and therefore the protein samples obtained from the affinity columns described in Section 3.1 were tested for the presence of phosphotyrosine-containing proteins by Western blotting. 5% of each eluant was separated by SDS-PAGE and transferred to nitrocellulose. The filters were probed with the RC20 anti-phosphotyrosine monoclonal antibody (Figure 3.3). It should be noted that the antibody used cross-reacts with the fusion protein used to prepare the column and which is present in the SDS-eluted samples. Detection of the fusion protein in indicated by an arrowhead in each case.

The abl SH2 domain and the chimaerin SH2 domain columns each bound a distinct set of phosphotyrosine-containing proteins (B and C respectively). The chimaerin column bound a number of phosphoproteins that resisted 1M NaCl and low and high pH treatments and were only released when SDS was applied to the column (lane 7). Prominent amongst these were proteins of around 50 kDa, 70 kDa and 100 kDa. There were also signals seen in the 0.1M NaCl, 0.2M NaCl and pH10 eluants of approximately 35kDa, 65 kDa and 55-60kDa respectively (C, lanes 1, 2 and 6). A number of phosphotyrosine-containing proteins were eluted at 0.5M NaCl, the most prominent being in the 55-60kDa region (C, lane 3). The level of phosphotyrosine detected in the abl SH2 domain-binding samples was low compared to that present in the samples purified on a chimaerin column. This was unexpected because the total quantity of protein bound to the abl column is higher than that bound to the chimaerin column (Figure 3.2). This suggests that the α2 chimaerin SH2 domain affinity column selected target phosphoproteins specifically from a complex mixture of rat brain proteins.

132
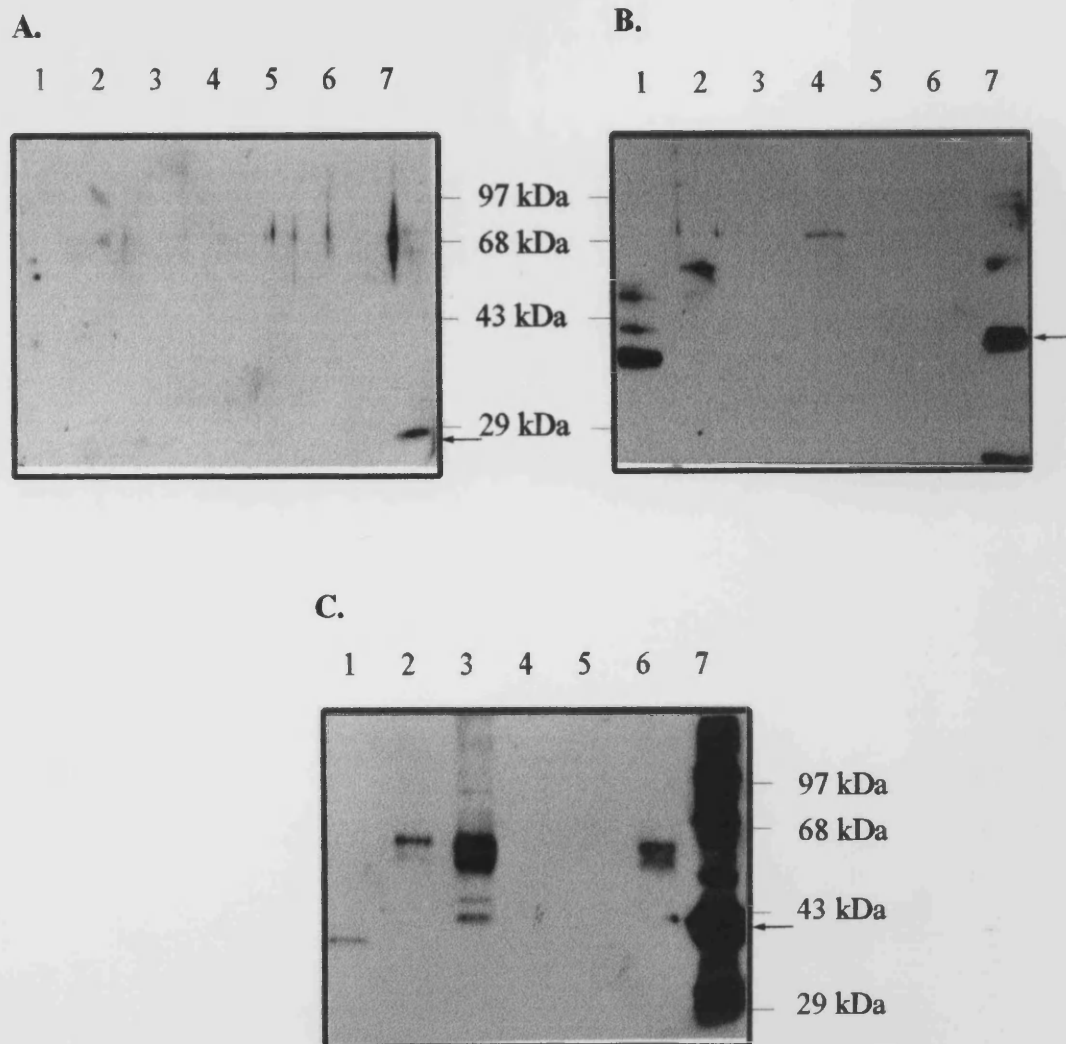
Figure 3.3: Phosphotyrosine–Containing Proteins Bind An α2 Chimaerin SH2 Domain Affinity Column

Rat brain proteins bound to GST (Figure A), GST-abl SH2 domain (Figure B) and GST-α2 chimaerin SH2 domain (Figure C) affinity columns were eluted sequentially with 0.1M NaCl (lane 1), 0.2M NaCl (lane 2), 0.5M NaCl (lane 3), 1.0M NaCl (lane 4), pH 3.6 buffer (lane 5), pH 10 buffer (lane 6) and 0.5% SDS (lane 7). A 5% sample of each eluant was separated by SDS-PAGE, transferred to PVDF membrane and probed with an anti-phosphotyrosine antibody (RC20, diluted to 1/5000). Phosphotyrosine-containing proteins were visualised by ECL. The antibody cross-reacted with the fusion protein present in the samples eluted with SDS (lanes 7, Figures A, B and C). In each case this is indicated by arrows.

3A.3 Optimisation of Target Protein Purification

The elution of bound protein from a column in a series of steps has the advantage that binding proteins will be separated into different eluant samples and will effectively be obtained at a higher purity. If the quantity of a protein binding an affinity column is very low and if it elutes in more than one fraction as has been the case for a number of proteins binding a chimaerin affinity column (Figure 3.2), it is possible that its concentration in each of these fractions may be beneath the limit of detection. To circumvent this problem it was decided to alter the elution conditions and, rather then perform a series of elutions with increasing concentrations of salt, simply apply 1M NaCl to the column. This sample would then contain all the proteins previously separated between the 0.1M NaCl, 0.2M NaCl, 0.5M NaCl and 1.0M NaCl eluants but there would be the concomitant disadvantage that there would be no indication of the strength of the association of the proteins with the column.

Rat brain extract (prepared in 0.05% Triton-X-100) was applied to a GST column and a GST-α2-chimaerin SH2 domain column. The columns were washed and bound proteins eluted in five column volumes of 1M NaCl. The proteins binding each column were analysed by SDS-PAGE (Figure 3.4, lanes G1 and C1, GST and chimaerin columns respectively). The columns were not treated with SDS but an aliquot of each column matrix after elution was also analysed (Figure 3.4, lanes G2 and C2). Silver staining revealed that the chimaerin SH2 domain column was binding both a greater number and quantity of proteins than could be detected in the previous experiment (Figure 3.2). Silver-staining did not detect any proteins in the sample eluted from the GST column. It was concluded that, of the proteins associating with the GST α2 SH2 domain column under the conditions used, the majority were specifically selected by the chimaerin moiety of the fusion rather than the GST. To obtain peptide sequence information it was necessary to obtain microgram rather than nanogram quantities of the purified protein and it was therefore decided to continue eluting bound proteins from the affinity column in a single step at 1M NaCl in further experiments.

3A.4 The Purification of a Potential Target of the Chimaerin SH2 Domain

It is a possibility that, in tissues in which α2 chimaerin is expressed, it will be complexed to its target and, as such, that target will not be free to interact with chimaerin *in vitro*. If this
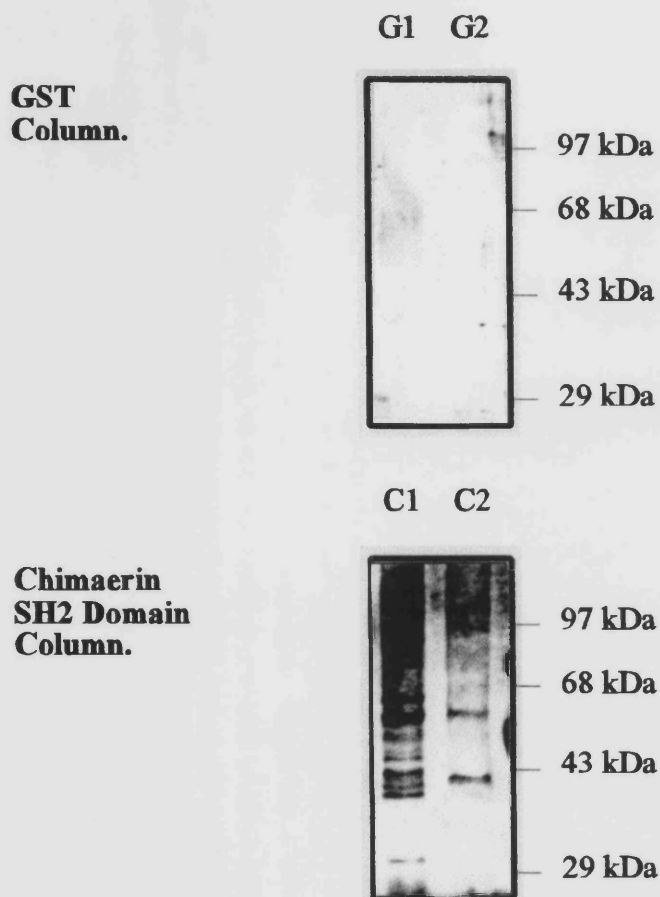
Figure 3.4: Rat Brain Proteins Bound to GST and α2 Chimaerin SH2 Domain Affinity Columns Eluted in a Single Step

Approximately 90ml of rat brain extract (prepared in 0.05% Triton-X-100) was passed down either a GST affinity column or a GST-α2 chimaerin SH2 domain affinity column as indicated. Bound proteins were eluted with 1M NaCl (lanes G1 and C1), separated by SDS-PAGE and visualised by silver staining. A 10μl aliquot of each column matrix after elution was also analysed (lanes G2 and C2).

were so then a tissue in which the protein of interest is not expressed might be a more suitable starting material for an affinity purification. Unlike the closely-related β2 chimaerin, α2 chimaerin is not expressed in the adult cerebellum (Leung et al, 1994 and Hall et al, 1993) and therefore extracts from rat cerebellum were tested.

Rat cerebellum proteins were extracted in 0.05% Triton-X-100 and applied to an α2 chimaerin SH2 domain affinity column. The column was washed and bound proteins eluted with a single application of five column volumes of 1M NaCl. The column was then stripped with 0.5% SDS. 5% of the total protein obtained in these two steps was analysed by SDS-PAGE (Figure 3.5). Coomassie staining revealed a protein of approximately 30 kDa (p30) in the 1M NaCl eluant. This was the only prominent band in this sample and this the first purification in which there was sufficient protein obtained to detect with Coomassie stain. There is a band of a similar size in the SDS-eluted sample but this may derive from fusion protein breakdown. Approximately 1μg of the p30 was present in the sample analysed and there would therefore be a total of around 20μg present in the total eluant. The total protein content of the cerebellum extract used for the purification was 400mg and therefore, if 100% of the p30 available bound to the column, it must constitute approximately 0.005% of the total cytosolic protein. There were at least two other proteins, of approximately 55kDa and 65 kDa, associating with the affinity column in relatively large amounts. These were eluted with 0.5% SDS.

To ensure that the 30kDa protein eluted at 1M NaCl was not GST, the samples were Western-blotted and probed with anti-GST (Figure 3.6). If fusion protein breakdown had occurred during the course of the experiment it was possible that GST would then be present at this stage, but this was not found to be the case. Recombinant GST was detected (lane 1) as was the GST fusion and a putative breakdown product in the SDS-eluted sample (lane 4) but no protein that could be detected by the antibody was present in the sample eluted from the chimaerin affinity column with 1M NaCl.

3A.5 Two-Dimensional SDS-PAGE Analysis of Binding Proteins

Analysis of the proteins binding an affinity column by SDS-PAGE may not indicate the full diversity of proteins present as some may co-migrate. 25% of the total protein eluted at 1M NaCl from the chimaerin SH2 domain affinity column described in section 3.4 was further
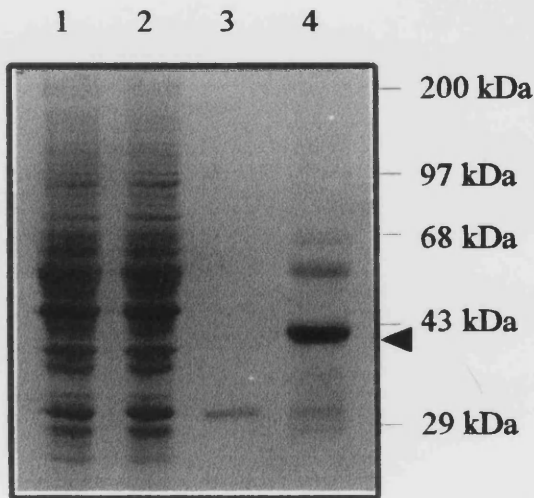
Figure 3.5: Analysis of Rat Cerebellar Proteins Binding a Chimaerin SH2 Domain Affinity Column

50ml of rat cerebellum extract (prepared with 0.05% Triton-X-100) was applied to a GST-α2 chimaerin SH2 domain column. The flow through was collected and proteins associated with the column eluted firstly with 1M NaCl and secondly with 0.5% SDS. Samples of brain extract and flow through containing approximately 50μg total protein (lanes 1 and 2 respectively) and 5% of the total protein eluted with 1M NaCl and 0.05% SDS (lanes 3 and 4 respectively) were analysed by SDS-PAGE and stained with Coomassie Blue. The fusion protein on the column was removed with SDS and this is indicated by an arrowhead.

137
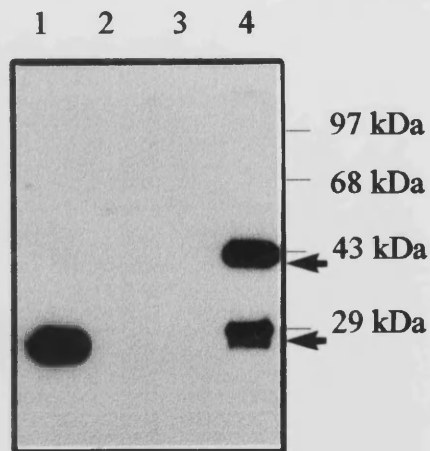
Figure 3.6: The 30kDa Rat Brain Protein Purified on a Chimaerin SH2 Domain Affinity Column is not GST

1µg of GST (lane 1), a sample of rat brain extract containing approximately 50µg of total protein (lane 2) and 5% of the rat brain proteins eluted from a GST-α2 chimaerin SH2 domain column with 1M NaCl and 0.5% SDS (lanes 3 and 4 respectively) were separated by SDS-PAGE, transferred to nitrocellulose and probed with a mouse monoclonal anti-GST antibody (Santa Cruz, diluted 1/1000). When the column was treated with SDS, fusion protein (FP) and GST breakdown products (G) were released. These react with the antibody as indicated by the arrows.

analysed by two-dimensional SDS-PAGE, a technique with greater resolving power than conventional SDS-PAGE. Proteins present in this sample and in rat cerebellum extract (approximately 100µg total protein) were separated on the basis of pI on iso-electric focusing (IEF) gels prior to separation on the basis of molecular mass. The 30kDa protein eluted from the affinity column at 1M NaCl is resolved into 2 separate spots (Figure 3.7). The pI of this protein lies in the basic range of the gel. There is only one spot in the cerebellum extract seen at a similar position on the 2D gel and it is not possible to be certain whether this represents the same protein as the p30 seen in the chimaerin-binding sample. Other eluted proteins, for example tubulin and actin, are also present at lower levels: several of these can be identified provisionally on the basis of their 2D SDS-PAGE mobility and this is indicated in the figure. Peptide mapping would be required to identify these proteins conclusively.

3A.6 Identification of a Potential Target by Peptide Sequencing

The affinity purification of the 30kDa potential target of the chimaerin SH2 domain from rat cerebellum extract was repeated until sufficient protein was obtained for sequencing. The protein samples were separated by two dimensional SDS-PAGE, identified by Coomassie staining, excised from the gels and approximately 10µg was sent for peptide sequencing. The sequences of five tryptic peptides were obtained (Figure 3.8B) and the 30 kDa protein was identified as glyoxalase II following searches of the protein sequence database (Ridderstrom et al, 1996). An alignment of the peptide sequences and the human liver glyoxalase II sequence is shown (Figure 3.8B). The peptides were between 75% and 100% identical to the equivalent sequences in the human liver protein. This protein is 260 amino acids in length and has a predicted molecular mass of 29.4kDa and pI of 7.46.

Glyoxalase II is the second enzyme of the glyoxalase system which catalyses a two stage reaction in which methylglyoxyl is converted to D-lactic acid via the intermediate S-D-lactoylglutathione in a glutathione-dependent fashion. The glyoxalase II sequence was available in the form of Expressed Sequence Tag (EST) cDNA clones and this allowed a recombinant p30 to be produced (cloned by C.Monfries). Attempts to purify GST-p30 were of limited success; the fusion protein was very unstable and the low level of the full-length product obtained hindered further analysis of the putative chimaerin-glyoxalase interaction.

A.

B.
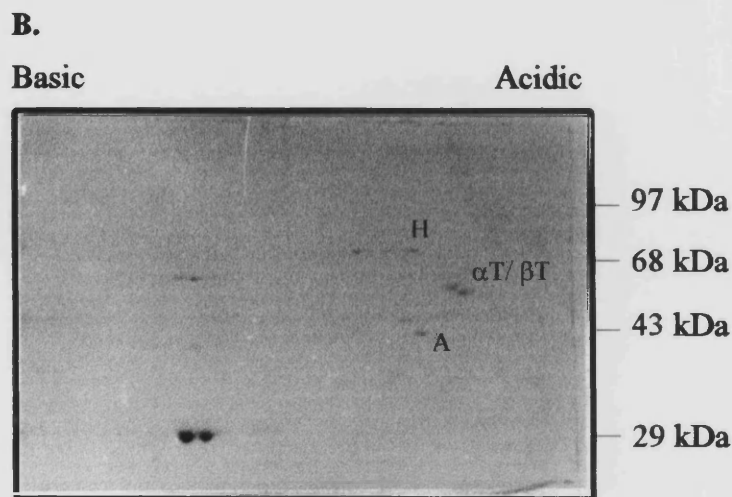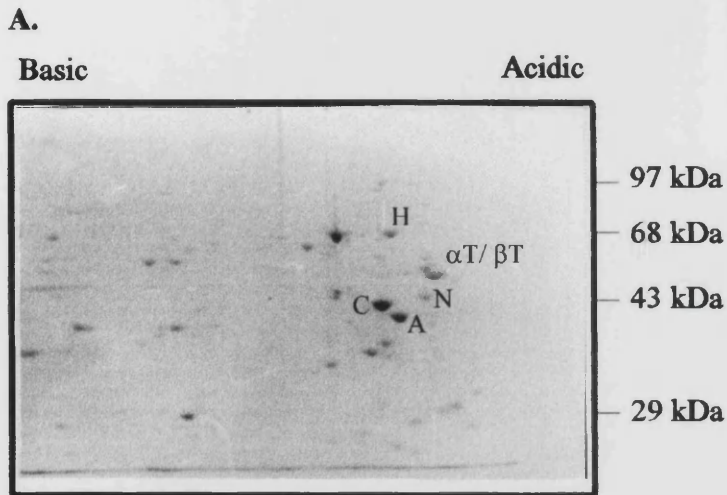
**Figure 3.7: Two-Dimensional SDS-PAGE Analysis of the Proteins Bound to a
Chimaerin SH2 Domain Affinity Column**

Rat brain proteins associating with a chimaerin SH2 domain affinity column were eluted with
1M NaCl and 25% of the total eluant analysed by 2-D SDS-PAGE (Figure B). A sample of
rat brain extract containing approximately 100µg total protein was also analysed (Figure A).
Separation in the first dimension, by isoelectric focusing, was over the range pH 3.6-10
(shown horizontally); the acidic and basic ends were as indicated. The separation in the
second dimension was by SDS-PAGE (shown vertically). The position of certain known
proteins are indicated: α and β-tubulin (αT and βT), actin (A), hsp70 (H), Neuron specific
enolase (N) and Creatine Kinase (C).

```
Peptide 1        QTTLTTHHH
Peptide 2        VVEPGNTAVQEK
Peptide 3        VTHLSTLQVGXLXVK
Peptide 4        LAWAK
Peptide 5        VYTGHEYTVNNLK
```

1

MKVEVLPALTDNYMYLVIDDETKEAAIVDPVQPQKVVDAARKHGVKL**TTV**

QTT

51

LTTHHHWDHAGGNEKLVKLESGLKVYGGDDRIGALTHK**I**THLSTLQVGSL

LTTHHH   VTHLSTLQVGXL

101

NVKCLATPCHTSGHICYFVSKPGGSEPPAVFTGDTLFVAGCGKFYEGTAD

XVK

151

EMCKALLEVLGRLPPDTR**VYCGHEYTINNLK**FARH**VEPGNAA**IREK**LAWA**

VYTGHEYTVNNLK   VVEPGNTAVQEK**LAWA**

201

KEKYSIGEPTVPSTLAEEFTYNPFMRVREKTVQQHAGETDPVTTMRAVRR

K

251   260

EKDQFKMPRD

Figure 3.8: A Potential Target of the α2 Chimaerin SH2 Domain, Glyoxalase II, is Identified on the Basis of Peptide Sequence Information

A 30 kDa potential target protein of the chimaerin SH2 domain was purified from rat brain extract by affinity chromatography and tryptic peptides derived from the protein sequenced using the Edman degradation method. Five peptide sequences were obtained, as shown. A search of the database with these sequences led to the identification of the 30kDa protein as Glyoxalase II. An alignment of the peptides with the human liver Glyoxalase II sequence is shown.

Any binding protein purified by affinity chromatography may be associating with column indirectly and at this time there is no definitive data supporting a direct interaction between the chimaerin SH2 domain and glyoxalase II. Several different approaches have been used to clarify this point, including the unsuccessful use of MBP-α2 SH2 domain affinity columns to purify glyoxalase II and the use of partly-purified glyoxalase II (obtained commercially from Sigma) columns to purify native α2-chimaerin, but no unequivocal data has been obtained and whether glyoxalase II interacted specifically with α2 chimaerin has not been established.

3B The Use of Non-Covalent Affinity Columns to Identify Potential Target Proteins

Although covalent linkage of a protein to an affinity matrix permits the use of stringent washing and eluting conditions, the physical constraints imposed by the interaction of the 'bait' protein with the matrix may impede the binding of target proteins. Furthermore, to produce an affinity column, it is necessary to purify the fusion protein using glutathione agarose beads, dialyse, concentrate and re-bind to the activated affinity agarose. If the fusion protein loses its structural integrity during this handling it may interact sub-optimally with potential target proteins. During purification of GST-α2 chimaerin SH2 domain significant breakdown of the full length fusion protein was observed routinely between the purification of the protein on glutathione agarose beads and the final elution and concentration of the protein. It was decided to directly utilise the freshly-purified glutathione agarose-associated protein as an affinity column.

3B.1 Small-Scale Non-Covalent Affinity Columns Can Select Binding Proteins from Rat Brain Extract

To determine whether the use of affinity columns with non-covalently rather than covalently-linked 'bait' protein facilitated the purification of α2 chimaerin target proteins, a small scale experiment was performed using approximately 50μg GST-α2 SH2 domain associated with around 25μl bed volume of glutathione agarose (GA) to select interacting protein from rat brain extract (prepared in 0.05% Triton-X-100). A GST column was used as a control. The protocol used for analytical scale affinity chromatography is described in Section 2B.29B. Proteins binding the columns were eluted in Protein Sample Buffer and were analysed by

142
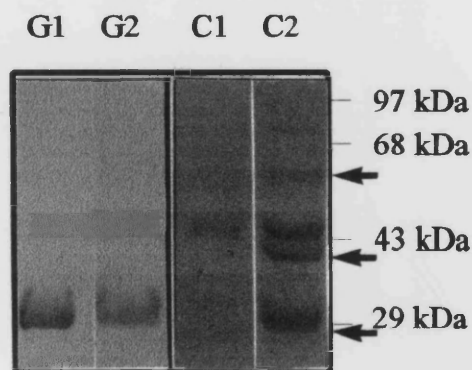
Figure 3.9: The Purification of Rat Brain Proteins on Small-Scale Glutathione Agarose Affinity Columns

1ml of rat brain extract (prepared with 0.05% Triton-X-100) was incubated with glutathione agarose-bound GST (sample set G) or GST-α2 chimaerin SH2 domain (sample set C). Approximately 25μl of glutathione agarose with a protein concentration of around 1mg/ml was used. The resin was washed and the fusion protein and any bound proteins eluted in Protein Sample Buffer. The samples were analysed by SDS-PAGE and proteins visualised with Coomassie Blue. The protein content of the eluants (lanes G2 and C2) was compared to that of the fusion protein on the column (G1 and C1). Proteins of approximately 30, 40 and 55 kDa bound the chimaerin column and are indicated by arrowheads.

SDS-PAGE in parallel with aliquots of the GA-GST and GA-GST-$\alpha2$ SH2 domain preparations used in the experiment (Figure 3.9).

Little protein associated with the GST column, whereas the chimaerin SH2 domain column bound proteins of approximately 30 kDa, 40 kDa and 55 kDa (lane C2). These are indicated by arrowheads in the figure. Similar quantities of the first two proteins were present in the sample relative to the amount of fusion protein present whereas the level of the 55 kDa protein was somewhat lower. Despite the small scale of this experiment, large quantities of proteins relative to the amount of 'bait' fusion protein were purified and the next step was to attempt a larger scale purification.

3B.2 The Purification of Target Proteins by Affinity Chromatography on Glutathione Agarose Columns

A large-scale purification of potential target proteins of the chimaerin SH2 domain using glutathione agarose (GA) affinity columns was performed so that sufficient protein material could be obtained for peptide sequencing. Rat brain extract (prepared in 0.05% Triton-X-100) was applied to a chimaerin SH2 domain affinity column and a GST control column and bound proteins eluted with a stepwise salt gradient prior to stripping the fusion protein and any tightly associated proteins by treatment with 0.5% SDS. 5% of the total protein bound to each column was analysed by SDS-PAGE(GST-binding, Figure 3.10A and chimaerin-binding, Figure 3.10B). An aliquot of the untreated column matrix (GST, lane G and chimaerin SH2 domain, lane C) was also analysed for comparison with the proteins present in the SDS elutions (A and B, lane 7).

No proteins were detected in the salt elutions (0.1M-1.0M) from either the GST or the chimaerin column using Coomassie Blue stain. However, a protein of around 55 kDa specifically bound the chimaerin column and was eluted with SDS. The amount of this protein was approximately half that of the fusion protein in the sample. Further bands were also seen, most notably in the 30kDa region; one of these proteins may be glyoxalase II. Alternatively, these may be the product of fusion protein breakdown. Any other bands detected were faint.

To examine the proteins that were associating with the chimaerin column in smaller amounts, the different salt elutions were combined and this and the SDS-eluted sample were

A.

B.
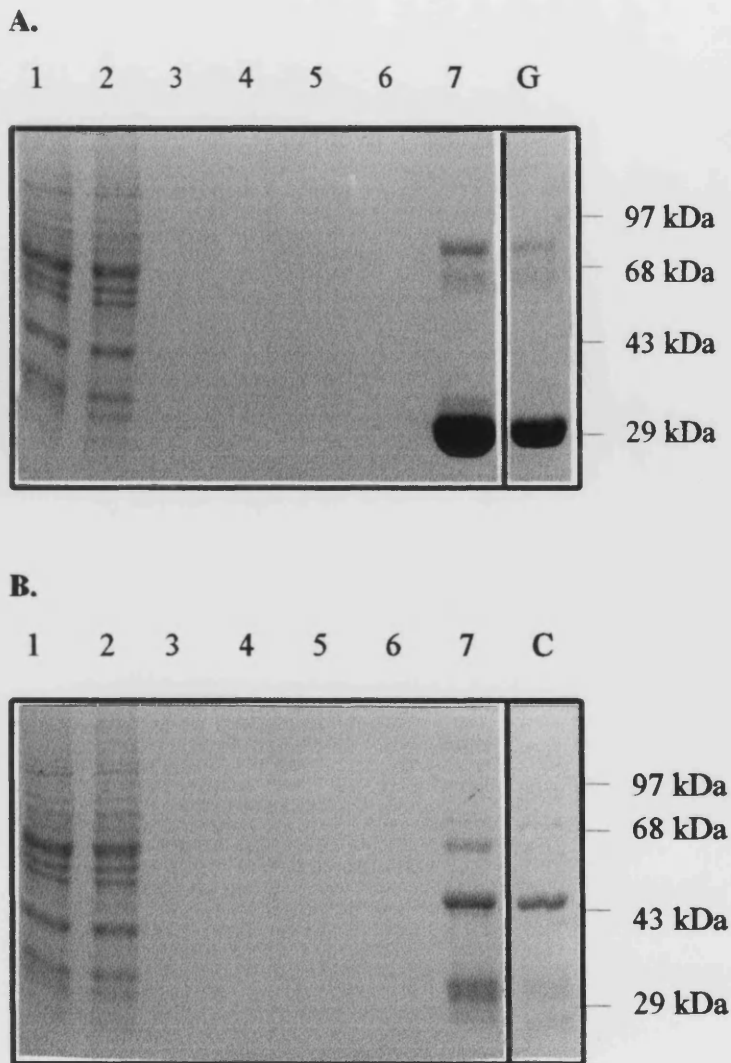
Figure 3.10: The Isolation of α2 Chimaerin SH2 Domain Target Proteins from Rat Brain Extract by Affinity Chromatography on Glutathione Agarose Columns

90ml of rat brain extract (prepared with 0.05% Triton-X-100, lane 1) was applied to GST (Figure A) and GST α2 chimaerin SH2 domain (Figure B) affinity columns. In each case there was approximately 1mg of fusion protein associated with a glutathione agarose matrix. The flow through was collected (lane 2) and bound proteins eluted with 0.1M NaCl (lane 3), 0.2M NaCl (lane 4), 0.5M NaCl (lane 5), 1M NaCl (lane 6) and 0.5% SDS (lane 7). 5% of each eluant was analysed by SDS-PAGE and proteins were stained with Coomassie Blue; an aliquot of the fusion protein used to prepare the affinity column was also tested (GST, lane G and GST-chimaerin SH2 domain, lane C).

further concentrated such that up to 50% of the total could be analysed at any one time. The samples of proteins eluted from the GST column were treated in the same manner for purposes of comparison. Coomassie staining of the concentrated samples identified two proteins, one of approximately 100 kDa (p100) present in the combined salt fraction, and one of higher molecular mass, labelled p200, present in the SDS eluant, that appeared to bind specifically to the chimaerin column. The appropriate bands were excised and sent for peptide sequencing. Unfortunately the quantities of proteins available were very low and hence the peptide data that could be obtained was limited, especially in the case of the p200. Four peptides from the p100 and one from the p200 were sequenced. The peptide sequences are shown in Figures 3.11A and B respectively. When the protein sequence database was searched with the sequences no proteins were identifed whose apparent molecular corellated with either p100 or p200 and hence these proteins are presumed to be novel.

3B.3 Tubulin and Actin Bind a GST-α2 Chimaerin SH2 Domain Affinity Column

Proteins of approximately 40kDa and 55kDa were found routinely to preferentially associate with chimaerin SH2 domain affinity columns. The apparent molecular masses of these proteins indicate that they could be actin and tubulin respectively, both of which were shown previously to be present in samples bound to an Affigel column (Figure 3.7). A set of samples from an affinity purification of rat brain proteins on GA-GST or GA-GST-α2 chimaerin SH2 domain columns equivalent to that shown in Figure 3.10, was transferred to nitrocellulose and probed with either anti-actin (Figure 3.12) or anti-β-tubulin (Figure 3.13). The results indicate that both actin and tubulin are present amongst the proteins eluted from the chimaerin column with SDS, but are not present in any of the samples eluted from the GST column. Again it is not known whether these interactions are direct.

3C The Use of Filter Binding Assays to Identify Targets of the α2-Chimaerin SH2 Domain

Radioactively-labelled proteins were used to probe rat tissue extract proteins immobilised on nitrocellulose filters to identify binding proteins. If any particular tissue extract, whole or fractionated, contained a high level of α2 chimaerin binding activity, this would be a suitable starting material for a purification procedure. The pGEX-2TK bacterial expression

p100 Peptide Sequences:

Peptide 1   XLVQPTYGXG

Peptide 2   HAEYIAK

Peptide 3   ALLEGGSSSX

Peptide 4   IPEDGK

Protein Sequence	Percentage Identity to Protein Sequence in the Database			
	Peptide 1	Peptide 2	Peptide 3	Peptide 4
Inositol 1,4,5-triphosphate receptor 1 (rat)	82.4	76.5	51.7	53.5
Inositol 1,4,5-triphosphate receptor 1 (human)	82.4	76.5	51.7	53.5
Dynein Heavy Chain, Cytosolic	82.4	44.1	65.5	60.5
Titin, Cardiac Muscle Human (human)	79.4	58.8	65.5	44.2

Figure 3.11A: Peptide Sequences Obtained from 100kDa Protein Purified on an α2 Chimaerin SH2 Domain Affinity Column

p200 Peptide Sequences:

Peptide 1   XLVVKSA

Protein Sequence	Percentage Identity to Protein Sequence in the Database	
	Peptide 1	
Cytosolic Thymidine Kinase (rat, mouse, human)	100	
Rho-associated Kinase α mRNA (Xenopus Laevis)	93.5	
Ten Different C. elegans Cosmid Clones	93.5	

Figure 3.11B: Peptide Sequence Obtained from 200kDa Protein Purified on an α2 Chimaerin SH2 Domain Affinity Column

Two potential target proteins of the α2 chimaerin SH2 domain, one of approximately 100 kDa and the other 200 kDa (p100 and p200 respectively) were purified by affinity chromatography. The peptide sequences obtained from the p100 are shown in Figure A and from the p200 in Figure B. An 'X' in the sequence indicates that it was not possible to identify the amino acid residue at this position. The peptide sequences were used to search the protein sequence database and the best matches are detailed in the tables in Figures A and B.
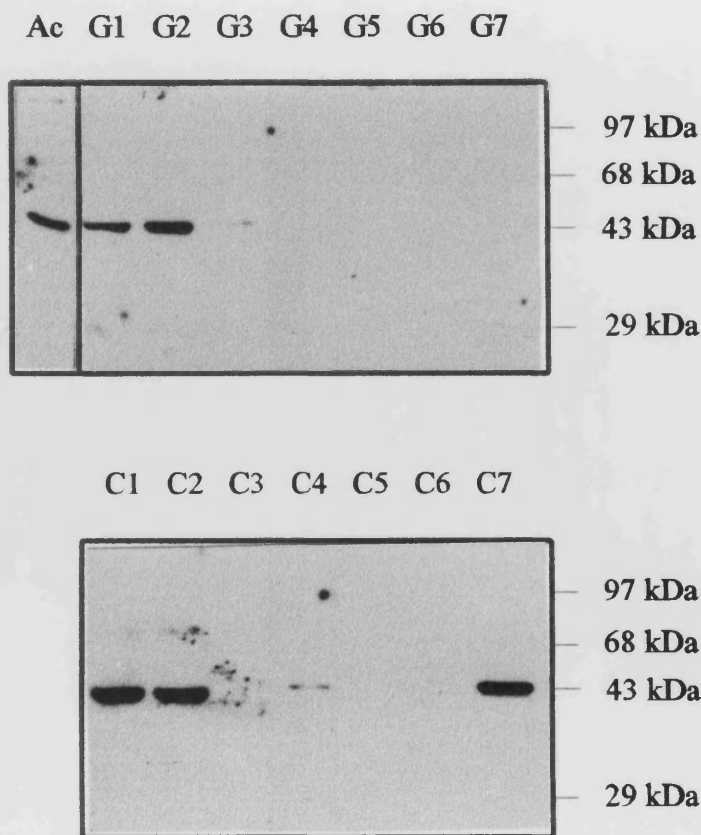
Figure 3.12: Actin Binds a Chimaerin SH2 Domain Affinity Column

Rat brain extract was applied to GST (sample set G) and GST-α2 chimaerin SH2 domain (sample set C) glutathione agarose affinity columns. Bound proteins were eluted with 0.1M NaCl (lanes G3 and C3), 0.2M NaCl (lanes G4 and C4), 0.5M NaCl (lanes G5 and C5), 1.0M NaCl (lanes G6 and C6) and 0.5% SDS (lanes G7 and C7), separated by SDS-PAGE and transferred to nitrocellulose filters. The immobilised proteins were probed with a mouse monoclonal anti-actin antibody (Amersham, diluted 1/1000). Brain extract (lanes G1 and C1) and flow through samples (lanes G2 and C2) containing approximately 50µg total protein were also tested. 1µg of monomeric actin (Ac) was tested as a control.
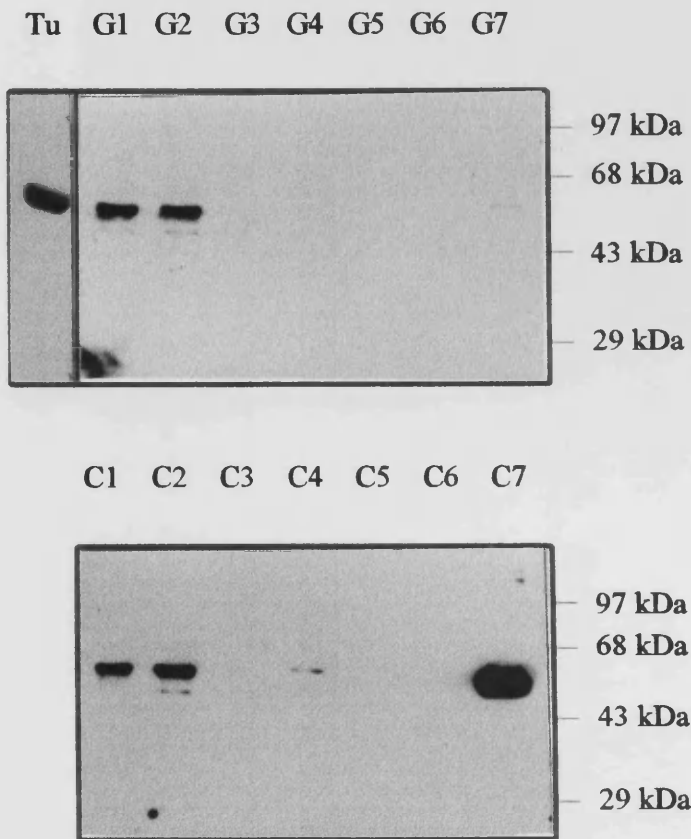
Figure 3.13: Tubulin Binds a Chimaerin SH2 Domain Affinity Column

Rat brain extract was applied to GST (sample set G) and GST-α2 chimaerin SH2 domain (sample set C) glutathione agarose affinity columns. Bound proteins were eluted with 0.1M NaCl (lanes G3 and C3), 0.2M NaCl (lanes G4 and C4), 0.5M NaCl (lanes G5 and C5), 1.0M NaCl (lanes G6 and C6) and 0.5% SDS (lanes G7 and C7), separated by SDS-PAGE and transferred to nitrocellulose filters. The immobilised proteins were probed with a mouse monoclonal anti-β tubulin antibody (Sigma, diluted 1/5000). Brain extract (lanes G1 and C1) and flow through samples (lanes G2 and C2) containing approximately 50μg total protein were also tested. 5μg of tubulin (Tu) was used as a control.

vector encodes a Protein Kinase A phosphorylation site and the α2 chimaerin SH2 domain sequence was cloned into this vector (by C.Monfries) to allow the fusion protein to be labelled with γ^{32}P-ATP to a high specific activity. The chimaerin sequence included forty-eight residues amino-terminal to the SH2 domain (Appendix 1). The fusion protein was radio-labelled whilst associated with glutathione agarose beads as described in Section 2B.33A. A sample of the labelled protein was analysed by SDS-PAGE (Figure 3.14A) and it was found that a 30kDa breakdown product, presumed to be GST, was also present. The protocol used for the binding assays described in this chapter involved blocking any unbound sites on the filter with a complex mixture of bacterial proteins, including GST (GST Blocking Buffer, see Section 2B.34A). GST Blocking Buffer was also present during the hybridisation of probe to the filter and hence any binding of labelled GST to the bound proteins should be competed by protein in the buffer. Indeed, when a GST probe was tested, it did not detect any binding proteins in rat brain extract (data not shown).

3C.1 Analysis of α2 Chimaerin Binding Proteins in Different Rat Tissue Extracts

α2-chimaerin is highly expressed during development it is possible that its *in vivo* targets are similarly expressed and therefore adult rat brain may not be the most appropriate source of binding proteins. Brain extracts were prepared from rats of different ages, ranging from one day to adult and extracts were also obtained from testis and small intestine, two non-neuronal tissues in which α2-chimaerin is expressed (Hall et al, 1993). 25μl of each of these extracts (prepared from a 20% w/v homogenate) were separated by SDS-PAGE, transferred to nitrocellulose filters and probed with ^{32}P-GST-α2 chimaerin SH2 domain. Binding activity was detected by autoradiography (Figure 3.14).

A binding activity of approximately 55 kDa is detected in all the tissue samples except the small intestine (lane 15); data from previous experiments suggest that this may be tubulin. Other than this no further distinct interactions were observed, with the possible exception of some faint activity in the 70-80kDa region in the 1% Triton-X-100 extract of fourteen day old rat brain. The results did not suggest that use of rat brain younger than adult would lead to dramatic improvement in the purification of target proteins of chimaerin SH2 domain.

Figure 3.14: The Detection of Proteins in Rat Tissue Extracts that Bind the Chimaerin SH2 Domain Using a Filter Binding Assay

A.

GST-α2 chimaerin SH2 domain was phosphorylated by the catalytic subunit of PK-A in the presence of γ^{32}P-ATP. The probe was analysed by SDS-PAGE and visualised by autoradiography.

B.

Rat tissue extracts containing approximately 100μg total protein were separated by SDS-PAGE and transferred to nitrocellulose filters, denatured and slowly renatured prior to probing with radiolabelled GST-α2 chimaerin SH2 domain. The rat tissue extracts tested were:

Lane 1, 12 hours post-natal brain extracted in 0.05% Triton-X-100

Lane 2, 1 day post-natal brain

Lane 3, 1 day post-natal brain extracted in 1% Triton-X-100

Lane 4, 7 day post-natal brain

Lane 5, 7 day post-natal brain extracted in 1% Triton-X-100

Lane 6, 11 day post-natal brain

Lane 7, 11 day post-natal brain extracted in 1% Triton-X-100

Lane 8, 14 day post-natal brain

Lane 9, 14 day post-natal brain extracted in 1% Triton-X-100

Lane 10, 21 day post-natal brain extracted in 0.05% Triton-X-100

Lane 11, 30 day post-natal brain extracted in 0.05% Triton-X-100

Lane 12, adult brain

Lane 13, adult brain extracted in 1% Triton-X-100

Lane 14, adult testis extracted in 0.05% Triton-X-100

Lane 15, adult intestine extracted in 0.05% Triton-X-100.

If no Triton-X-100 concentration has been given, the extraction buffer did not contain detergent.

A.
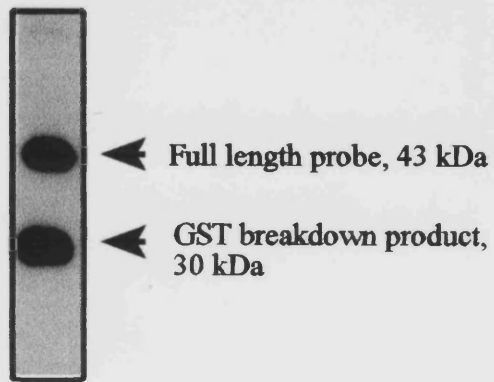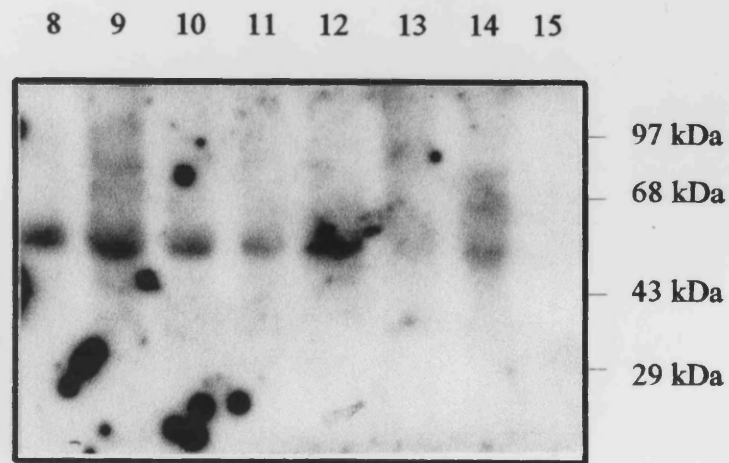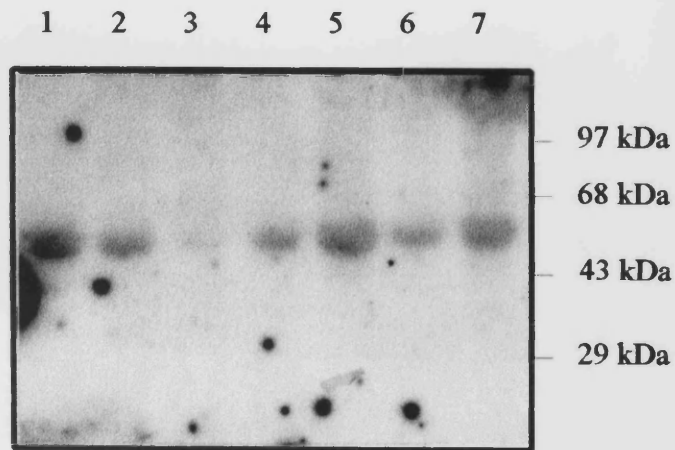
Full length probe, 43 kDa

GST breakdown product, 30 kDa

B.

1   2   3   4   5   6   7

97 kDa
68 kDa
43 kDa
29 kDa

8   9   10 11 12 13 14 15

97 kDa
68 kDa
43 kDa
29 kDa

153

Conventional protein purification protocols rely on a series of protein separations on the basis of such characteristics as ionic charge, hydrophobicity or molecular mass. The enrichment of a particular protein is monitored by biochemical assay at each step. The quantity of protein selected from rat brain extracts by affinity columns has generally been very low and therefore *in vitro* binding assays were used to identify different fractions of rat brain extract that contained α2-chimaerin SH2 domain binding proteins with the intention that these could then be purified further by affinity chromatography.

Rat brain extract, prepared in 0.05% Triton-X-100, was fractionated on either an S Sepharose (Figure 3.15) or Q Sepharose Column (Figure 3.16). Bound proteins were eluted with increasing concentrations of salt in a stepwise manner (0.1M to 0.5M in 0.1M increments). The unfractionated extract, the flow-through on the appropriate column and the eluants were analysed by SDS-PAGE and proteins visualised with Coomassie Blue (Figures 3.15A and 3.16A). Two duplicates of each set of samples were also electrophoresed and transferred to nitrocellulose and the filters probed with either γ^{32}P-GST-α2 SH2 domain (Figures 3.15B and 3.16B) or anti-β-tubulin (Figures 3.15C and 3.16C).

The chimaerin probe detected a protein of approximately 55kDa and, more weakly, two others of 35kDa and 40 kDa in rat brain extract used for the fractionation on S Sepharose but the same set of signals was seen in the flow-through from this column and the S Sepharose fractions themselves did not contain any detectable chimaerin binding activities. That the 55kDa protein detected in the brain extract and flow through samples might be tubulin was indicated by the results of the Western blot in Figure 3.15C: the vast majority of tubulin in rat brain extract is not retained on an S Sepharose column and its mobility during SDS-PAGE appears similar to that of the protein detected in the binding assay. Several different binding activities were detected in the Q Sepharose fractions, most prominently in the 0.2M NaCl eluant where bands of approximately 35kDa (also seen in the 0.3M NaCl eluant), 40kDa and 97kDa were seen. A strong signal at around 55 kDa was present in the 0.4M NaCl eluant; when the same fractions were probed with anti-β-tubulin it was found that the majority of the tubulin bound to the column was eluted at this salt

Figure 3.15: The Identification of Potential Targets of the α2 Chimaerin SH2 Domain in Rat Brain Extract Fractionated on S Sepharose.

50 ml rat brain extract (prepared in 0.05% Triton-X-100) was loaded onto a 2.5ml S Sepharose column and the flow through collected. The column was washed and proteins eluted with a stepwise salt gradient (two column volumes). 20µl samples were separated by SDS-PAGE and proteins visualised with Coomassie Blue stain (Figure A) or transferred to nitrocellulose and probed with either ^{32}P-labelled GST-α2 chimaerin SH2 domain (Figure B) or anti β-tubulin (Sigma, diluted 1/5000; Figure C). The samples tested were:

(Ex), rat brain extract

(FT), the unbound material

(1), the 0.1M NaCl elution

(2), the 0.2M NaCl elution

(3), the 0.3M NaCl elution

(4), the 0.4M NaCl elution

(5), the 0.5M NaCl elution
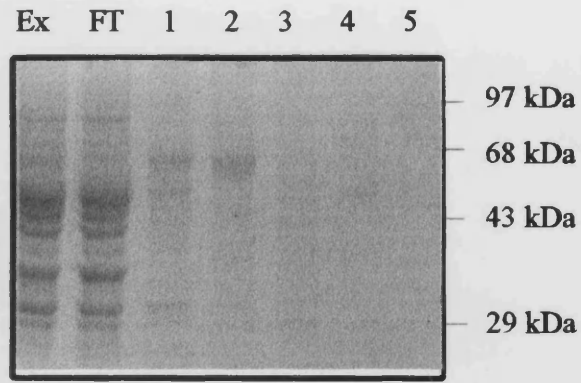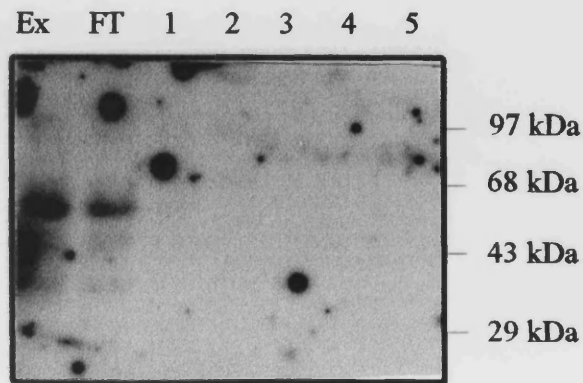
Figure 3.16: The Identification of Potential Targets of the α2 Chimaerin SH2 Domain in Rat Brain Extract Fractionated on Q Sepharose.

50 ml rat brain extract (prepared in 0.05% Triton-X-100) was loaded onto a 2.5ml Q Sepharose column and the flow through collected. The column was washed and proteins eluted with a stepwise salt gradient (two column volumes). 20μl samples were separated by SDS-PAGE and proteins visualised with Coomassie Blue stain (Figure A) or transferred to nitrocellulose and probed with either ^{32}P-labelled GST-α2 chimaerin SH2 domain (Figure B) or anti β-tubulin (Sigma, diluted 1/5000; Figure C). The samples tested were:

(Ex), rat brain extract
(FT), the unbound material
(1), the 0.1M NaCl elution
(2), the 0.2M NaCl elution
(3), the 0.3M NaCl elution
(4), the 0.4M NaCl elution
(5), the 0.5M NaCl elution

A.

Ex FT 1 2 3 4 5

97 kDa
68 kDa
43 kDa
29 kDa

B.

Ex FT 1 2 3 4 5

97 kDa
68 kDa
43 kDa
29 kDa

C.

Ex FT 1 2 3 4 5

97 kDa
68 kDa
43 kDa
29 kDa
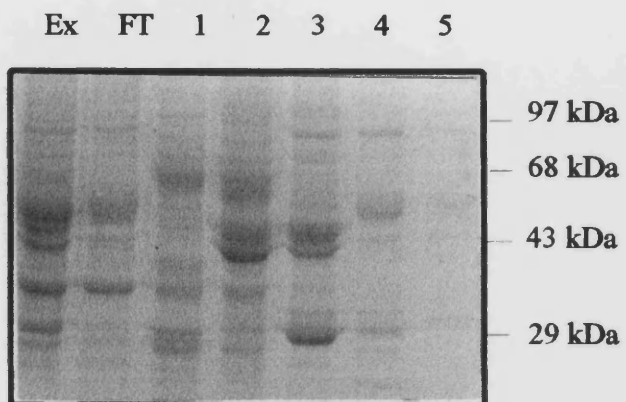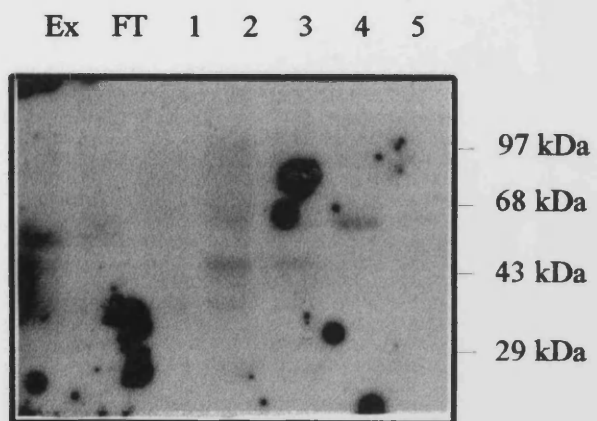
concentration.

3D Summary

Three potential targets of the α2 chimaerin SH2 domain have been affinity-purified from rat brain extract. One of these, a 30kDa protein, has been identified from its peptide sequence as glyoxalase II, but attempts to demonstrate a direct interaction with α2 chimaerin *in vitro* have not been successful to date. The peptide sequences obtained for the two other potential targets, p100 and p200, did not match any others in the database and, at the present time, these proteins are presumed to be novel.

Actin and tubulin have been shown to bind α2 chimaerin SH2 domain columns and results from filter binding assay studies suggested that tubulin interacted directly with the α2-chimaerin SH2 domain. Attempts to identify more abundant sources of target proteins of the chimaerin SH2 domain by probing rat brain proteins that had been fractionated on either Q or S Sepharose with radio-labelled α2-chimaerin indicated that Q Sepharose 0.2M NaCl fraction of rat brain extract contained the highest level of detectable chimaerin binding activities, including proteins of approximately 35 kDa, 40 kDa and 97 kDa.

CHAPTER FOUR

RESULTS II

Investigation of the Enzyme Activities Associated with Chimaerin SH2 Domain Affinity Columns

SH2 domains bind specific phosphotyrosine-containing peptide sequences and the expression of a chimaerin splice variant with an SH2 domain could place this protein within kinase signalling pathways. If $\alpha 2$ chimaerin interacts with a kinase, either directly or indirectly, it would be possible to attempt purification of the kinase by following its activity through multiple chromatographic steps. Chimaerin may also be a substrate for phosphorylation; if this were so, it may serve to regulate chimaerin activity. Identification of the optimal starting material and use of an affinity column as the final step of the purification procedure would maximise the protein yield which might then allow sufficient peptide sequence information to be obtained to permit identification of the kinase. Protein kinase activity may be dependent upon phosphorylation. To preserve the phosphorylated state of the native proteins, rat brain extracts were prepared in buffer containing phosphatase inhibitors.

4A Analysis of the Kinase Activities Selected by the $\alpha 2$ Chimaerin SH2 Domain

4A.1 An $\alpha 2$-Chimaerin SH2 Domain Column Can Bind Kinases

To determine whether $\alpha 2$ chimaerin bound a kinase, rat brain proteins selected by affinity chromatography were tested for *in vitro* kinase activity. Brain extract was applied to a GST-$\alpha 2$ chimaerin SH2 domain Affigel affinity column as described in Section 3A.1 and the bound proteins eluted with an increasing salt gradient. GST and GST-abl SH2 domain columns were used as controls. 5% of the total protein eluted from each column at 0.1M NaCl, 0.2M NaCl, 0.5M NaCl and 1.0M NaCl was assayed for *in vitro* kinase activity in the manner detailed in Chapter 2. Approximately 2μg of full length $\alpha 2$ chimaerin (cleaved from the GST moiety of the full length fusion protein) was added to each reaction mix as a potential substrate. The results are shown in Figure 4.1. The samples contain a mixture of proteins, possibly including both a kinase and its substrate, and hence the signals seen may indicate either kinase autophosphorylation or phosphorylation of substrates. Serine/threonine and tyrosine kinases can be detected using this technique.

Kinases which phosphorylate the chimaerin substrate, which has an apparent molecular mass of 43 kDa, bound each column. Phosphorylation of this protein is indicated
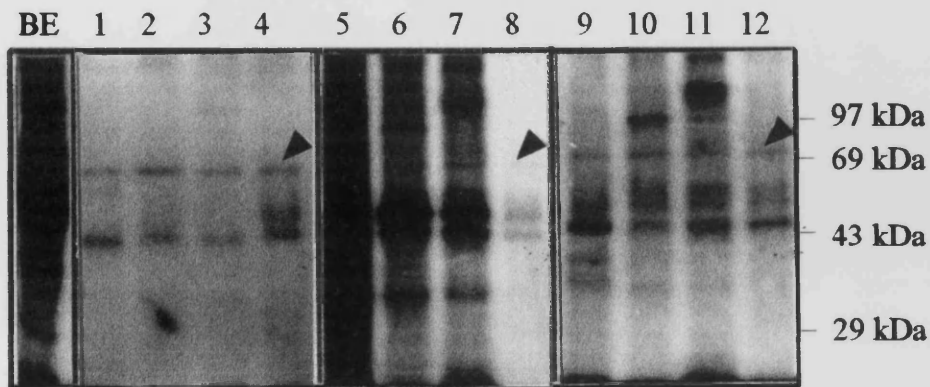
Figure 4.1: Kinases Bind A Chimaerin SH2 domain Affinity Columns

Rat brain extract was applied to GST (lanes 1-4), GST-abl SH2 domain (lanes 5-8) and GST-α2 chimaerin SH2 domain (lanes 9-12) affinity columns. Bound proteins were eluted sequentially with 0.1M NaCl (lanes 1, 5, 9), 0.2M NaCl (lanes 2, 6, 10), 0.5M NaCl (lanes 3, 7, 11) and 1.0M NaCl (lanes 4, 8, 12). 5% of each eluant was assayed for *in vitro* kinase activity. A sample of rat brain extract (BE) containing approximately 40µg total protein was also tested. 2µg of recombinant full-length α2 chimaerin was added to each reaction as a potential kinase substrate and its phosphorylation is indicated in the Figure using arrowheads.

by an arrow head to the left of the figure. There was a further band at 69 kDa which may represent phosphorylation of full-length GST-α2 chimaerin, present in low levels in the cleaved protein preparation, or phosphorylation of a bacterial protein present as a contaminant in the protein preparation. The level of kinase activity bound to the GST column (lanes 1-4) was relatively low and little phosphorylation other than that of the chimaerin substrate occurred. In each of the samples from the abl SH2 domain column (lanes 5-8) and the chimaerin SH2 domain column (lanes 9-12) multiple phosphorylated bands were seen. The overall level of phosphorylation in the abl SH2 domain-bound proteins was higher than in the chimaerin SH2 domain-bound proteins. The results indicate that, relative to a GST control, the chimaerin SH2 domain can preferentially select one or more kinase activities from rat brain extract.

4A.2 Analysis of the Associated Kinase Activities by In-Gel Kinase Assay

In order to establish the apparent molecular masses of the kinases binding to the α2 chimaerin SH2 domain, rat brain protein samples obtained by affinity chromatography were assayed for in-gel kinase activity in the manner described in Chapter 2. It is possible to determine the apparent molecular mass of a kinase using this technique because the mixture of proteins present in the samples is separated by SDS-PAGE prior to the kinase assay and hence any signals seen represent kinase autophosphorylation unless kinase and substrate exactly co-migrate. If the assumption is made that the latter is improbable then this technique allows estimation of the size of the kinase. Again, GST-binding and GST-abl SH2 domain-binding proteins were used as controls. Each set of samples used was equivalent to those described in sections 3A.1 and 4A.1. Exogenous kinase substrates, myelin basic protein (MBP, Figure 4.2A) or full-length α2 chimaerin (Figure 4.2B), were polymerised into the gel to expand the detectable range of kinase activities. Proteins such as receptor tyroine kinases which require oligomerisation for activity will not be detected under the conditions used and this technique is generally used to monitor the activity of serine/threonine kinases.

No kinases active in this assay were detected amongst the proteins purified on the GST column (lanes 1-4, Figures 4.2A and 4.2B) but both the abl and chimaerin SH2 domain columns (lanes 4-8 and 9-12 respectively, Figures 4.2A and 4.2B) did bind kinases that could phosphorylate either MBP or α2 chimaerin. The chimaerin column bound a kinase of

Figure 4.2: Rat Brain Proteins Binding Affinity Columns Assayed for In-Gel Kinase Activity

Rat brain extract (prepared with 0.05% Triton-X-100) was applied to GST (samples 1-4), GST-abl SH2 Domain (samples 5-8) and GST-α2 chimaerin SH2 domain (samples 9-12) affinity columns and bound proteins eluted with 0.1M NaCl (lanes 1, 5, 9), 0.2M NaCl (lanes 2, 6, 10), 0.5M NaCl (lanes 3, 7, 11) and 1M NaCl (lanes 4, 8, 12). Proteins present in brain extract (BE, approximately 40µg total protein) and 5% of the total protein in each eluant were separated by SDS-PAGE and assayed for in-gel kinase activity. The gels were prepared with 1mg/ml Myelin Basic Protein (Figure A) or 0.1mg/ml cleaved recombinant full length α2 chimaerin (Figure B) polymerised into the gel matrix as potential kinase substrates. Samples of proteins binding the GST-α2 chimaerin SH2 domain column were also assayed for in-gel kinase activity in the absence of added protein substrate (Figure C).
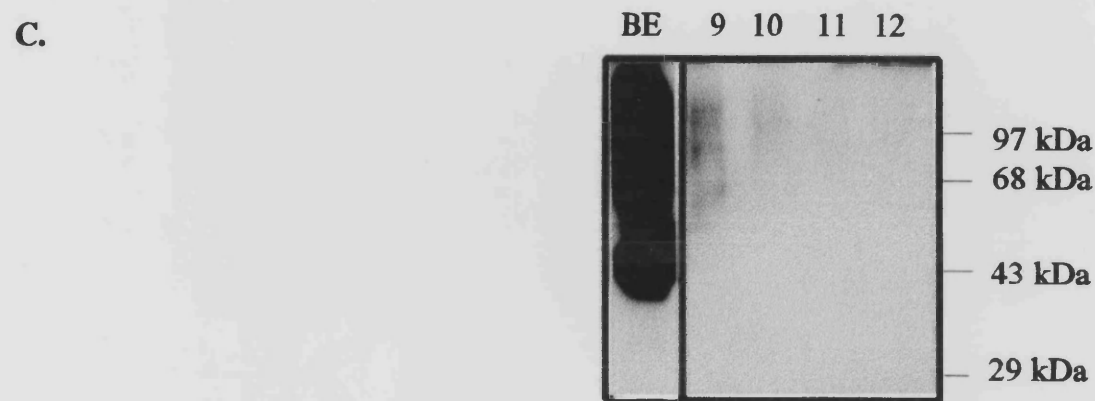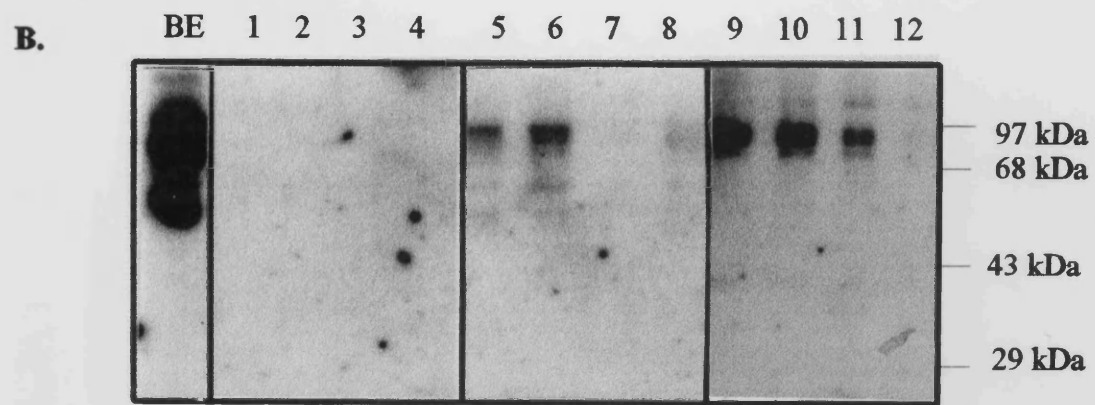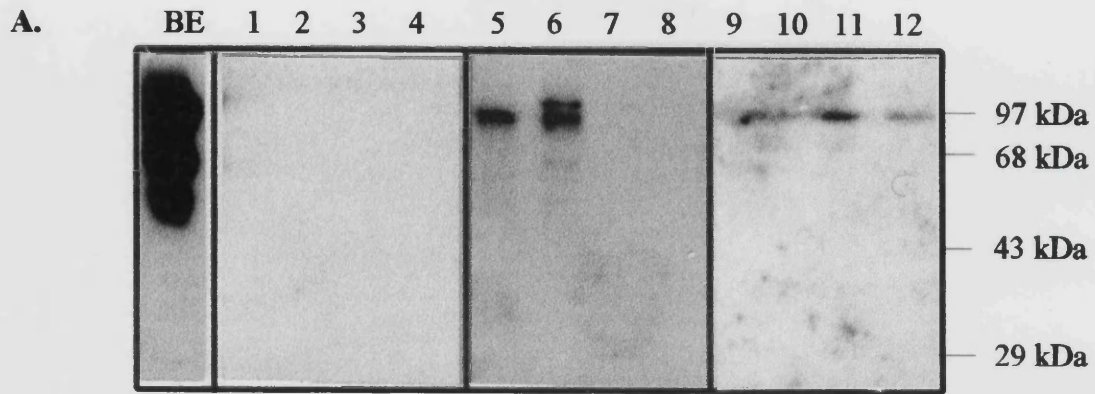
approximately 97 kDa that phosphorylated MBP and was present in the samples eluted with 0.2M NaCl (lane 10), 0.5M NaCl (lane 11) and 1.0M NaCl (lane 12) but the strongest activity was in the 80-95 kDa region towards the α2 chimaerin substrate; at least three bands were detected in this region. These kinase activities were present in the samples eluted with 0.1M NaCl, 0.2M NaCl and 0.5M NaCl. A kinase of higher molecular mass also bound the chimaerin column. It was present in both the 0.2M and 0.5M NaCl samples (lanes 10 and 11 respectively). A faint signal was seen at around 40kDa in the 0.1M NaCl (lane 9) and 0.2M NaCl (lane 10). The detection of chimaerin-binding kinases appeared to depend in part upon the presence of an appropriate substrate in the gel matrix. In the absence of substrate far lower levels of kinase activity were seen (lanes 9-12, Figure 4.2C). A number of very faint signals were observed in the proteins eluted with 0.1M and 0.2M NaCl (lanes 9 and 10) respectively. The predominant kinase activities detected in the samples eluted from the abl column were of similar apparent molecular mass to those present in the eluants from the chimaerin column although the actual pattern of bands observed differed. It should be noted that the treatment of the gels during the experiments caused considerable shape distortion and this complicates direct comparisons of the exact position of signals between gels. Furthermore, slight differences in the handling of the proteins samples may possibly have profound effects on kinase activity.

The processing involved in the production of a covalently-linked affinity column may result in a loss of enzymatic activity. Fusion proteins are affinity-purified on a glutathione agarose column and, rather than eluting, concentrating and freezing the protein, it can be used directly to affinity purify target proteins whilst associated with this resin. Proteins purified from rat brain extract on a GST-α2 chimaerin SH2 domain glutathione agarose (GA) affinity column were tested for in-gel kinase activity. A GA-GST column was used as a control and the sampled assayed in parallel. Figure 3.10 shows an equivalent set of samples that have been stained with Coomassie Blue. Gels either without added protein substrate (Figure 4.3A) or with full-length α2 chimaerin polymerised into the gel matrix were used in this experiment (Figure 4.3B). As previously, there is very little kinase activity associated with the GST column (lanes G1-G5) but the samples associated with the chimaerin column (lanes C1-C5) contained at least one highly active kinase of approximately 97 kDa that was capable of autophosphorylation. Since, in the experiments shown in Figure 4.2, no kinase
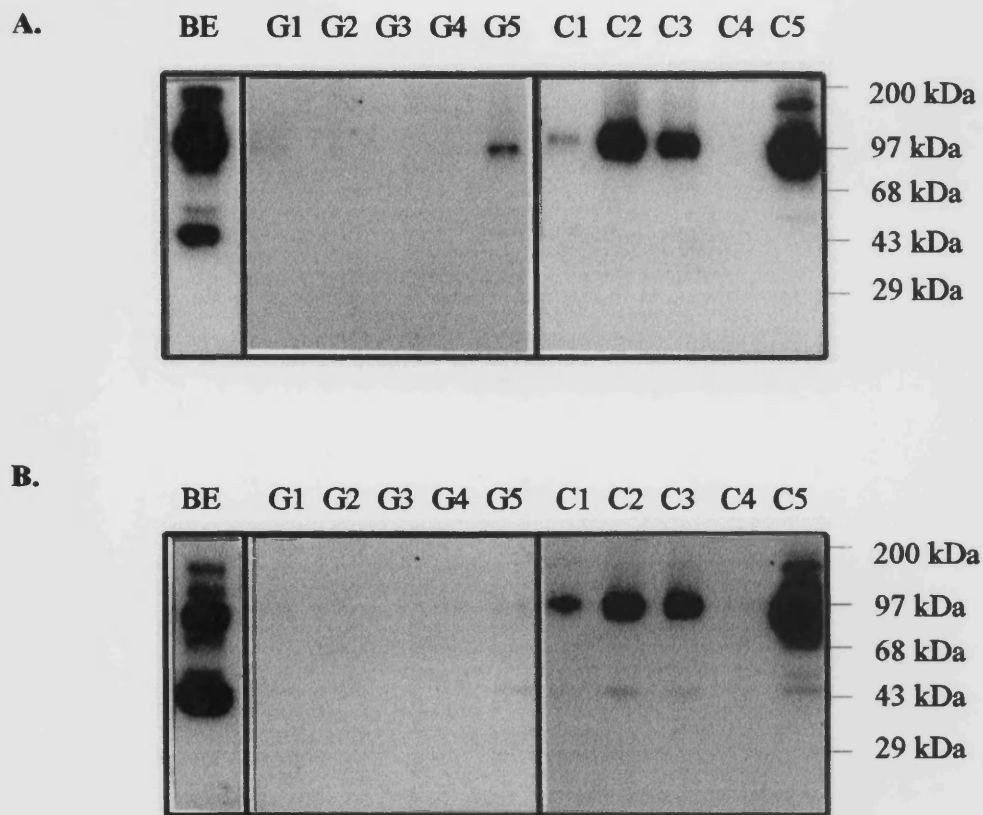
Figure 4.3: The In-Gel Kinase Activity of the Rat Brain Proteins Bound To Chimaerin SH2 Domain and GST Affinity Columns

Rat brain extract (prepared with 0.05% Triton-X-100) was applied to glutathione agarose-GST (sample set G) and GST-α2 chimaerin SH2 domain (sample set C) columns. Bound proteins were eluted with 0.1M NaCl (lanes G1 and C1), 0.2M NaCl (lanes G2 and C2), 0.5M NaCl (lanes G3 and C3), 1.0M NaCl (lanes G4 and C4) and 0.5% SDS (lanes G5 and C5). Approximately 40µg of rat brain extract (BE) and 5% of each eluant were assayed for in-gel kinase activity in the absence (Figure A) or presence (Figure B) of 0.1mg/ml full length recombinant α2 chimaerin (polymerised into the gel matrix).

167

activity was seen when a protein substrate was omitted from the gel, it may that this 97 kDa kinase is distinct from those of a similar size present in the samples purified on the Affigel affinity column. Alternatively, if the kinase were fully phosphorylated prior to the kinases assay, it is possible that autophosphorylation would not be detected in the assay. A kinase of approximately 43 kDa bound the chimaerin and was eluted predominantly in the 0.2M and 0.5M NaCl fractions (lanes C2 and C3). It was not detected in the absence of chimaerin substrate, although the signals obtained in the presence of α2 chimaerin were weak and the possibility of autophosphorylation cannot be excluded. Both the 43 kDa and the 97 kDa kinases were present in the SDS-eluant, indicating that a fraction of the total of each kinase bound associated with the column very tightly. A further kinase of high molecular mass (around 150 kDa) was also present in this sample.

The signal strength from the 97kDa kinase in this experiment would be sufficient to mask those produced by any weaker, less active kinases, and would obscure the results. An attempt to resolve these possibly different activities by two-dimensional SDS-PAGE separation prior to the in-gel kinase assay was unsuccessful; presumably the kinases do not renature sufficiently to regain activity following denaturation in 9M urea during the iso-electricfocussing step of 2D SDS-PAGE. Amongst the other kinase activities detected in the samples eluted from the chimaerin column was one with an approximate molecular mass of 43kDa that was detected only when α2 chimaerin was present as a substrate. However the signal was weak and it is possible that whilst this kinase can autophosphorylate it is present at such low levels that this reaction is below the limit of detection.

4B Further Purification of Kinase Activities

It was known that at least one kinase that was highly active in an in-gel assay could be purified on a GA-GST-α2 chimaerin SH2 domain affinity column but no protein of the appropriate size was seen when the samples were stained with Coomassie Blue, indicating that it must be present in very small amounts. This being so, it was necessary to develop a more involved purification strategy if sufficient protein were to be purified to permit peptide sequencing. Two approaches were used: the first was to test different fractions of rat brain extract to identify those enriched in kinase activities of around 97 kDa which could than be used as the starting material for an affinity purification, and the second was to use testis

extract rather than brain. α2 chimaerin is present in both brain and testis and it is possible that certain target proteins may be expressed at a higher level in testis, facilitating purification.

4B.1 Fractionation of Rat Brain Extract Prior to the Affinity Purification of Kinase Activities

Rat brain extracts were fractionated on S Sepharose and Q Sepharose columns. Proteins were eluted from the columns with a stepwise salt gradient (0.1M-0.5M NaCl in 0.1M increments); Coomassie Blue stains of the fractions after SDS-PAGE separation were shown in Figures 3.15 (S Sepharose) and 3.16 (Q Sepharose). 20μl of each fraction was assayed for in-gel kinase activity and it was found that the highest levels of kinase activity were detected in the 80-100 kDa range (S Sepharose, Figure 4.4A and Q Sepharose, Figure 4.5A). The greatest enrichment of kinase activities of around 97 kDa in the two experiments was seen in the S Sepharose 0.5M NaCl fraction (lane 7) and the Q Sepharose 0.2M/0.3M/0.4M NaCl fractions, which were subsequently pooled (lanes 4, 5 and 6). these were therefore selected for further purification. Each was divided equally between a GA-GST and a GA-GST-α2 chimaerin SH2 domain column from which bound proteins were eluted with 0.1M NaCl, 0.2M NaCl, 0.5M NaCl, 1.0M NaCl and 0.5% SDS. The starting material, the flow through and 5% of each of the fractions were tested for in-gel kinase activity. The results for the proteins purified from the S Sepharose fraction are shown in Figure 4.4B and from the Q Sepharose fraction in Figure 4.5B (in each case the 'B1' figure refers to the samples purified on the GST column and the 'B2' figure those purified on the chimaerin column).

In both sets of experiments the highest level of kinase activity was present in the SDS-eluted samples (B1 and B2, lane 7) and a kinase of approximately 97 kDa bound both the GST and chimaerin columns. Whether a single kinase associated with both columns in a non-specific manner or whether the signals seen emanated from different kinases of similar molecular mass could not be determined. A number of relatively faint kinase signals, specifically bound to the chimaerin column, were detected in the Q Sepharose-separated proteins eluted with 0.1M, 0.2M and 0.5M NaCl (B2, lanes 3, 4, and 5). These included at least two kinases of 90-100 kDa and one kinase of 43kDa.

Figure 4.4A: Brain Proteins, Fractionated on S Sepharose, Assayed for In-Gel Kinase Activity.

100ml of rat brain extract (prepared in 0.05% Triton-X-100) was fractionated on a 2.5ml S Sepharose column. The flow through was collected, the column washed and proteins eluted in two column volumes with a stepwise salt gradient. 10µl samples were assayed for in-gel kinase activity.

Lane 1, rat brain extract
Lane 2, the unbound material
Lane 3, proteins eluted with 0.1M NaCl
Lane 4, proteins eluted with 0.2M NaCl
Lane 5, proteins eluted with 0.3M NaCl
Lane 6, proteins eluted with 0.4M NaCl
Lane 7, proteins eluted with 0.5M NaCl

Figure 4.4B: Analysis of the In-Gel Kinase Activity of Fractionated Rat Brain Extract Proteins Further Purified by Affinity Chromatography

Equal quantities of the rat brain extract proteins eluted from an S Sepharose column at 0.5M NaCl were applied to glutathione agarose-GST (B1) and glutathione agarose-GSTα2 SH2 domain (B2) columns. The columns were washed and bound proteins eluted. 10µl of the starting material and the flow through and 5% of the total protein eluted under different conditions was tested for in-gel kinase activity.

Lane 1, S Sepharose 0.5M NaCl fraction (the starting material)
Lane 2, the unbound material
Lane 3, proteins eluted with 0.1M NaCl
Lane 4, proteins eluted with 0.2M NaCl
Lane 5, proteins eluted with 0.5M NaCl
Lane 6, proteins eluted with 1.0M NaCl
Lane 7, proteins eluted with 0.5% SDS

A.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |

97 kDa
68 kDa
43 kDa
29 kDa
18 kDa

B1.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |

97 kDa
68 kDa
43 kDa
29 kDa
18 kDa

B2.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |

97 kDa
68 kDa
43 kDa
29 kDa
18 kDa
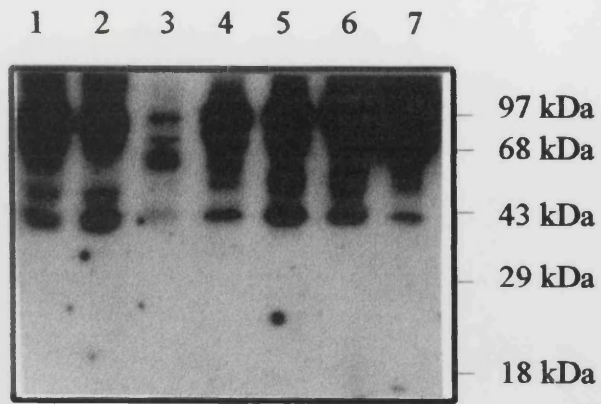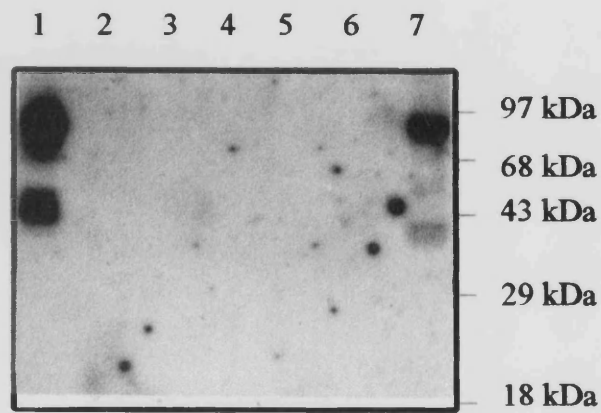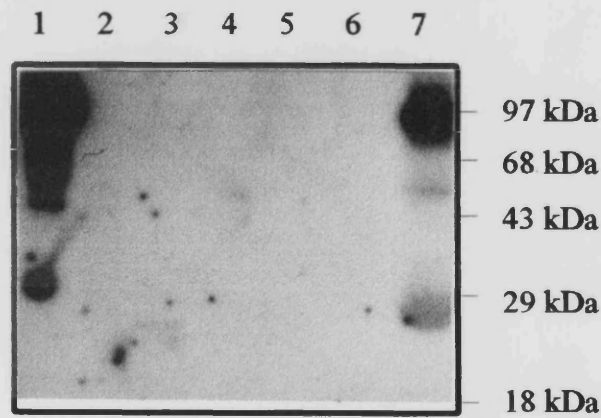
Figure 4.5A: Brain Proteins, Fractionated on Q Sepharose, Assayed for In-Gel Kinase Activity.

100ml of rat brain extract (prepared in 0.05% Triton-X-100) was fractionated on a 2.5ml Q Sepharose column. The flow through was collected, the column washed and proteins eluted in two column volumes with a stepwise salt gradient. 10µl samples were assayed for in-gel kinase activity.

Lane 1, rat brain extract
Lane 2, the unbound material
Lane 3, proteins eluted with 0.1M NaCl
Lane 4, proteins eluted with 0.2M NaCl
Lane 5, proteins eluted with 0.3M NaCl
Lane 6, proteins eluted with 0.4M NaCl
Lane 7, proteins eluted with 0.5M NaCl

Figure 4.5B: Analysis of the In-Gel Kinase Activity of Fractionated Rat Brain Extract Proteins Further Purified by Affinity Chromatography

The rat brain extract proteins eluted from a Q Sepharose column at 0.2M, 0.3M and 0.4M NaCl were pooled and equal quantities were applied to glutathione agarose-GST (B1) and glutathione agarose-GSTα2 SH2 domain (B2) columns. The columns were washed and bound proteins eluted. 10µl of the starting material and the flow through and 5% of the total protein eluted under different conditions was tested for in-gel kinase activity.

Lane 1, Q Sepharose 0.2M/0.3M/0.4M NaCl fraction (the starting material)
Lane 2, the unbound material
Lane 3, proteins eluted with 0.1M NaCl
Lane 4, proteins eluted with 0.2M NaCl
Lane 5, proteins eluted with 0.5M NaCl
Lane 6, proteins eluted with 1.0M NaCl
Lane 7, proteins eluted with 0.5% SDS

172

A.
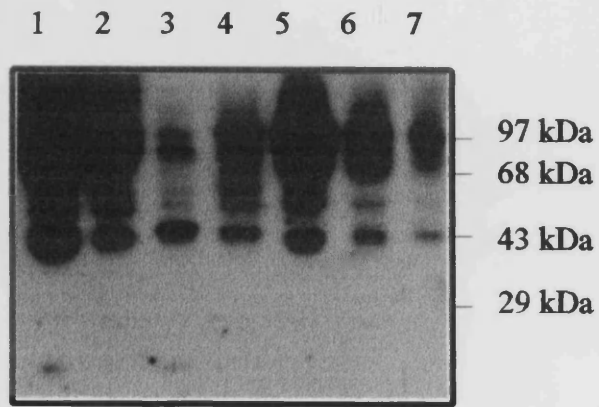
B1.
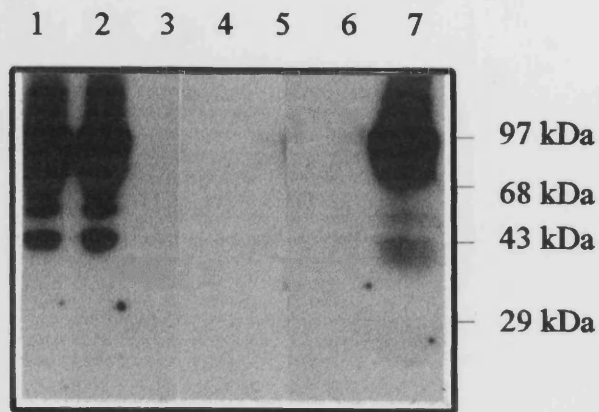
B2.
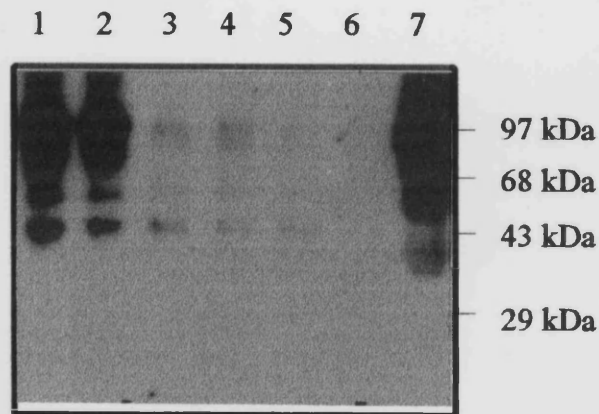
The low levels of kinase activity detected after the second step of the purification could reflect either a low protein yield or possibly a loss of enzymatic activity due to the additional handling involved in performing two consecutive chromatographic separations. Protein phosphorylation is labile and a loss during purification may directly affect kinase activity or remove essential determinents of target binding, for example phosphotyrosine in the case of an SH2 domain. If the kinase activities detected in this experiment were the same as those purified directly from whole rat brain extract then no improvement in the yield of catalytically active protein was achieved. For these reasons no further purification was attempted.

Proteins in the Q Sepharose 0.2M-0.4M NaCl fractions that bound GST and chimaerin affinity columns were also separated by SDS-PAGE and visualised with Coomassie Blue. These gels are shown in Figure 4.6. Whilst this two-step purification did not appear to enrich for any kinase activity that specifically bound the α2-chimaerin SH2 domain, the total number of proteins present in the sample eluted from this column with SDS (A, lane 7) was greater than that in the equivalent sample from the GST column (B, lane 7). However, since the amount of the fusion protein on the aliquot of the untreated chimaerin column analysed (Figure 4.6B, lane 8) was so much lower than that in the SDS-eluted sample (lane 7), it is possible that these proteins are actually the product of fusion protein breakdown or are bacterial contaminants. When peptide sequence information was obtained for several of these proteins this was found to be the case (data not shown).

4B.2 The Purification of Kinases from Rat Testis Extract

To establish whether testis might be a better source of α2 chimaerin target proteins than brain, preparative scale affinity purifications were performed and the purifiedproteins tested for in-gel kinase actvity. The experimental protocol reflected that used in previous experiments with the exception that rat testis, rather than brain, extract (prepared with 0.05% Triton-X-100) was applied to a GA-α2 chimaerin SH2 domain affinity column. A GA-GST column was used as a control. 5% of the total protein in each eluant was tested for in-gel kinase activity in the absence of exogenous substrate (Figure 4.7A) and in the presence of full length recombinant α2-chimaerin (Figure 4.7B). With one exception which will be described later (A, lane C3), the highest level of kinase activity was detected in the
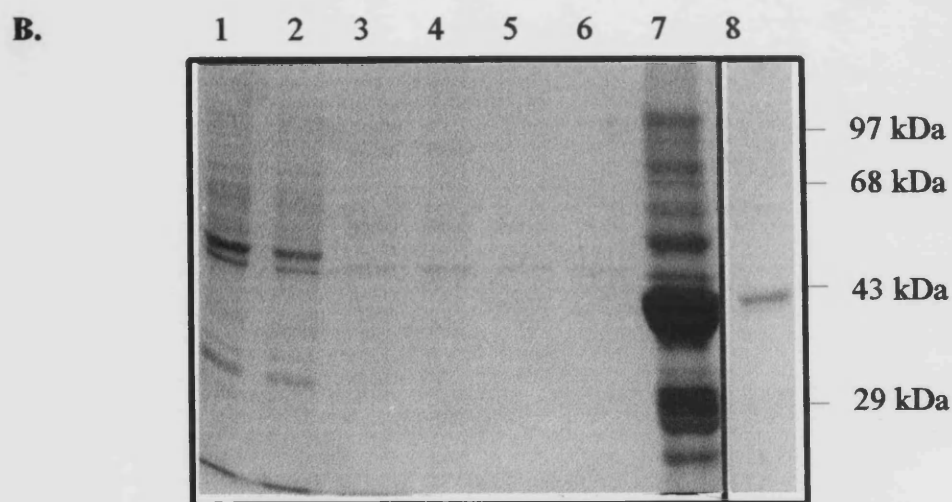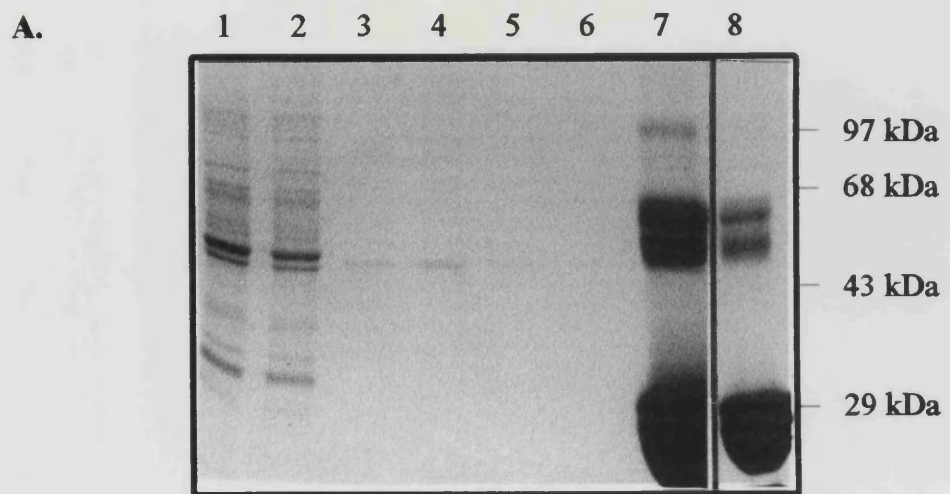
Figure 4.6: Rat Brain Proteins Fractionated on a Q Sepharose Column Further Purifed by Affinity Chromatography

100ml of rat brain extract was fractionated on a Q Sepharose column and the proteins eluted at 0.2M NaCl, 0.3M NaCl and 0.4M NaCl pooled and applied to either a glutathione agarose-GST column (A) or a glutathione agarose-GST α2 chimaerin SH2 domain column (B). 10µl of the starting material (Q sepharose 0.2/0.3/0.4 M NaCl eluant mix, lane 1) and the flow through on each affinity column (lane 2) and 5% of the total eluant obtained at 0.1M NaCl (lane 3), 0.2M NaCl (lane 4), 0.5M NaCl (lane 5), 1.0M NaCl (lane 6) and at 0.5% SDS (lane 7) were analysed. 10µl of the untreated column matrix was run in parallel (lane 8). Samples were separated by SDS-PAGE and proteins visualised using Coomassie Blue.

175

samples eluted with SDS, as in previous experiments. There did not appear to be any specific association of a 97 kDa kinase with the chimaerin SH2 domain; signals of this size were detected in the eluants from both the chimaerin and the GST columns. However, the the total kinase activity of the samples eluted from GST and the α2 chimaerin SH2 domain columns with SDS differed, being slightly greater in the case of chimaerin.

A 43 kDa kinase activity visible only in the presence of the α2 chimaerin substrate bound only the chimaerin column. This was was found in the samples eluted with 01.M, 0.2M, 0.5M and 1.0M NaCl (B, lanes C1-C4). This result reflects that obtained using brain extract as the starting material for the affinity purification (Figure 4.3), although in this case the signals seen were somewhat stronger. Immunoblotting indicated that this kinase was not JNK (data not shown) but there are a large number of other serine/threoine kinases, for example the ERKs, of approximately this size and the possible identity of this kinase will be discussed further in Chapter 7.

The highest level of kinase activity detected in the absence of added substrate was in the protein sample eluted from the chimaerin column with 0.5M NaCl (Figure 4.7A, lane C3). Similar results were not observed when the sample was assayed in the presence of gel-bound substrate (Figure 4.7B, lane C3). If not due to error or artefact, this result may reflect the presence of an α2 chimaerin-dependent phosphatase in the sample or alternatively an inhibition of kinase activity by α2 chimaerin. The results obtained using testis extract were not significantly better than those obtained using brain extract and so time constraints precluded the repetition of this experiment to investigate further this anomaly.

4C The Phosphatase Activity of Proteins Bound to a Chimaerin SH2 Domain Affinity Column

The regulation of signalling through phosphorylation involves not only kinases but phosphatases. A simple phosphatase assay was devised to investigate whether there was any phosphatase activity present amongst the proteins bound by an α2-chimaerin affinity column. The set of samples used was prepared by the same procedure as those shown in Figure 3.2. Myelin Basic Protein (MBP), known to be a substrate for many kinases, was phosphorylated in the presence of γ^{32}P-ATP by either p65-pak or src, serine/threonine and tyrosine kinases respectively. The labelled MBP was then incubated with either an affinity-

A.
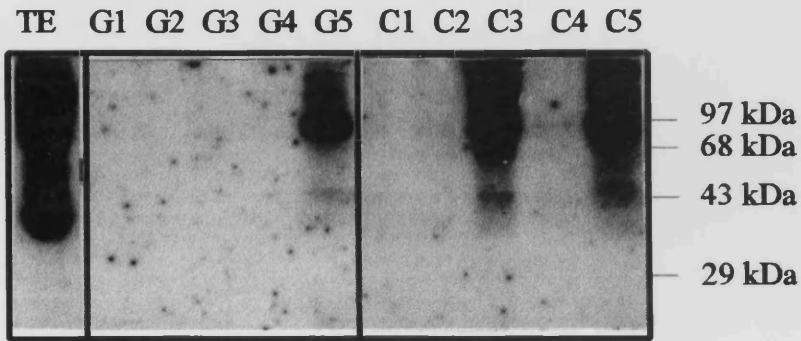
B.
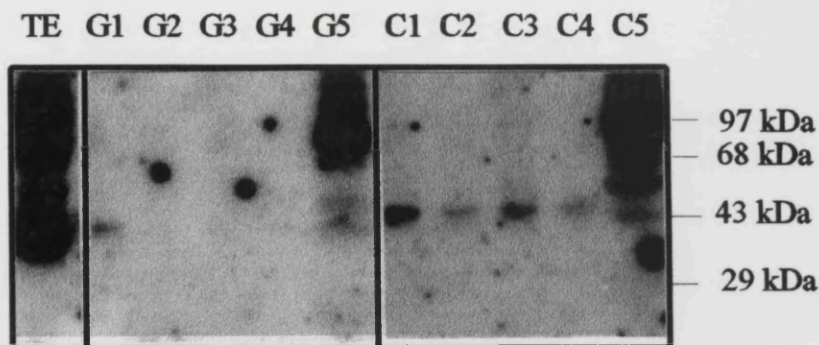
Figure 4.7: The In-Gel Kinase Activity of the Rat Testis Proteins Associated with GST and Chimaerin SH2 Domain Affinity Columns

Rat testis extract (prepared with 0.05% Triton-X-100) was applied to glutathione agarose-GST (sample set G) and GST-α2 chimaerin SH2 domain (sample set C) columns. Bound proteins were eluted with 0.1M NaCl (lanes G1 and C1), 0.2M NaCl (lanes G2 and C2), 0.5M NaCl (lanes G3 and C3), 1.0M NaCl (lanes G4 and C4) and 0.5% SDS (lanes G5 and C5). Approximately 40μg of rat testis extract (TE) and 5% of each eluant were assayed for in-gel kinase activity in the absence (Figure A) or presence (Figure B) of 0.1mg/ml full length recombinant α2 chimaerin (polymerised into the gel matrix).

purified protein sample or, as controls, with rat brain extract and buffer. It was anticipated that the presence of phosphatase activity could be detected by decreased MBP phosphorylation; if the loss was from the p65-pak-labelled substrate it would be indicative of a serine/threonine phosphatase (Figure 4.8A) and if it were from the src-labelled substrate, a tyrosine phosphatase (Figure 4.8B).

It was found that rat brain extract contained both serine/threonine and tyrosine phosphatases that were active under the conditions used because it caused a decrease in the phosphorylation of both substrates (lane 2, figures A and B). However, whilst there was possibly a slight reduction in serine/threonine phosphorylation in the presence of proteins eluted from a GST-α2 chimaerin SH2 domain column with 0.2M NaCl (Figure 4.8A, lane 1), the results did not indicate unequivocally that the α2 chimaerin SH2 domain bound a rat brain phosphatase. This assay system was unlikely to be sufficiently sensitive to detect low levels of enzyme activity and the kinase/substrate combination used may not produce an optimal substrate for any phosphatase present in the samples. Unidentified phosphatase activities are more difficult to investigate relative to unidentified kinase activities, a slight loss of radiolabel being more difficult to detect than a slight gain, and the possibility of a chimaerin/phosphatase interaction was not pursued further.

4D Summary

In vitro kinase assays indicated that α2 chimaerin SH2 domain affinity columns could bind kinases in rat brain extract which could phosphorylate full length α2 chimaerin. Attempts were made to investigate this further using in-gel kinase assays and it was found that serine/threonine kinase activities of approximately 97 kDa, 43 kDa and 150 kDa could reproducibly be purified from rat brain extract. The 43kDa kinase was specifically selected by an α2 chimaerin SH2 domain affinity column and only detected in the presence of an in-gel α2 chimaerin substrate, although an ability to autophosphorylate has not been eliminated. A kinase of the same apparent molecular mass was also present in testis. Low levels of a higher molecular mass kinase (150 kDa) that preferentially bound a chimaerin rather than control column were also detected. It was unclear whether the 97kDa kinase activity did in fact interact specifically with the chimaerin SH2 domain. Attempts to purify these kinases further by use of a second chromatographic step or a different starting material were not

A.

B.
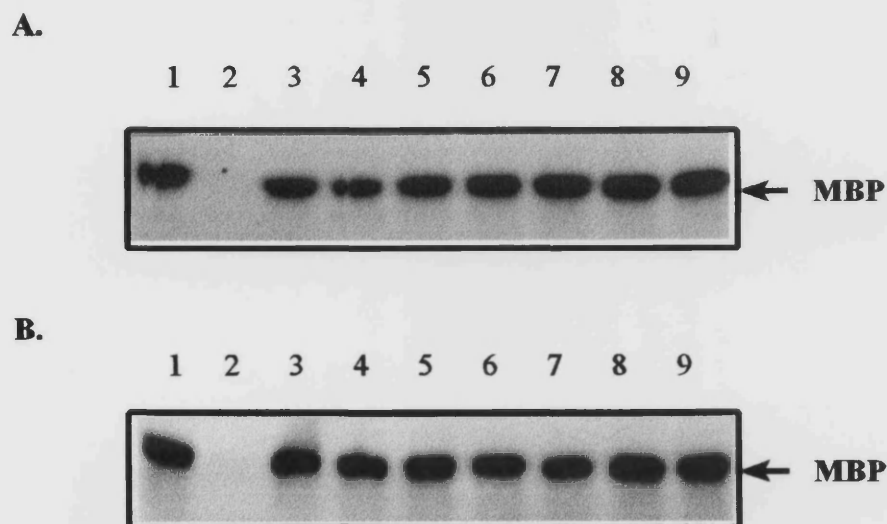
Figure 4.8: Assaying the Proteins Bound To an α2 Chimaerin SH2 Domain Affinity Column for Phosphatase Activity

Myelin basic protein (MBP) was phosphorylated either on serine/threoine residues by β-pak (Figure A) or on tyrosine residues by Src (Figure B) in the presence of γ^{32}P-ATP and the radiolabelled protein used as a potential phosphatase substrate. Rat brain proteins binding a GST-α2 chimaerin SH2 domain affinity column were eluted under a series of different conditions and a 5% aliquot of each eluant incubated with the radioactively-labelled MBP. The samples were electrophoresed and the level of MBP phosphorylation detected by autoradiography. In both sets of assays, the samples incubated with the MBP substrate were:
Lane 1, buffer only
Lane 2, Rat brain extract (lane 2)
Lane 3, proteins eluted with 0.1M NaCl (lane 3),
Lane 4, proteins eluted with 0.2M NaCl (lane 4),
Lane 5, proteins eluted with 0.5M NaCl (lane 5),
Lane 6, proteins eluted with 1.0M NaCl (lane 6),
Lane 7, proteins eluted with pH 3.6 buffer (lane 8)
Lane 8, proteins eluted with and pH 10 buffer (lane 9).
Lane 9, proteins eluted with 0.5% SDS (lane 7)

successful. Assaying for phosphatase activity associate with the chimaerin SH2 domain affinity column gave inconclusive results; the possibitity has not been excluded but a more sensitive and selective technique may be required.

CHAPTER FIVE

RESULTS III

Mutational Analysis of the Interaction of the α2 Chimaerin SH2 domain With Target Proteins

Previous studies have shown that mutation of specific amino acid residues in the src SH2 domain (Bibbins K et al, 1993) can have profound effects on the interaction of the SH2 domain with its phosphotyrosine-containing targets. The α2 chimaerin SH2 domain is atypical in that its first amino acid residue, that at the βA1 position, is glutamate rather than tryptophan; only three other SH2 domains, one of which is β2 chimaerin (Leung et al, 1994), do not have an amino terminal tryptophan (Thompson et al, 1996 and Hatada et al, 1995) and in at least one of these cases there is a radical modification of the mechanism of interaction with phosphotyrosine. Point mutations were made in the chimaerin SH2 domain at sites equivalent to those known to be of importance for src/substrate interactions to further investigate whether the SH2 domain of α2-chimaerin behaves as a conventional SH2 domain. Recombinant proteins containing the altered amino acid residues were expressed and used to study the interaction between the chimaerin SH2 domain and two putative target proteins.

5A   Site-Directed Mutagenesis of the α2-Chimaerin SH2 Domain

5A.1   Mutation of the Chimaerin SH2 Domain and Expression of Recombinant Proteins

Point mutations were made at four positions within the chimaerin SH2 domain sequence as described in Chapter 2. The positions of these alterations in the protein sequence and the substitutions made are shown in Figure 5.1A which shows an alignment of the src and chimaerin SH2 domain sequences. Resolution of the crystal structures of different SH2 domains has allowed SH2 domains to be described and compared in terms of their secondary structure but it should be noted that, in the case of the chimaerin SH2 domain this is predicted rather than proven. The substitutions made in the chimaerin SH2 domain were analogous to mutations that have previously been studied in the src SH2 domain (Figure 5.1B). The first amino acid residue of the chimaerin SH2 domain, a glutamate, was altered to the tryptophan (E49W) found at this position in src and virtually all known SH2 domains. In the src SH2 domain structure this amino acid residue is in the hydrophobic interior of the

A. An Alignment of the Sequences of the Src and α2-Chimaerin SH2 Domains

βA AA   αA   AB   βB   BC   βC   CD

```
Src  WYFGKITRRESERLLLNPENPRGTFLVRESETTKGAYCLSVSDFDNAKGL
α2   EFHGMISREAADQLLI V  AEGSYLIRESQRQPGTYTLALRF  GSQ
```

E49W   R56L   R73L

βD   βD' DE βE   EF βF FB   αB   BG   βG

```
Src  NVKHYKIRKLDSGGFYITSRTQFSSLQQLVAYYSKHADGLCHRLTNVCPTS
α2   TRNFRLY Y DGKHFV GEKRFESIHDLV      TDGLIT L YIETKL
```

N94H
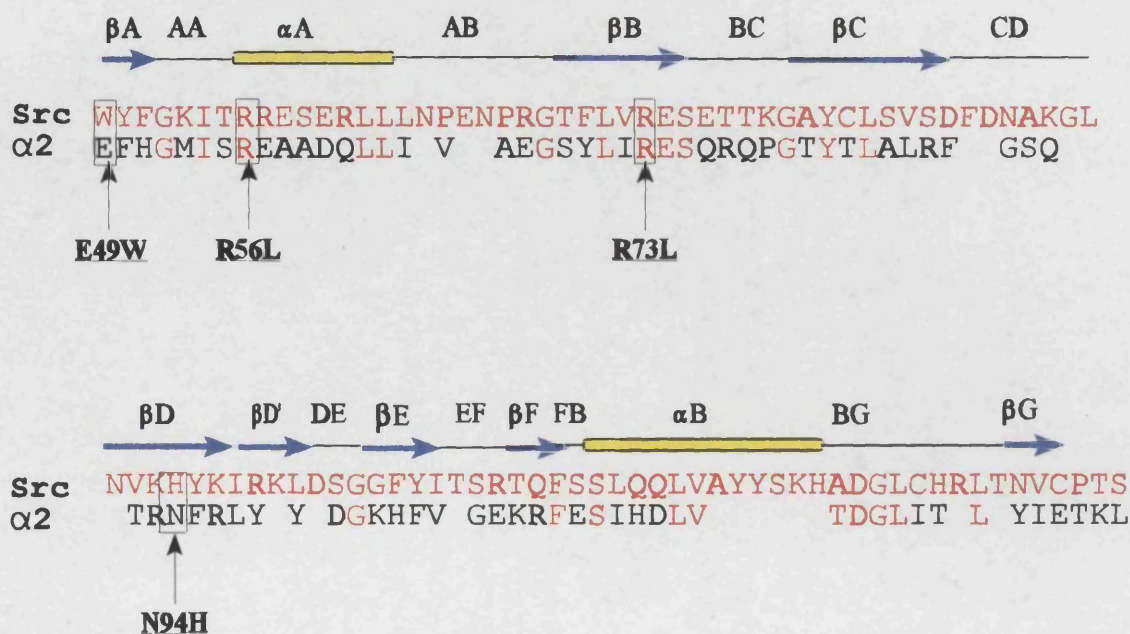
B. Mutational Analysis of the Src SH2 Domain

Mutant	Position of residue	Conserved residue	Coordinates with phospho-tyrosine	Binding to phospho-peptide	α2-Chimaerin Mutations
Wild Type	-	-	-	1	-
W14E	βA1	++	-	<0.1	E49W
R155A	αA2	+	+	0.8	R56L
R175L	βB5	++	+	<0.1	R73L
H201R	βD4	+	-	0.8	N94H

Figure 5.1: Site Directed Mutagenesis of the α2 Chimaerin SH2 Domain

The chimaerin and Src SH2 domain sequences have been aligned in Figure 5.1A. The Src sequence and identical residues in the chimaerin SH2 domain are marked in red. The secondary structure of the src SH2 domain is shown: α-helices are in yellow and β-strands in blue. The four amino acid substitutions made in the chimaerin SH2 domain are indicated. Figure 5.1B is adapted from Bibbins et al, 1993, and shows the effect of certain point mutations in the Src SH2 domain on phosphotyrosine binding. The chimaerin sequence was altered at equivalent sites.

183

protein and, when mutated to glutamate, binding to phosphotyrosine is effectively abolished. Two arginine residues at the αA2 and βB4 positions, the first highly conserved and the second invariant and known in src to be involved in co-ordinating the phosphate group of the phosphotyrosine target, were mutated to leucine (R56L and R73L). Both these mutations might be predicted to drastically reduce binding to phosphotyrosine but in fact analysis of the equivalent src SH2 domain mutants showed that only mutation of the invariant arginine (R175A) had a profound effect. The R155A mutant retained approximately 80% of the wild-type binding capacity. The fourth position altered in the chimaerin SH2 domain was an arginine (N94H) which was altered to the histidine found at the equivalent position in src. Structural analysis predicts that this amino acid residue is found at the mouth of the phosphotyrosine-binding pocket and is important for maintainance of active site geometry. Figure 5.1B summarises this information.

The Transformer Site-Directed Mutagenesis Kit (Clonetech) was used to introduce specific nucleotide changes into the α2 chimaerin SH2 domain sequence. The nucleotides altered to achieve the desired amino acid residue substitutions are descibed in Chapter 2 and in Appendix 2A. The protocol used is outlined in Appendix 2B. The procedure relied upon the simultaneous introduction of a mutation at a unique restriction site outside the α2 chimaerin sequence, in this case converting the Bluescript Sac1 site to an Aat2 site. Clones that contained this mutation were further analysed. Analysis of the restriction sites proximal to the altered nucleotide using the DNASTAR MapDraw software indicated that there were restriction site changes associated with the introduction of the E49W, the R56L and the R73L mutations that could be used for analytical purposes. This facilitated identification of clones containing these mutations because it became neccessary to sequence only a limited number of clones. Approximately 5% of the clones tested contained the correct mutations. The nucleotide change used to produce the N94H amino acid residue substitution did not cause concomitant changes in the restiction map of the surrounding sequence that could be exploited as an analytical tool. In this case it was necessary to sequence a number of randomly-selected clones to identify one that contained the mutation.

Point mutations were introduced into the full length α2 chimaerin sequence. It was therefore possible to use this to produce constructs containing the point mutation and different amounts of the α2 chimaerin sequence following a single mutagenesis step.

Sequences encoding either the SH2 domain (plus the amino-terminal forty-eight amino acid residues, as indicated in Appendix 1) or the full length protein from each of the mutated clones. These were sub-cloned into the pGEX-2TK vector expressed as bacterial fusion proteins. The set of wild-type and mutant proteins were purified and full-length protein expression confirmed by SDS-PAGE (Figure 5.2) analysis. The apparent molecular mass of the SH2 domain proteins was approximately 43 kDa and of the full length proteins approximately 70 kDa. In all the samples shown, protein expression had been induced in one litre of bacterial culture and the fusion protein purified was ultimately concentrated to approximately 600μl of which a 10μl aliquot was analysed. The expression level of the SH2 domain constructs, both the wild-type and mutant, was low; in general less than 100μg of these fusion proteins could be purified from one litre of culture (Figure 5.2A). Expression of the full length α2 chimaerin constructs gave higher yields (Figure 5.2B), up to 4mg per litre of culture, although less of the wild-type protein was generally obtained. The full-length fusion protein/breakdown ratio was higher in the preparations of the SH2 domain proteins than the full length α2 chimaerin proteins.

The GST-α2 chimaerin fusion proteins with point mutations in their SH2 domains were assayed for GAP activity by measuring the loss of γ^{32}P-GTP from GST-Rac1 (Figure 5.3). The wild-type protein was also tested. Two sets of experiments were performed, the first using 0.5μg of chimaerin GAP and the second 0.1μg (these quantities refer the total protein and made no allowance for differences in the amount of fusion protein breakdown in the preparation). No significant differences in the GAP activities of the wild-type and mutant proteins was detected. 0.1μg of GAP stimulated the intrinsic GTPase activity of Rac1 approximately two-fold: 35-40% GTP remained bound to the p21 after four minutes as opposed to around 75% (Graph B). This quantity of GAP was not saturating and when the assays were repeated using 0.5μg the stimulation of GTPase activity was closer to fourfold (Graph A).

5A.2 The Interaction of α2 Chimaerin with Phosphotyrosine

One potential consequence of the unusual sequence features of the α2 chimaerin SH2 domain is an abrogation of its anticipated phosphotyrosine-binding ability. SH2 domains can interact with isolated phosphotyrosine, albeit with lower affinity than with their specific
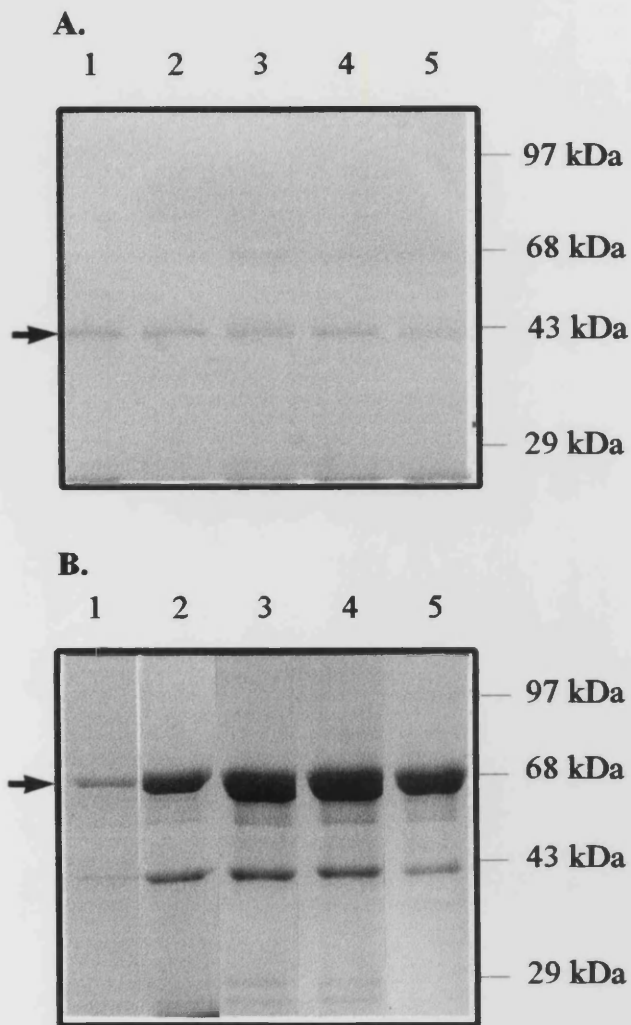
Figure 5.2: Point Mutation of the Chimaerin SH2 Domain and Expression of Recombinant Proteins

Four residues in the chimaerin SH2 domain were altered by site-directed mutagenesis and sequences encoding either the isolated SH2 domain (Figure A) or the full-length protein (Figure B) were subcloned into the bacterial expression vector pGEX-2TK. GST fusion proteins were purified, analysed by SDS-PAGE and visualised with Coomassie Blue. Full-length protein expression was confirmed by comparison of the apparent molecular mass of the product with that of the wild type protein (lane 1). The amino acid substitutions made were E49W (lane 2), R56L (lane 3), R73L (lane 4) and N94H (lane 5). The full length protein product is indicated by an arrowhead at the left of each figure.

A. 0.5ug GAP
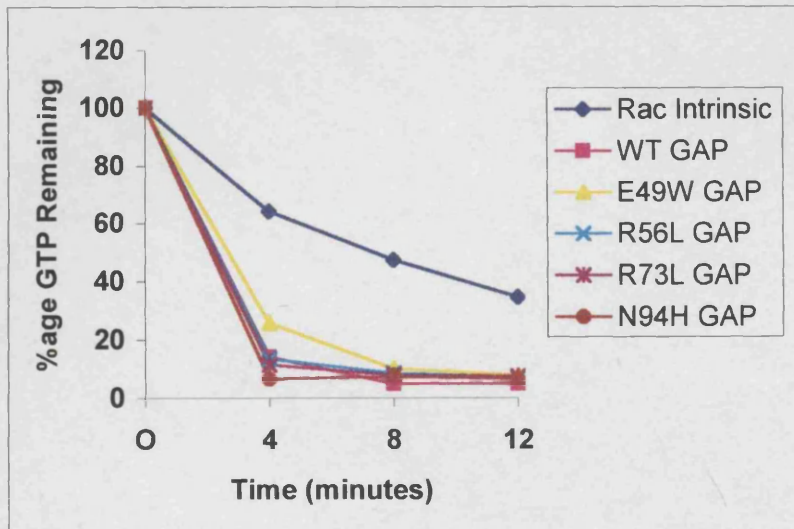
B. 0.1ug GAP
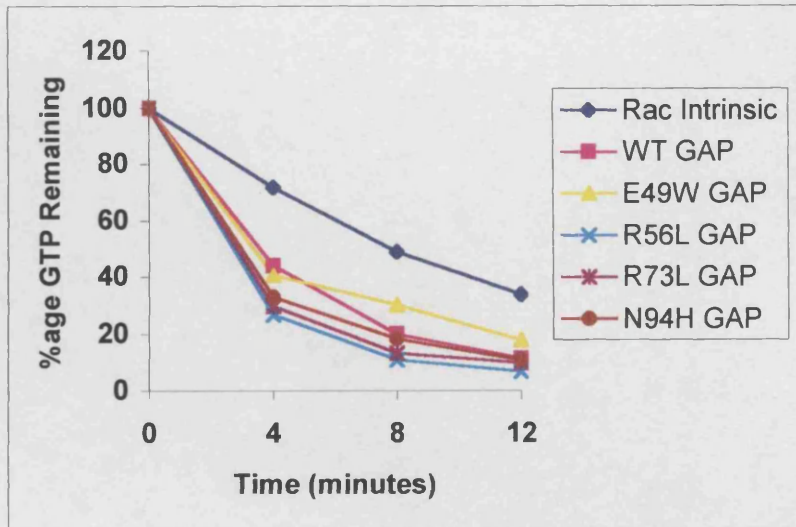
Figure 5.3: GAP Activity of α2 Chimaerin Proteins with Altered SH2 Domains

γ^{32}P-GTP –loaded GST-rac1 was incubated in GAP assay reaction buffer either alone, to measure its intrinsic GTPase activity, or in the presence of 500ng or 100ng GAP (Figures A and B respectively). Aliquots of the reaction mix were removed at 0, 4, 8 and 12 minutes, dotted onto nitrocellulose, the filters washed and the remaining radioactivity measured in a scintillation counter. To visualise the rate of loss of GTP in the different reactions, the percentage of GTP remaining was plotted against time (time zero = 100%). Wild type (WT) GST-α2 chimaerin and the four GST-α2 chimaerin proteins with a point mutation in their SH2 domain (at E49W, R56L, R73L or N94H) were tested.

phosphopeptide target, and retention of an SH2 domain on a phosphotyrosine column is a convenient method to establish whether such an interaction occurs. When using GST fusion proteins an equivalent purification on glutathione agarose can be performed, allowing an estimate to be made of the relative amount of the total available protein that can bind phosphotyrosine.

Analytical scale affinity chromatography was carried out as described in Chapter 2 using purified GST fusion proteins. The α2 chimaerin SH2 domain (Construct A, with ten amino acid residues amino-terminal to the SH2 domain, as indicated in Appendix 1) and full length α2 chimaerin were both tested. GST and GST-abl SH2 domain were used as controls. The results are shown in Figure 5.4. In each case the protein bound to glutathione agarose is shown in lane 3 and that bound to phosphotyrosine agarose in lane 5. It was found that whilst GST did not interact significantly with the phosphotyrosine column, both the abl and the chimaerin SH2 domain proteins bound either column equally well, indicating that up to 100% of the total protein in the preparation was functionally active in its ability to bind phosphotyrosine. However, this was not the case for full length α2 chimaerin: only approximately 50% of the available protein was retained by a phosphotyrosine column. This suggests the possibility of an intra-molecular interaction that reduces the availability of the phosphotyrosine binding site. The results confirm that the α2 chimaerin SH2 domain can interact with phosphotyrosine.

Affinity chromatography on phosphotyrosine agarose is an approach that has been used to purify recombinant SH2 domains from bacterial cell extracts (Koegl et al, 1994). Wild-type and mutated α2 chimaerin SH2 domain proteins were purified in this way to establish whether point mutation affected the interaction of the protein with phosphotyrosine. A similar set of experiments to those described above was performed, using bacterial cell lysates as the starting material for the purifications rather than purified fusion proteins. The results were analysed in two ways: visualisation of proteins with Coomassie Blue following SDS-PAGE (Figure 5.5A) and Western Blotting and probing with an anti-α2 chimaerin antibody (Figure 5.5B).

In Figure 5.5A the protein binding glutathione agarose is shown in lane 3 of each gel and this is assumed to be the total protein available. The protein binding to phosphotyrosine is shown in lane 5. The non-specific association of bacterial proteins with
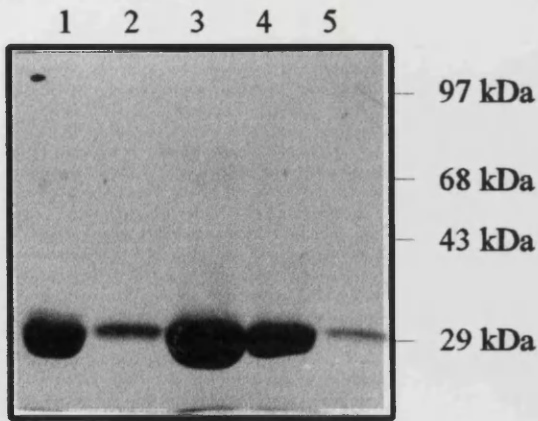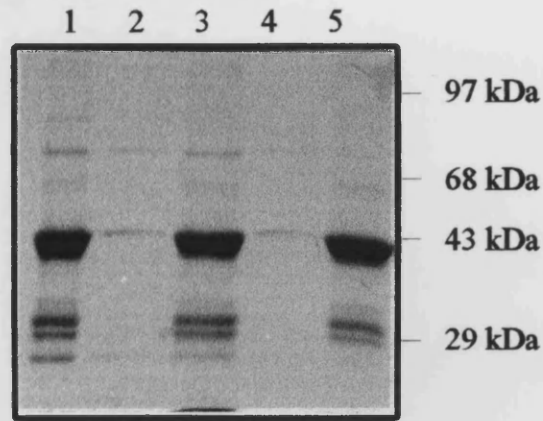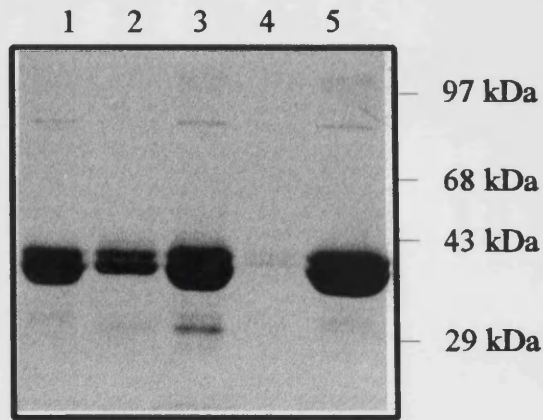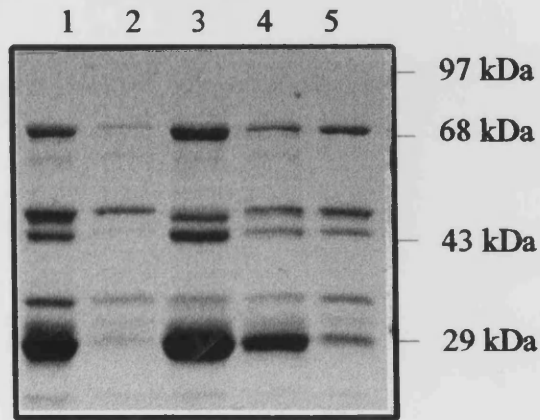
Figure 5.4: The Chimaerin SH2 Domain Can Bind a Phosphotyrosine Column

Approximately 25μg of GST, GST-abl SH2 domain, GST-α2 SH2 domain and GST-α2 chimaerin were incubated with either 50μl of glutathione agarose or 50μl of phosphotyrosine agarose. Unbound material was collected and the resin washed. Proteins were eluted from each column in 50μl of Protein Sample Buffer. Aliquots of the starting material, flow through and bound protein were analysed by SDS-PAGE and proteins were visualised with Coomassie Blue. In each case the samples tested were:

Lane 1, 25% of the amount of the fusion protein applied to each column

Lane 2, 25% of the unbound material on glutathione agarose

Lane 3, 50% of the protein bound to the glutathione agarose column

Lane 4, 25% of the unbound material on phosphotyrosine agarose

Lane 5, 50% of the protein bound to the phosphotyrosine agarose column

each column resulted in a high level of background staining and the position of the fusion protein is indicated by an arrowhead. As with the previous set of experiments it was found that GST did not bind phosphotyrosine agarose. However, in this case, the amount of wild-type GST-α2-chimaerin SH2 domain retained by the phosphotyrosine column was only around 20% of that binding the glutathione agarose column whilst the previous results obtained using purified protein indicated that the α2 chimaerin SH2 domain could bind glutathione agarose and phosphotyrosine agarose equally well. This may reflect a difference in the α2-chimaerin SH2 domain construct used: that used in the experiment shown in Figure 5.4 contained only ten amino acid residues amino terminal to the SH2 domain rather than the full amino terminal sequence of α2-chimaerin. The SH2 domain with the E49W mutation was found to bind the phosphotyrosine column at least as well as, if not better than the wild-type (possibly up to 50% of the available protein retained) whereas the protein with the R73L mutation did not bind appreciably to the phosphotyrosine column, as might be predicted from the results obtained with the src SH2 domain. Mutation of the chimaerin SH2 domain at the N94 position did not appear to affect the ability of the SH2 domain to bind phosphotyrosine; this protein interacted with the phosphotyrosine column at least as well as the wild-type protein although the high level of background staining complicated analysis of the results.

It was not possible to determine the phosphotyrosine-binding capacity of the R56L SH2 domain mutant due to its poor expression: very little fusion protein bound the glutathione agarose column and, if only half the available protein were to bind a phosphotyrosine agarose column it may be below the limits of detection using Coomassie. To clarify this point, the samples of the wild-type α2 SH2 domain and the E49W, the R56L and the R73 mutants that had bound either the glutathione (Figure 5.5B, lane 1) or the phosphotyrosine column (Figure 5.5B, lane 2) were separated by SDS-PAGE, Western blotted and probed with anti-α2 chimaerin antiserum. This had been affinity-purified on a GST column and should not cross-react significantly with GST or contaminating bacterial proteins. Detection of the fusion protein is indicated by an arrow. The interaction of the wild-type and E49W proteins with phosphotyrosine was confirmed and also the failure of the R73L mutant to interact. Interestingly, the R56L mutant was severely compromised in its ability to bind phosphotyrosine. The equivalent mutation in the src SH2 domain reduced

Figure 5.5A: The Purification of Wild-Type and Mutated GST-α2 Chimaerin SH2 Domain Proteins on a Phosphotyrosine Agarose Column.

A cleared cell lysate was prepared from 250ml bacterial culture in which expression of GST or GST-α2 SH2 domain proteins (the wild-type, WT, and SH2 domain with different point mutations, as labelled) had been induced. This lysate (approximately 10ml) was divided into two aliquots and one incubated with 50μl glutathione agarose and the other with 50μl phosphotyrosine agarose. The unbound material was collected, the resin washed and proteins associated with each column eluted in 50μl of Protein Sample Buffer. In each case, samples were analysed by SDS-PAGE and proteins visualised with Coomassie Blue.

Lane 1 10μl of the cleared cell lysate

Lane 2 10μl of the flow through on glutathione agarose

Lane 3 50% of the protein bound to the glutathione agarose column

Lane 4 10μl of the flow through on phosphotyrosine agarose

Lane 5 50% of the protein bound to the phosphotyrosine agarose column

Arrowheads indicate the fusion protein.
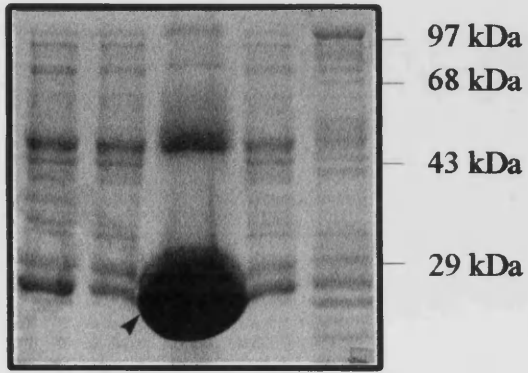
191

GST.

1   2   3   4   5

97 kDa
68 kDa
43 kDa
29 kDa

WT α2 SH2.

1   2   3   4   5

97 kDa
68 kDa
43 kDa
29 kDa

E49W α2 SH2.

1   2   3   4   5

97 kDa
68 kDa
43 kDa
29 kDa

R56L α2 SH2.

1   2   3   4   5

97 kDa
68 kDa
43 kDa
29 kDa

R73L α2 SH2.

1   2   3   4   5

97 kDa
68 kDa
43 kDa
29 kDa

N94H α2 SH2.

1   2   3   4   5

97 kDa
68 kDa
43 kDa
29 kDa

192
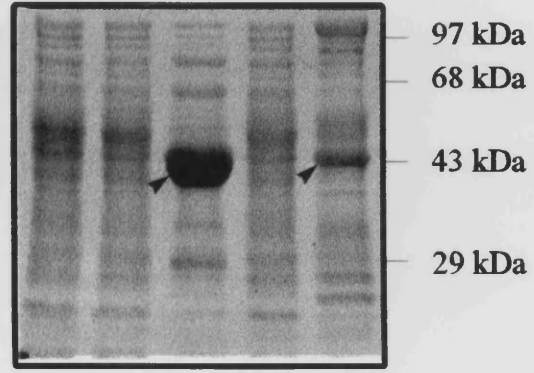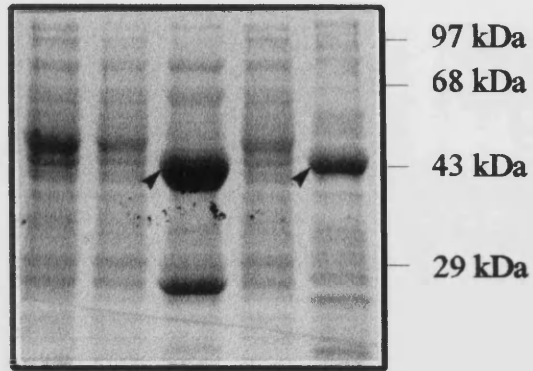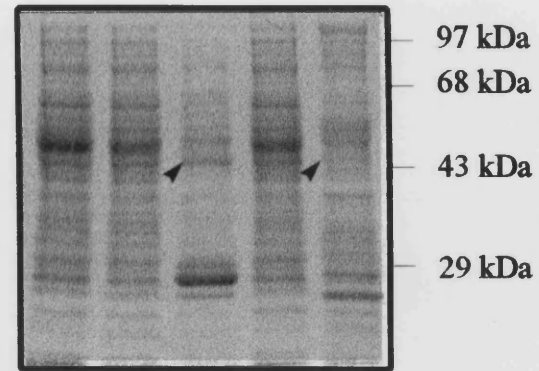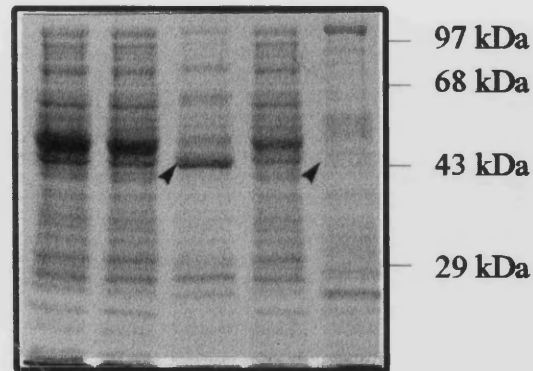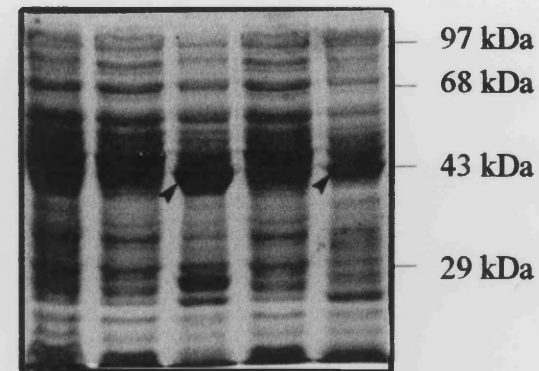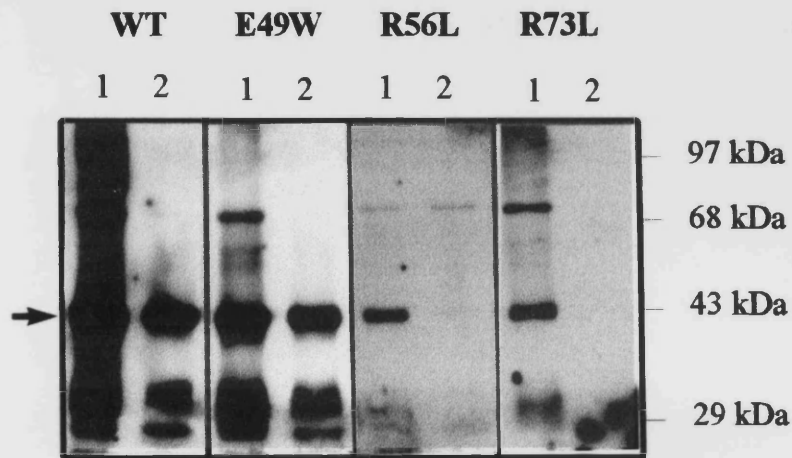
Figure 5.5B: The Purification of Wild-Type and Mutated GST-α2 Chimaerin SH2 Domain Proteins on a Phosphotyrosine Column

GST-α2 chimaerin SH2 domain proteins, the wild-type (WT) and those with single amino acid substitutions (E49W, R56L, R73L), were purified from bacterial cell lysates on either glutathione agarose (lane 1) or phosphotyrosine agarose (lane 2) affinity columns, as described in the legend to Figure 5.5A. Approximately 8% of the total protein bound by each column was separated by SDS-PAGE and transferred to nitrocellulose filters. These were probed with affinity-purified polyclonal anti-α2 chimaerin antiserum (obtained from G.Michaels, used at a 1/10000 dilution). The detection of full-length GST-α2 chimaerin SH2 domain proteins is indicated by an arrow.

phosphotyrosine binding by only around 20%. This difference may suggest that the mechanisms by which the Src and chimaerin SH2 domains interact with phosphotyrosine differ.

5B The Interaction of the α2 Chimaerin SH2 Domain With Two Putative Target Proteins, B13 and TOAD-64

Two potential target proteins of the chimaerin SH2 domain, B13 and TOAD-64, were isolated by members of our group. B13 was selected by the α2-chimaerin SH2 domain using the yeast two hybrid system (C.Monfries) and identified as the B13 subunit of the inner mitochondrial membrane Complex 1 NADH-oxidoreductase. Recombinant B13 was available in a bacterial expression vector (pGEX, obtained from C.Monfries). TOAD-64 was purified from rat brain extract using conventional chromatography (M.Teo, Ph.D Thesis, 1994). Peptides derived from the purified protein were sequenced and the protein identified on the basis of sequence homology as TOAD-64, a brain-specific phosphoprotein. The availability of appropriate Expressed Sequence Tag clones enabled construction of a recombinant TOAD-64 (C.Monfries). Recombinant TOAD-64 and B13 are referred to as p64 and p13 respectively.

5B.1 Preparation of Recombinant Proteins and Probes

The interaction of wild-type and mutated α2-chimaerin SH2 domain proteins with the two potential target proteins, B13 and TOAD-64 , was investigated using a filter binding assay in which recombinant B13 and TOAD-64 were immobilised on nitrocellulose filters and detected with radioactively-labelled chimaerin proteins. It should be noted that tyrosine kinases are not present in E.coli and hence bacterially-expressed recombinant proteins are not tyrosine phosphorylated. Thus, if p64 and p13 do interact with chimaerin the implication is that these interactions cannot be phosphotyrosine-dependent. However, there are examples in the literature of phosphotyrosine-independent SH2 domain interactions and B13 was initially isolated from the yeast two-hybrid system in the absence of mammalian tyrosine kinases.

Chimaerin SH2 domain proteins were radioactiveley labelled by PK-A whilst bound to a glutathione agarose column according to the protocol described in Section 2B.33A.
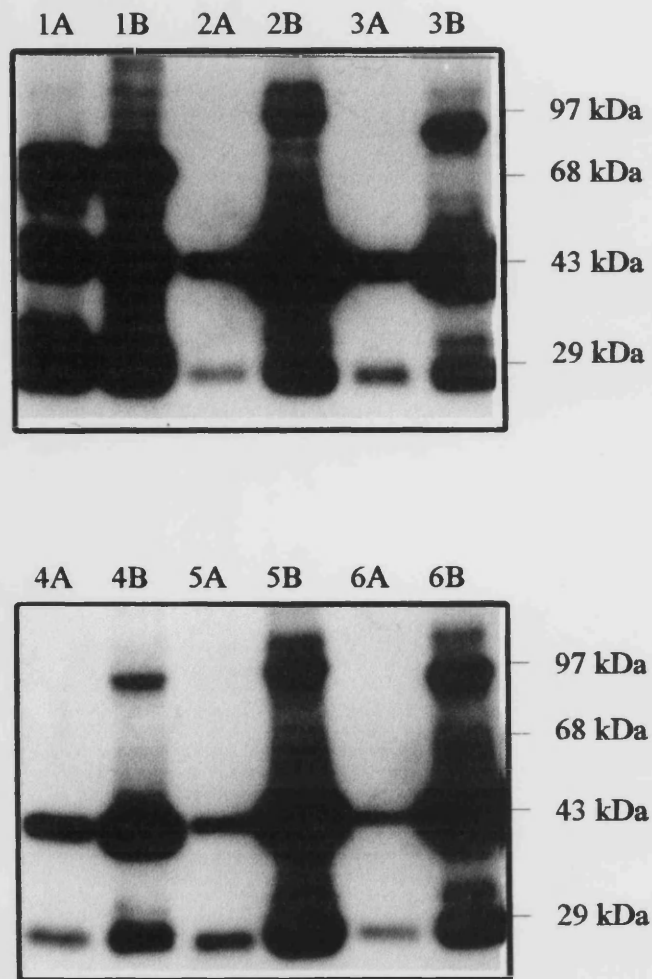
Figure 5.6: Radioactive Labelling of Recombinant Proteins Expressed as GST-2TK Fusions

Fusion proteins were radioactively labelled with ^{32}P-ATP by PK-A whilst associated with glutathione agarose beads and the labelled protein was eluted with glutathione. Approximately 10μg of full length α2 chimaerin (1) and the wild type (2) and mutated SH2 domain constructs (3, E49W; 4, R56L; 5, R73L; 6, N94H) were phosphorylated whilst bound to approximately 100μl of glutathione agarose. Each probe was eluted in a final volume of 1ml and 10μl samples of this (sample set A) and the resin after elution (sample set B) were analysed by SDS-PAGE.

A.
Purification of
Recombinant B13
(p13)
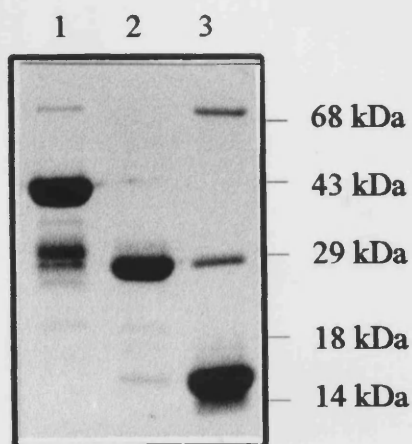
B.
Purification of
Recombinant TOAD-64
(p64)
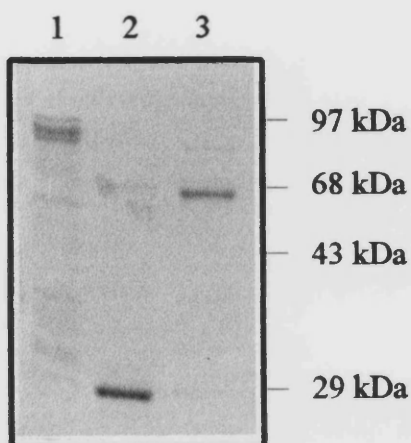
Figure 5.7: Purification of Recombinant B13 and TOAD-64

GST-p13 and GST-p64 were expressed in E.coli and purified from bacterial cell lysates by affinity chromatography on glutathione agarose beads. The protein of interest was cleaved from the GST moiety by proteolysis with thrombin and the purified protein concentrated to a final volume of approximately 600µl. 10µl samples of the full fusion protein associated with glutathione agarose (lane 1), the resin after thrombin cleavage (lane 2) and the purified, concentrated recombinant protein (lane 3) were analysed by SDS-PAGE. Gels containing 12% or 9% v/v acrylamide were used for analysis of p13 and p64 preparations respectively. Proteins were visualised with Coomassie Blue.

Radio-labelled full length GST-α2 chimaerin, wild-type GST-α2 SH2 domain and the set of four GSTα2 SH2 domain proteins with point mutations in their SH2 domains (four proteins mutated at E49W, R56L, R73L or N94H) were eluted and analysed by SDS-PAGE/ autoradiography. (Figure 5.6, 'A' lanes). The glutathione agarose beads after elution were also tested to allow estimation of the efficiency of the elution (Figure 5.6, 'B' lanes). The quantity of the agarose analysed was approximately tenfold greater than that of the probe (i.e. 10% of the total as opposed to 1%). All six proteins were phosphorylated by PK-A. In all cases labelled breakdown products were present; the sizes of these reflected those seen in the Coomassie stained proteins (Figure 5.2). It was estimated that, for the SH2 domain proteins, the elution was at least 50% efficient and for the full-length protein it was closer to 90% efficient.

Recombinant B13 and TOAD-64 were expressed as GST fusion proteins and, in some cases were cleaved with thrombin to release the target protein. Typical purifications of the two proteins are shown in Figure 5.7. GST-p13 has an apparent molecular mass of approximately 43 kDa and the cleaved protein 15 kDa. Occasionally doublets of bands were seen. GST-p64 is 95 kDa and cleaved p64 around 65 kDa. The yield of GST-p13 was routinely higher than that of GST-p64, up to 4mg/l bacterial culture versus 100μg/l.

5B.2 The Interaction of ^{32}P-Labelled Chimaerin Proteins with Recombinant B13 and TOAD-64

To investigate the interactions between chimaerin and the two potential target proteins B13 and TOAD-64, binding assays were performed using recombinant proteins to confirm that direct binding could occur *in vitro*. Two sets of experiments were performed using the chimaerin probes shown in Figure 5.6 to detect either GST-p13 or GST-p64 using the overlay assay protocol described in Section 2B.34A. The filters were blocked in a buffer containing GST and bacterial proteins to eliminate non-specific background binding. It was found that recombinant p13 and p64 could interact with both the SH2 domain and full length wild-type α2 chimaerin probes (Figures 5.8A and 5.9A, GST-p13 and GST-64 respectively) but that these interactions could be affected dramatically by specific point mutations in the chimaerin SH2 domain (Figures 5.8B and 5.9B, GST-p13 and GST-64 respectively). Both the R73L probe and the N94H probe had a very limited ability to interact with GST-13

A.

SH2   FL

→ GST-p13
43 kDa

B.

1   2   3   4   5
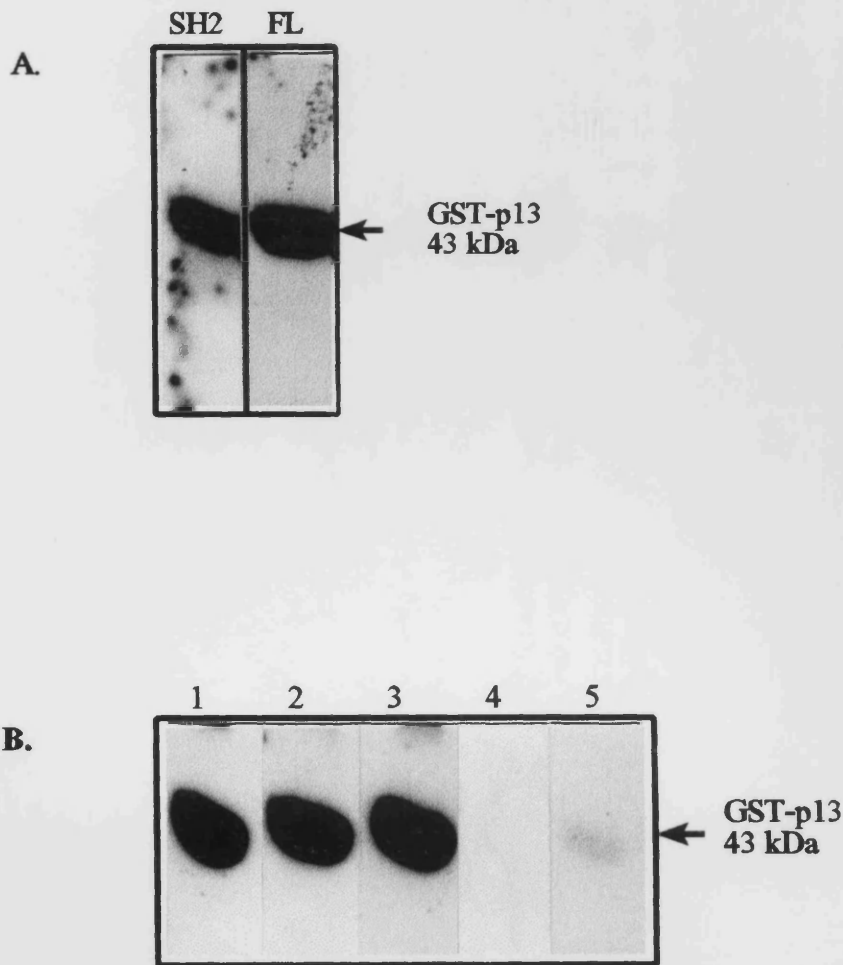
→ GST-p13
43 kDa

Figure 5.8: Detection of GST-p13 by ^{32}P-Labelled α2 Chimaerin Probes

Approximately 5µg GST-p13 samples were electrophoresed on an acrylamide gel and transferred to a nitrocellulose filter. The protein on the filter was denatured, slowly renatured and probed with ^{32}P-GST α2 SH2 domain (Figure A, SH2) or ^{32}P-GST α2 chimaerin (Figure A, FL). GST-p13 samples were also probed with ^{32}P-GST α2 chimaerin SH2 domain constructs with point mutations in their SH2 domains (Figure B). The probes used were: (1) the wild-type, (2) the E49W mutant, (3) the R56L mutant, (4) the R73L mutant and (5) the N94H mutant.

Figure 5.9: Detection of GST-p64 by ^{32}P-Labelled $\alpha 2$ Chimaerin Probes

Approximately 5µg GST-p64 samples were electrophoresed on an acrylamide gel and transferred to a nitrocellulose filter. The protein on the filter was denatured, slowly renatured and probed with ^{32}P-GST $\alpha 2$ SH2 domain (Figure A, SH2) or ^{32}P-GST $\alpha 2$ chimaerin (Figure A, FL). GST-p64 samples were also probed with ^{32}P-GST $\alpha 2$ chimaerin SH2 domain constructs with point mutations in their SH2 domains (Figure B). The probes used were: (1) the wild-type, (2) the E49W mutant, (3) the R56L mutant, (4) the R73L mutant and (5) the N94H mutant.

relative to the wild-type chimaerin probe, although the N94H-p13 interaction was a little stronger than the R73L-p13 interaction. The only point mutation in the chimaerin SH2 domain that reduced the ability of the probe to bind immobilised GST-p64 was the N94H substitution. The different results obtained with the two potential target proteins suggest that the molecular characteristics of their interactions with the chimaerin SH2 domain may differ.

In the experiments described above it was observed that the interaction of the wild-type chimaerin probe with GST-p13 invariably resulted in a stronger signal than the interaction of the probe with GST-p64. This could reflect a genuine difference in the affinities of the two interactions. However, the two proteins may not renature equally well under the conditions used leading to differences in their ability to interact with a probe. To investigate this further binding assays were carried out using cleaved p13 and p64, directly dotted onto squares of nitrocellulose filters. GST and GST-α2 chimaerin SH2 domain probes were prepared by PK-A-mediated phosphorylation of the purified fusion protein in solution as described in Section 2B.33B and the filter-immobilised proteins were not denatured and renatured prior to probing. The amino-terminal 192 amino acid residues of the neutrophil oxidase protein p67^{phox} was used as a control.

The GST probe did not bind any of the proteins whilst the chimaerin SH2 domain probe bound both p13 and p64 (Figure 5.10). Interestingly, p64 was detected as strongly as p13 in these experiments, perhaps indicating that p64/ chimaerin binding is dependent on the native conformation of p64 and that this is not recovered sufficiently after denaturation to permit optimal detection of the interaction *in vitro*. This suggests that the choice of binding assay may be critical in determining whether a substrate can be detected.

5C Further Investigation of the Interaction Between Recombinant TOAD-64 and α2 Chimaerin

5C.1 The Detection of Native TOAD-64 in Fractionated Rat Brain Extract

The first step in the purification of native TOAD-64 from rat brain extract was an S Sepharose fractionation. TOAD-64 was enriched in fractions eluted with 0.1M NaCl and 0.2M NaCl. To confirm that this result could be reproduced, rat brain extract, S Sepharose 0.1M NaCl and 0.2M NaCl fractions, a partly purified preparation of native TOAD-64 (produced by M.Teo) and recombinant p64 (cleaved from GST) were separated by SDS-

A.

B.
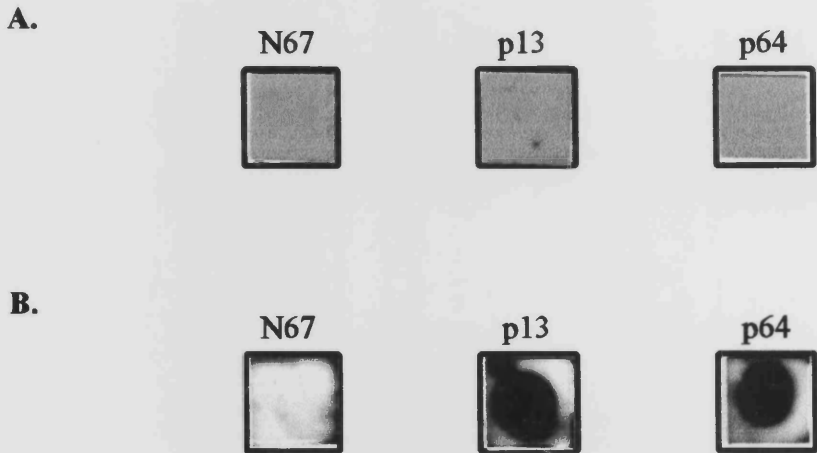
Figure 5.10: An α2 Chimaerin SH2 Domain Probe can Bind Recombinant B13 and TOAD-64 in a Dot Blot Assay

Recombinant B13 and TOAD-64 (marked p13 and p64 respectively) and the amino-terminal 192 amino acid residues of p67^{phox} (N67, obtained from E.Prigmore) were diluted to a concentration of 0.2mg/ml and 10μl of each dotted onto a nitrocellulose square. The filters were blocked with BSA before probing with either ^{32}P-labelled GST (Figure A) or GST-α2 SH2 domain (Figure B). The probes were diluted to give approximately 10^{6}cpm/ml incubation buffer. Each of the proteins tested had been bacterially expressed as GST fusions and cleaved from their GST moieties prior to use.

PAGE, transferred to nitrocellulose and probed with ^{32}P-labelled GST, GST-α2 SH2 domain and GST-α2 chimaerin (Figure 5.11, parts B, C and D respectively). A duplicate gel was run at the same time and stained with Coomassie Blue to visualise protein (Figure 5.11A). The SH2 domain probe bound both the purified native p64 and a protein of the same size in both the S Sepharose 0.1 M and 0.2M NaCl fractions. Other proteins were also detected in these samples, notably proteins of approximately 55 kDa and 30 kDa in the S Sepharose 0.1M NaCl fraction. Whether these correspond to the proteins of similar sizes, tubulin and glyoxylase II respectively, that purify on chimaerin SH2 domain affinity columns is not known. Multiple signals were seen when whole rat brain extract was probed. Interestingly, recombinant and native p64 are not identical in size: the recombinant protein has a slightly lower apparent molecular mass than the native. This was observed to be the case not only in the proteins detected in the binding assay but in the Coomassie stain of these two samples. This apparent difference in molecular mass may be due to phosphorylation of the native protein. Comparable results were obtained using the full length α2 chimaerin probe which bound both the native and recombinant p64 proteins and detected a similar set of proteins in the S Sepharose fractions, albeit not as strongly as the SH2 domain probe. No GST-binding proteins were detected.

Previous attempts to use an overlay assay to detect chimaerin-binding proteins in tissue extracts were of limited success (results shown in Figures 1.14 and 1.15 and 1.16). Several weak signals were detected, two of which were identified as tubulin and actin, but there was no indication of an interaction between chimaerin and a protein with the molecular mass of p64. The different results obtained using two different binding assays, one in which the protein on the filter is denatured in guanidinium chloride and one in which it is not, suggest that technical reasons may have led to the failure to detect native p64 in previous experiments.

5C.2 The Effect of Phosphorylation on the TOAD-64/ α2 Chimaerin SH2 Domain Interaction

Native TOAD-64 is a phosphoprotein and has previously been shown to be a substrate for MAP kinase-mediated phosphorylation (M.Teo, Ph.D Thesis). To determine whether recombinant p64 was a substate for this and other kinases, the experiment was repeated
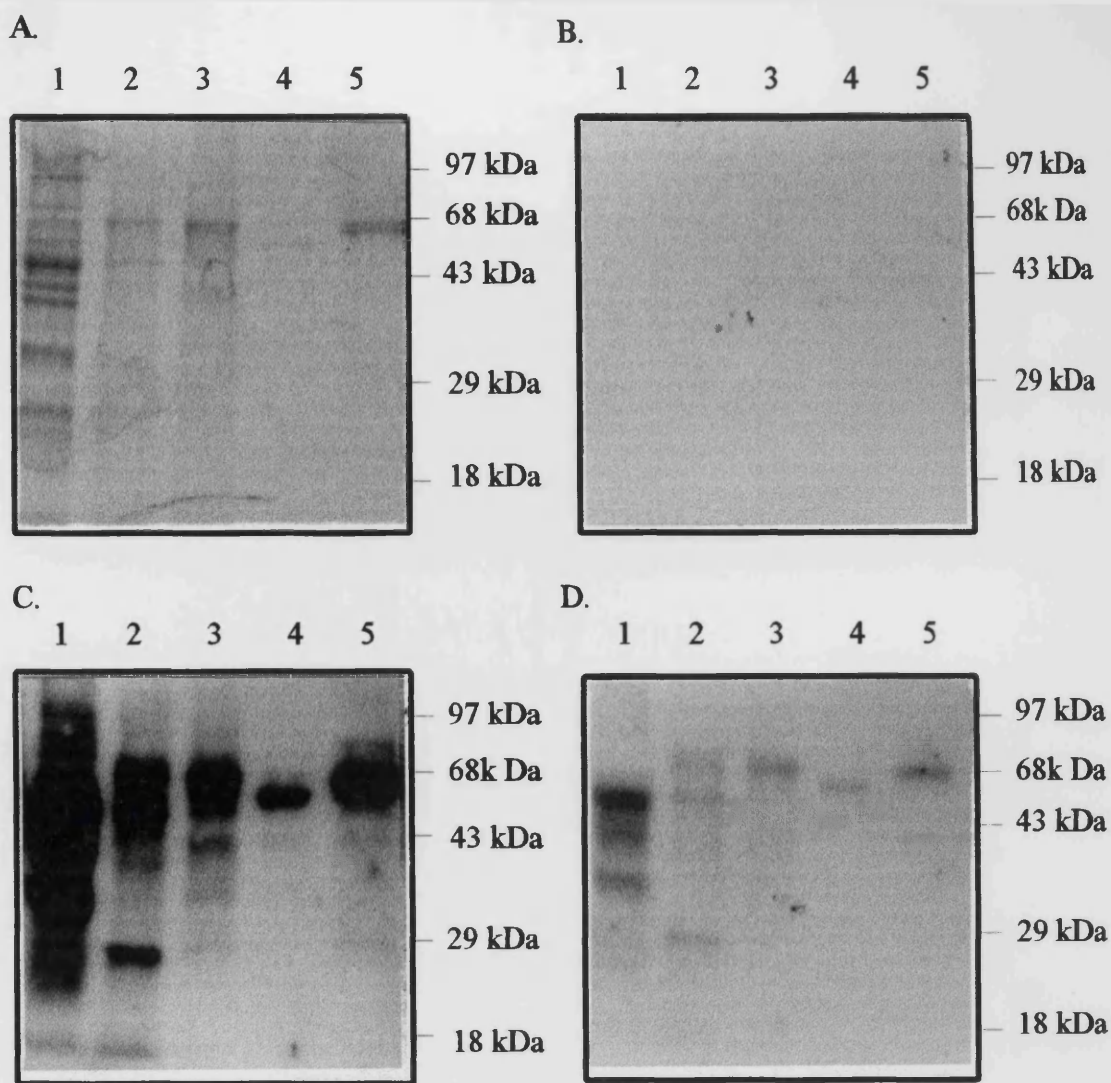
Figure 5.11: The Detection of Native TOAD-64 in Fractionated Brain Extracts

Rat brain proteins extracted in 0.05% Triton-X-100 (lane 1), proteins eluted from an S Sepharose column at 0.1M and 0.2M NaCl (lanes 2 and 3 respectively), recombinant TOAD-64 (lane 4) and a partly purified preparation of native TOAD-64 (produced by M.Teo, lane 5) were separated by SDS-PAGE and either stained with Coomassie Blue (Figure A) or transferred to nitrocellulose and probed with ^{32}P-GST (Figure B), ^{32}P-GST-α2 chimaerin SH2 domain (Figure C) or ^{32}P-GST-α2 chimaerin (Figure D). The brain extract sample analysed was an aliquot of that used for the S Sepharose fractionation. All samples were 20μl with the exception of the recombinant TOAD-64; 1μg of this protein was tested.

using both the recombinant and the native proteins. β-pak, src and brain extract were also tested for their ability to phosphorylate p64 *in vitro*. Approximately 1μg of histone (a general kinase substrate, lane 1), recombinant TOAD-64 (lane 2) and native TOAD-64 (lane 3) were used as kinase substates (Figure 5.12A). To determine whether or not phosphorylation of p64 affected its binding to the α2 chimaerin SH2 domain, a duplicate set of kinase assays were carried out using only 'cold' ATP: phosphorylation will still occur but the products will not be radiolabelled. These samples were then transferred to nitrocellulose and probed with γ^{32}P-labelled GST-α2 chimaerin SH2 domain (Figure 5.12B). If phosphorylation of TOAD-64 causes an increase or decrease in the ability of the protein to interact with α2 chimaerin this will be reflected in the size of the signal seen in the binding assay. For each experiment one set of samples was not treated with kinase; this allowed comparison of the results plus and minus phosphorylation.

It was found that recombinant TOAD-64 and native TOAD-64 were equally effective MAP kinase substrates but that neither was phosphorylated by β-pak. It was difficult to determine whether p64 was phosphorylated by src due to the similar molecular masses of the autophosphorylated kinase and the potential substrate. The results shown in Figure 5.12B demonstrate that the phosphorylation of TOAD-64, either native or recombinant, by MAP kinase does not in turn modulate the interaction of that protein with an α2 chimaerin SH2 domain probe. The only set of proteins whose chimaerin-binding capacity might have been modified by kinase treatment were those phosphorylated by src. However, the reduction in signal strength relative to the control samples was slight and since it was not shown unequivocally that src phosphorylated the TOAD-64 proteins, this point requires clarification

5D The Effect of Recombinant B13 and TOAD-64 on The GAP Activity of α2-Chimaerin

The GAP activity of GST-α2 chimaerin towards GST-Rac1 was determined in the presence of varying amounts of two potential targets of the chimaerin SH2 domain, p13 and p64 (Figures 5.13 and 5.14 respectively). 2μg of GAP, 2μg of the p21 and 1, 2 or 4μg of recombinant p13 or p64 were used in these experiments. It was found that p13 and p64 neither exerted any direct effect on the intrinsic GTPase activity of Rac1 nor modulated the

No Kinase

1   2   3

— 97 kDa
— 68 kDa
— 43 kDa
— 29 kDa

MAPK   β-pak   Src   Brain Extract
1   2   3   1   2   3   1   2   3   1   2   3

— 97 kDa
— 68 kDa
— 43 kDa
— 29 kDa
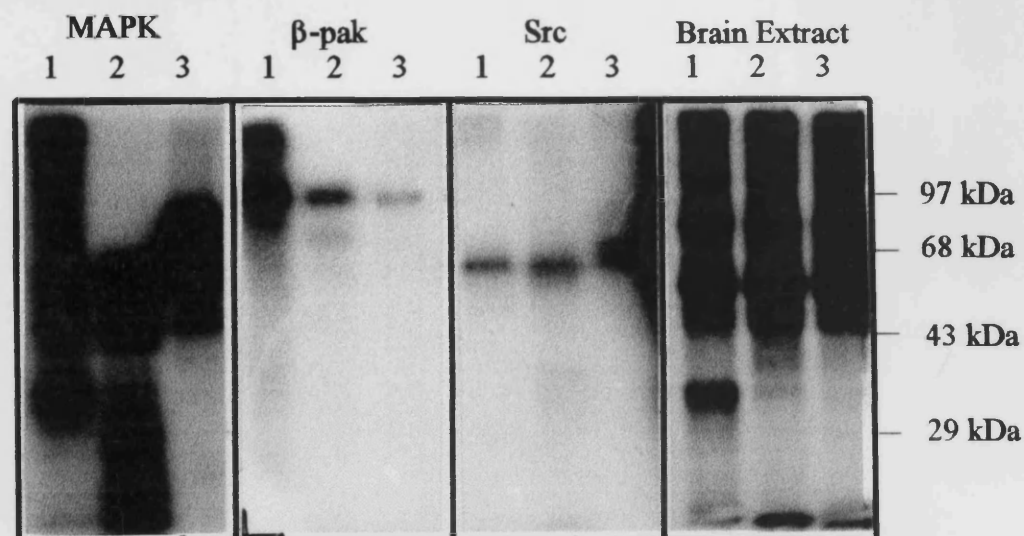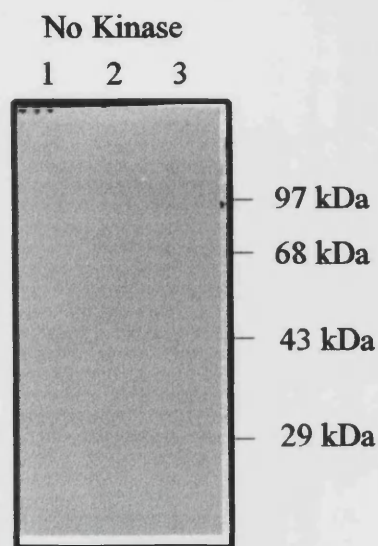
Figure 5.12A: TOAD-64 is a Substrate for Phosphorylation by MAP Kinase

1μg samples of (1) histone, (2) recombinant TOAD-64 and (3) part-purified native TOAD-64 (prepared by M.Teo) were diluted into Kinase Buffer and incubated with γ^{32}P-ATP in the presence of approximately 0.1μg of MAP Kinase, Src or β-pak. In addition, one set of samples was incubated with approximately 20μg rat brain extract and another without added kinase. The kinase reactions were ended by addition of Protein Sample Buffer, the samples separated by SDS-PAGE and the phosphorylation of the TOAD-64 substrates visualised by autoradiography.

No Kinase

1 2 3

— 97 kDa

— 68 kDa

— 43 kDa

— 29 kDa

MAPK   β-pak   Src   Brain Extract
1 2 3   1 2 3   1 2 3   1 2 3

— 97 kDa

— 68 kDa

— 43 kDa

— 29 kDa
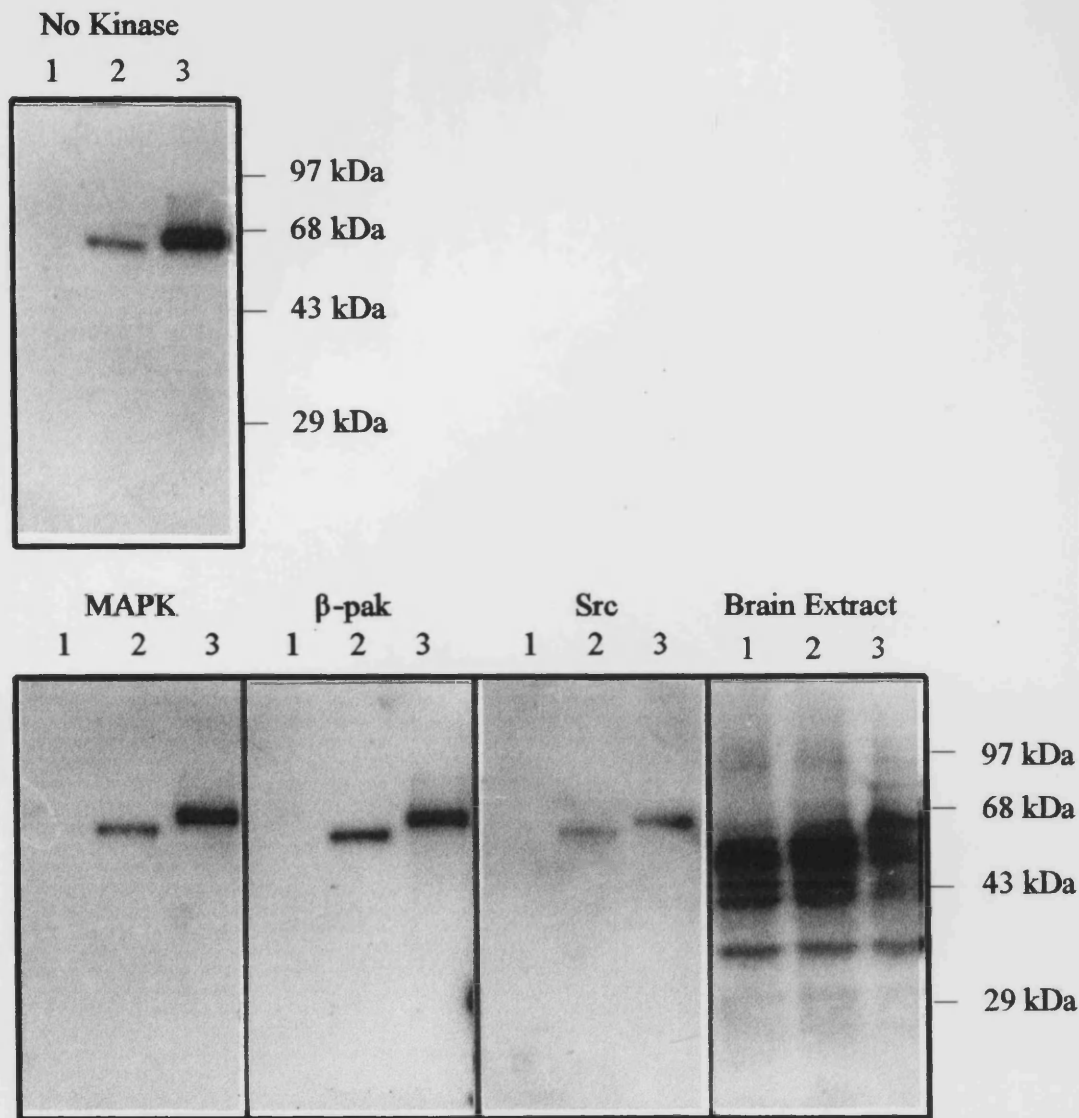
Figure 5.12B: Phosphorylation by MAP Kinase Does Not Affect the Interaction Between TOAD-64 and the α2 Chimaerin SH2 Domain

1μg samples of (1) histone, (2) recombinant TOAD-64 and (3) part-purified native TOAD-64 (prepared by M.Teo) were diluted into Kinase Buffer and incubated with 20μM ATP in the presence of approximately 0.1μg of MAP Kinase, Src or β-pak. In addition, one set of samples was incubated with approximately 20μg rat brain extract and another without added kinase. The kinase reactions were ended by addition of Protein Sample Buffer, the samples separated by SDS-PAGE and proteins transferred to nitrocellulose filters. The filters were blocked with BSA and then probed with ^{32}P-GST α2 chimaerin SH2 domain (diluted to give approximately 10^6 cpm/ml incubation buffer).
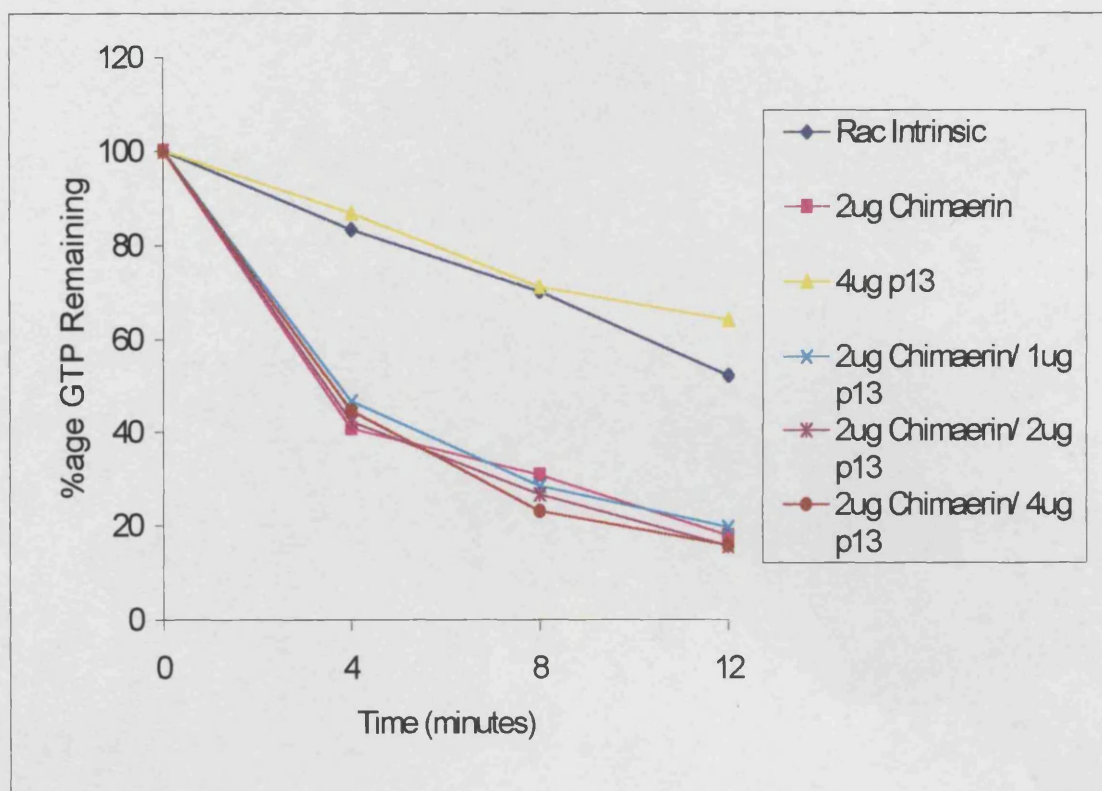
Figure 5.13: GAP Activity of α2 Chimaerin In the Presence of Recombinant B13

γ^{32}P-GTP–loaded GST-rac1 was incubated in GAP assay reaction buffer either alone, to measure its intrinsic GTPase activity, with 2µg GST-α2 chimaerin, with 4µg of recombinant B13 (p13), or with a combination of 2µg GST-α2 chimaerin and 1, 2 or 4µg of p13. Aliquots of the reaction mix were removed at 0, 4, 8 and 12 minutes, dotted onto nitrocellulose, the filters washed and the remaining radioactivity measured in a scintillation counter. To visualise the rate of loss of GTP in the different reactions, the percentage of GTP remaining was plotted against time (time zero = 100%).
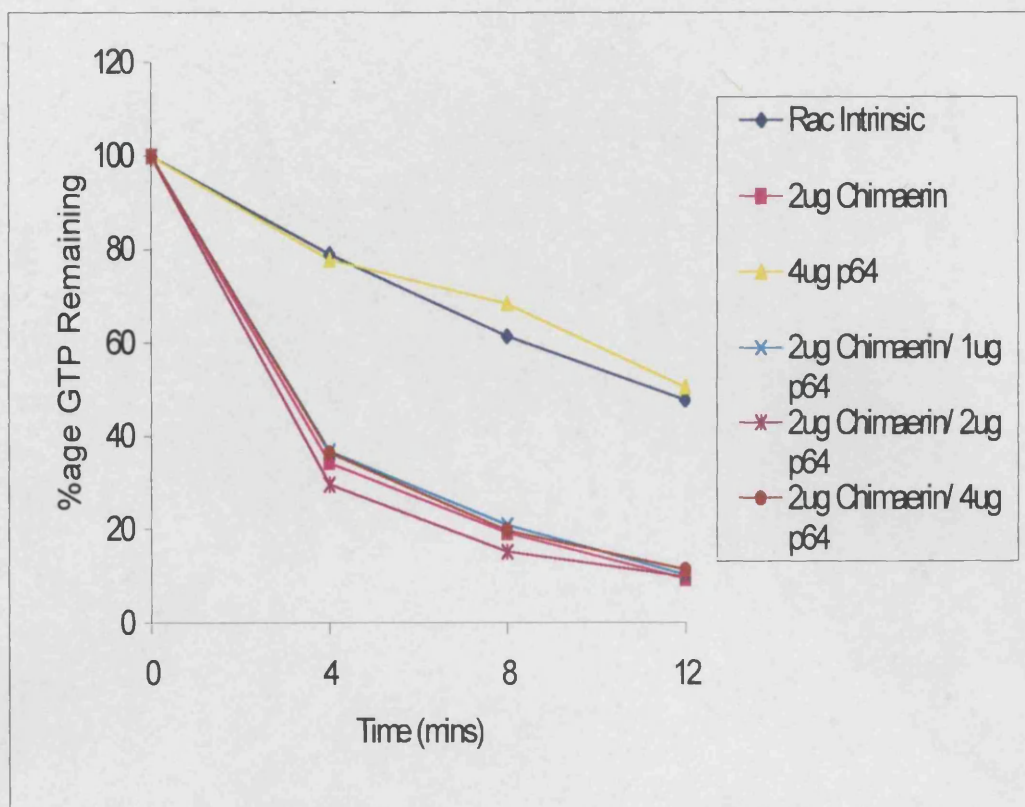
Figure 5.14: GAP Activity of α2 Chimaerin In the Presence of Recombinant TOAD-64

γ^{32}P-GTP–loaded GST-rac1 was incubated in GAP assay reaction buffer either alone, to measure its intrinsic GTPase activity, with 2μg GST-α2 chimaerin, with 4μg of recombinant TOAD-64 (p64), or with a combination of 2μg GST-α2 chimaerin and 1, 2 or 4μg of p64. Aliquots of the reaction mix were removed at 0, 4, 8 and 12 minutes, dotted onto nitrocellulose, the filters washed and the remaining radioactivity measured in a scintillation counter. To visualise the rate of loss of GTP in the different reactions, the percentage of GTP remaining was plotted against time (time zero = 100%).

GAP activity of α2-chimaerin. This is consistent with results obtained by M.Teo using purified native TOAD-64 preparations. However, the possibility that these proteins may modulate the lipid-binding activity of chimaerin or, alternatively, its GAP activity in the presence of lipid cannot be excluded.

5E Summary

Four single amino acid substitutions were made in the α2-chimaerin SH2 domain, the sequences subcloned into a bacterial expression vector and the isolated SH2 domain or the full length sequence expressed as a fusion protein. The ability of the chimaerin SH2 domain to bind a phosphotyrosine agarose column was abolished by R56L and R73L amino acid substitutions. It appeared that an SH2 domain construct with additional amino terminal chimaerin sequence did not bind phosphotyrosine as well as a shorter construct. Interactions between both the α2-chimaerin SH2 domain and the full length protein and two previously-identified putative target proteins, B13 and TOAD-64, were confirmed using radioactively-labelled chimaerin to probe recombinant proteins immobilised on nitrocellulose filters. It was found that point mutation of the chimaerin SH2 domain could drastically reduce these interactions: the R73L mutant did not interact with p13 and binding of the N94H mutant to both p13 and p64 was impaired. An α2-chimaerin SH2 domain probe detected native TOAD-64 in fractionated rat brain extract; the apparent molecular mass of this protein was slightly higher than that of the recombinant. Native and recombinant TOAD-64 proteins are substrates for phosphorylation by MAP kinase but this did not affect their interaction with the α2-chimaerin SH2 domain. No modulation of the GAP activity of α2-chimaerin by recombinant B13 or TOAD-64 was detected.

CHAPTER SIX

RESULTS IV

The Production of Polyclonal Antibodies to Two Potential $\alpha2$ Chimaerin Target Proteins

Polyclonal antibodies were raised in rabbits to two potential chimaerin target proteins, the B13 subunit of the mitochondrial Complex I oxidoreductase (p13) and TOAD-64 (p64). The availability of these reagents would facilitate study of the interaction of these proteins with chimaerin *in vivo*, in immunoprecipitation experiments, and also enable investigation of the cellular distribution of B13 and TOAD-64 themselves. Recombinant B13 and TOAD-64, termed p13 and p64, were expressed as fusion proteins in bacteria and subsequently cleaved from their GST affinity tags, and used as antigens. They were presented as water-in-oil emulsions with Titermax Classic Adjuvant. Unlike Freund's Adjuvant, Titermax does not contain whole M.tuberculosis particles and was recommended as being relatively unlikely to cause complications following injection. Each antigen was injected into two rabbits since the quality of antiserum obtained can vary between rabbits. Serum was prepared from blood samples taken 10-14 days after each of a series of seven injections and tested for the presence of antibodies that could specifically recognise the appropriate antigen by immunoblotting. Whole antiserum was used in these experiments. The different antisera were identified on the basis of the antigen used (p13 or p64) and the rabbit from which they were obtained (numbers 1-4); the four antisera were therefore labelled p13-1, p13-2, p64-3 and p64-4.

6A Confirmation of the Specific Detection of Antigen by p13 and p64 Antisera

To monitor antibody production serum was obtained after each injection at a time when the antibody titre might be expected to be at its peak (10-14 days post-injection) and used to probe antigen immobilised on a nitrocellulose filter. Significant titres of antigen-specific antibodies were observed from the fifth bleed onwards with one exception: the serum labelled p13-2 detected p13 very strongly from the third injection onwards. Approximately 1µg samples of p13 and p64 were Western blotted and probed with antiserum diluted into 1% Marvel/ PBS/ 0.1% Tween-20 (PBS-T20). Typical preparations of p13 and p64 are shown, Coomassie stained, in Figures 6.1A1 and 6.1A2 respectively. In addition to conventional immunoblottting, a duplicate set of experiments were performed using antiserum that had been pre-incubated with 5µg of the appropriate antigen to confirm that
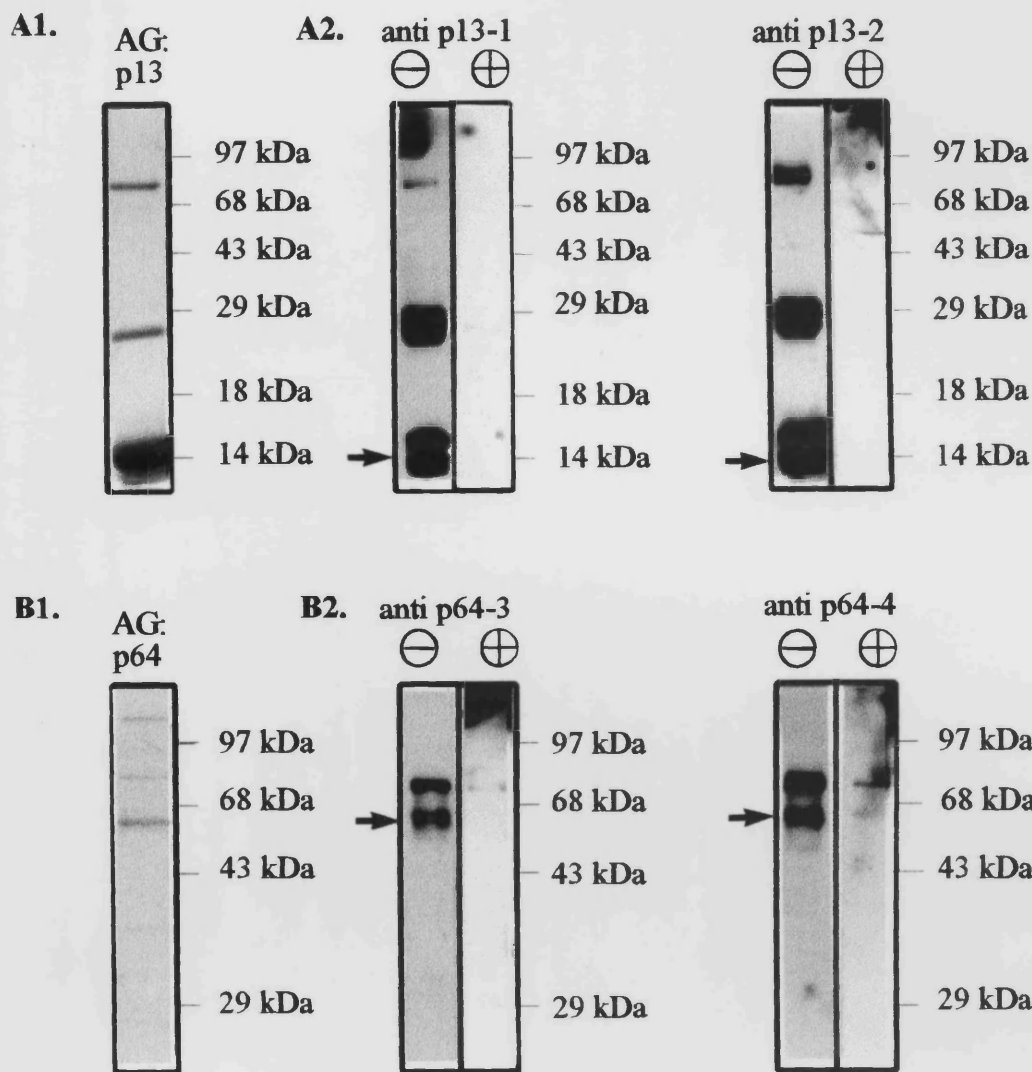
Figure 6.1: Polyclonal Antisera to Recombinant B13 and TOAD-64 can Specifically Detect the Antigens to which they were Raised

Rabbit polyclonal antibodies were raised to the putative chimaerin target proteins B13 (Figure A) and TOAD-64 (Figure B). The recombinant protein antigens (AG), p13 and p64, stained with Coomassie Blue, are shown in Figures A1 and B1. Two rabbits were injected with each antigen. The antisera (labelled 1-4) obtained after six injections of antigen, were used to probe approximately 1μg of antigen immobilised on a nitrocellulose filter (Figures A2 and B2). Duplicate experiments were performed using either the untreated antiserum (samples marked ⊖) or antiserum that had been pre-incubated with 5μg of antigen (samples marked ⊕). Anti p13-1 and anti p13-2 were used at a dilution of 1/5000 whereas anti p64-3 and anti p64-4 were diluted 1/4000. Arrowheads indicate the position of the proteins specifically detected by the antisera.

the antigen was being detected specifically. The antisera were diluted into 1% Marvel/ PBS-T20 and incubated for approximately 16 hours at 4°C either with or without antigen. These two treatments are marked ⊕ and ⊖ respectively in Figure 6.1. p13-1 and p13-2 were used at a dilution of 1/5000 (see Figure 6.1A1) and p64-3 and p64-4 at 1/4000 (see Figure 6.1B2). The antisera used in this experiment were after the sixth injection.

Each antiserum detected the appropriate antigen, as indicated by arrowheads in Figure 6.1, and moreover this could be blocked by pre-incubation with the antigen. The p13-1 and p13-2 antisera detected two distinct proteins of approximately 15kDa. SDS-PAGE analysis of recombinant p13 preparations very often showed a similar doublet of bands of this apparent molecular mass and these were presumed to be the cleaved protein product. It is possible that thrombin cleaves at a site within the p13 sequence of the GST-p13 fusion protein, so giving two protein products. These antisera also detect a doublet of proteins of approximately 29 kDa; this can be blocked by pre-incubation of the antisera with p13. These results would be consistent with the presence of a p13 dimer although there is no other evidence to suggest that this protein does indeed dimerise. Of the two antisera raised to p13, p13-2 gave better results than p13-1: this may reflect a higher titre of p13-specific antibodies, a higher level of antibodies with higher affinity for p13 or a combination of the two.

The two antisera raised to p64 both detect a protein of the expected size (apparent molecular mass of around 65 kDa) and, with equal intensity, a slightly larger protein (apparent molecular mass approximately 80 kDa). This is presumed to a bacterial contaminant present in the protein preparation; a protein of this size was also observed when a p64 preparation was analysed by SDS-PAGE and stained with Coomassie Blue (Figure 6.1B1). Pre-incubation with antigen blocked the detection of both these proteins, indicating that the antisera also contained antibodies specific for the presumed contaminant. p64-3 was less effective than p64-4 in recognising recombinant p64.

To determine the sensitivity of antigen detection, different amounts of recombinant p13 and p64 were Western blotted and probed with antiserum that had been obtained after the seventh, final injection. p13-1 and p13-2 were diluted to 1/5000 and p64-3 and p64-4 to 1/4000. A range of antigen amounts between 25ng and 1μg were probed with the p13 antisera and between 50ng and 1μg with the p64 antisera (Figure 6.2A and 6.2B

A.

Anti p13-1

1μg 0.25 μg 0.1μg 0.05μg 0.025μg

— 97 kDa
— 68 kDa
— 43 kDa
— 29 kDa
— 18 kDa
— 14 kDa

Anti p13-2

1μg 0.25 μg 0.1μg 0.05μg 0.025μg

— 97 kDa
— 68 kDa
— 43 kDa
— 29 kDa
— 18 kDa
— 14 kDa

B.

Anti p64-3

1μg 0.5μg 0.25μg 0.1μg 0.05μg

— 97 kDa
— 68 kDa
— 43 kDa
— 29 kDa

Anti p64-4

1μg 0.5 μg 0.25μg 0.1μg 0.05μg

— 97 kDa
— 68 kDa
— 43 kDa
— 29 kDa
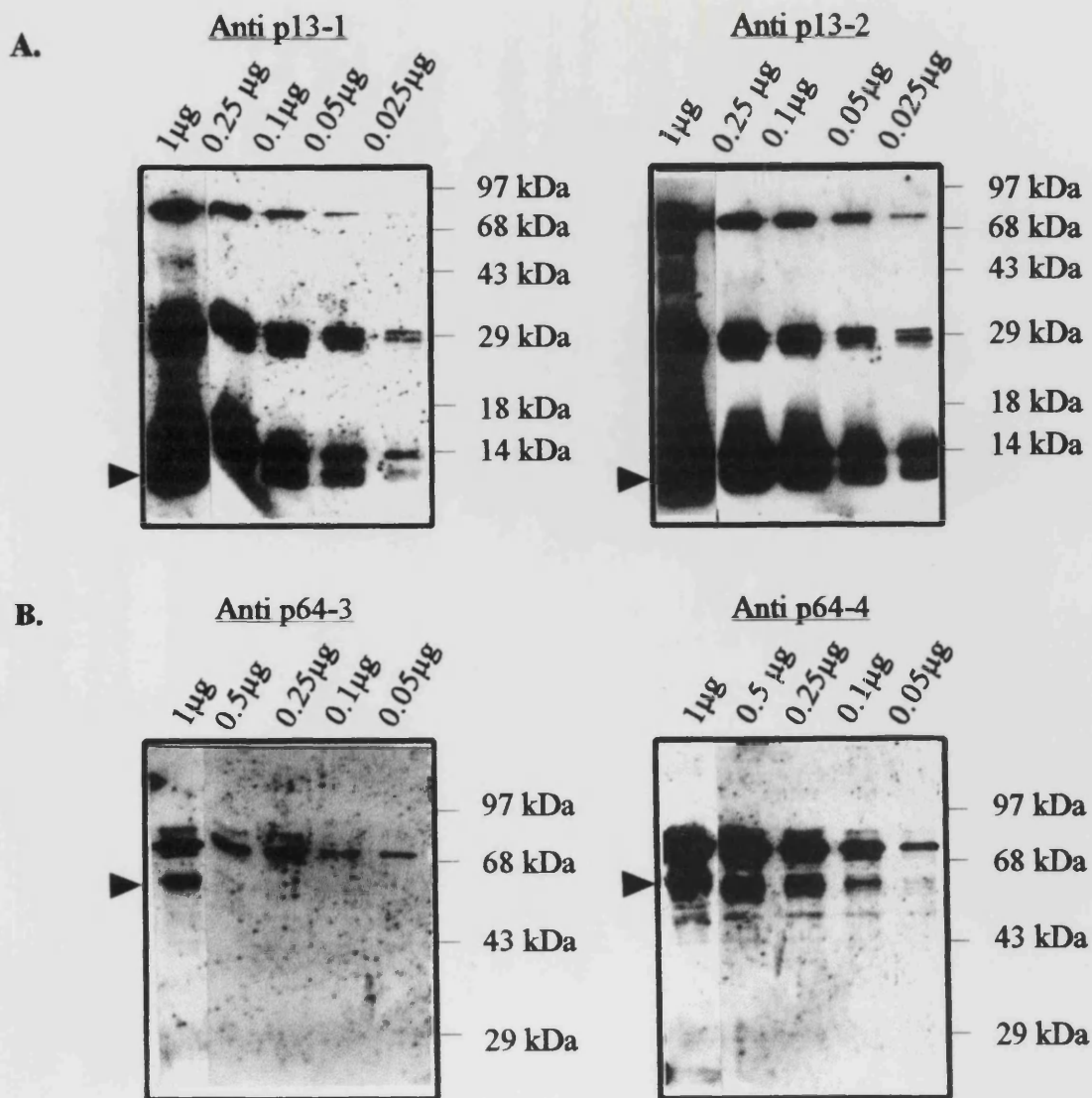
Figure 6.2: The Sensitivity of Anti-p13 and Anti-p64 Antisera Estimated by Immunoblotting

Different quantities of the appropriate antigen were electrophoresed, transferred to nitrocellulose and probed with the four different antisera. The antiserum used and the amounts of antigen tested are as shown in the figure. The antisera used were obtained after seven injections with antigen; p13-1 and p13-2 were used at a dilution of 1/5000 and p64-3 and p64-4 were used at a dilution of 1/4000. Arrowheads indicate detection of the antigen.

respectively). Both p13-1 and p13-2 could detect as little as 25ng of antigen and the strength of signal was such as to suggest that p13-2 at least could certainly detect considerably lower levels of the protein (the ECL results shown were visualised following a ten second exposure). It was found that the limit of detection of p64-4, at the dilution used, was between 50 and 100ng, whereas for p64-3 it was in the range 0.5-1µg. As was the case when using the antisera obtained after the sixth injection, a second protein of approximately 80kDa was detected by both p64-3 and p64-4. The p64-3 antiserum detected this protein with greater sensitivity than the antigen itself (as little as 50ng could be visualised). This suggests that the titre of p64-specific antibodies in the p64-3 antiserum was actually at its peak following the sixth injection. It may be possible to increase the sensitivity and specificity of the p64 antisera by further purification; used in the unpurified state the level of non-specific binding to the filters is relatively high and this precludes the use of exposure times greater than 5-10 minutes.

That the antisera raised against p64 appear to be less sensitive to their antigen than those raised against p13 may reflect differences in the amounts of antigen used for injection: the rabbits injected with p13 received around five-fold more protein per injection than did those injected with p64 (100µg versus 20µg). Alternatively p64 may simply have fewer highly antigenic epitopes than p13.

6B The p13 and p64 Antisera Can Detect Native Antigen

It was confirmed that the p13 and p64 antisera could detect the antigens, bacterially-expressed recombinant proteins, to which they had been raised but, prior to any further studies it was necessary to establish whether they could also recognise a native form of the antigen.

The p13-2 antiserum (obtained after the sixth injection) was used to probe rat brain extract prepared in different concentrations of Triton-X-100 (Figure 6.3 A) and also different sub-cellular rat brain fractions (these were obtained from C.Hall) (Figure 6.3 B). The antiserum was used at a dilution of 1/2500. Rat brain proteins extracted in 0.05% Triton-X-100 and 1% Triton-X-100 were Western blotted and probed with the antiserum (Figure 6.3A, lanes 1 and 2). The 1% Triton-X-100-insoluble fraction was also tested (lane 3). A protein of the appropriate molecular mass (approximately 15 kDa) was detected in the

A.

B.
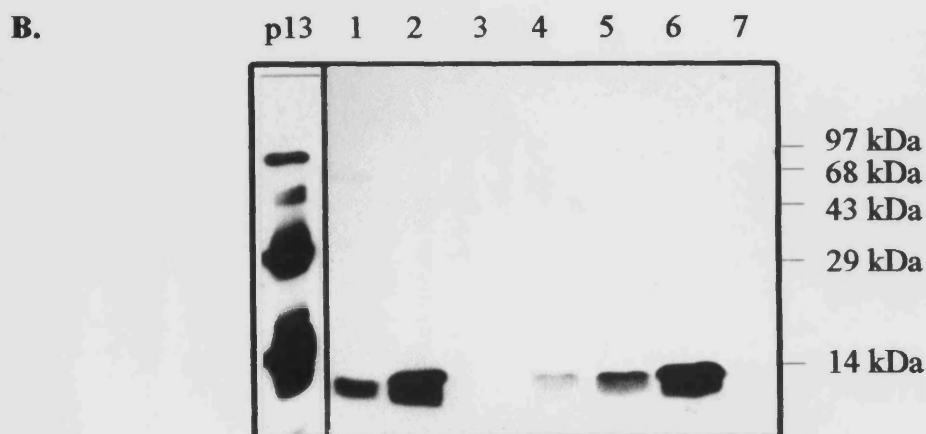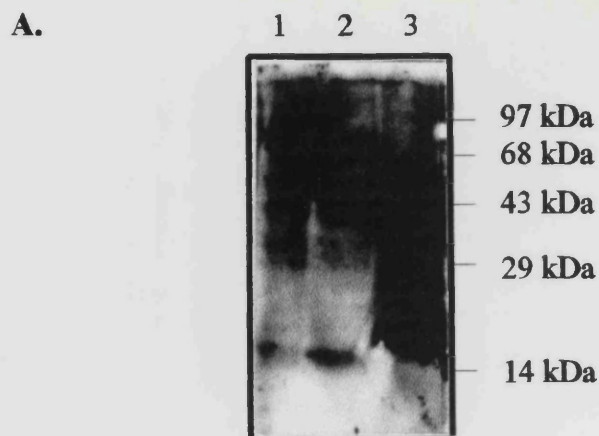
Figure 6.3: The Detection of Native B13

Approximately 80µg of rat brain proteins extracted in 0.05% Triton-X-100 or 1% Triton-X-100 (Figure A, lanes 1 and 2 respectively) were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti p13-2. Approximately 10µl of the rat brain material insoluble in 1%Triton-X-100 was further solubilised by boiling in Protein Sample Buffer and analysed in the same manner (Figure A, lane 3). Figure B shows the results obtained when different sub-cellular fractions prepared from rat brain (obtained from C.Hall) were probed with anti p13-2. The volumes of the different samples were adjusted such that each contained 50µg total protein. The fractions tested were: crude nuclei (lane 1), purified mitochondria (lane 2), synaptic plasma membrane A (SPM A, lane 3), SPM B (lane 4), SPM C (lane 5), intra-terminal mitochondria (lane 6) and cytosol (lane 7). 1µg of recombinant B13 (lane marked 'p13') was also tested. The antiserum was used at a dilution of 1/2500.

1% Triton-X-100 fraction and, less strongly in the sample prepared in 0.05% Triton-X-100. These bands are indicated by arrowheads in Figure 6.3. A number of other proteins in the 30-70 kDa range were also detected. It was not possible to interpret the results obtained when the proteins present in the Triton-X-100-insoluble fraction due to their anomalous electrophoretic mobility, although a high level of signal was seen and it may be that the highest concentrations of p13 were present in this sample.

p13 was identified as the B13 subunit of the mitochondrial Complex I oxidoreductase (C.Monfries). It was predicted that it should be possible to detect the protein in purified mitochondria. Different subcellular fractions were probed with p13-2 and a protein of the correct size was detected in the mitochondrial sample and the interterminal mitochondrial sample (Figure 6.3B, lanes 2 and 6 respectively). Interestingly, a doublet signal was seen, reflecting the results obtained using recombinant protein. Since it cannot be argued that this result may be caused by thrombin cleavage of the full length protein as it could in the previous instance, the results may reflects the existence of differently phosphorylated forms of p13 or different isoforms. Lower levels of the native p13 were also detected in the crude nuclei fraction and the synaptic plasma membrane fraction (lanes 1 and 5 respectively).

TOAD-64 is a neuronally-expressed phosphoprotein. To determine whether the antisera raised against the recombinant protein could detect a native form of the protein, samples of rat brain extract (prepared in 0.05% Triton-X-100 and containing approximately 40µg of total protein were separated by SDS-PAGE, transferred to nitrocellulose and probed with p64-3 and p64-4 antisera that had been obtained after six injections with antigen. The antisera were diluted to 1/4000 and the results are shown in Figure 6.4. A single protein of approximately 68 kDa was detected in the rat brain extract sample by each of the antisera (lane 2). In each case this effect was blocked by pre-incubation of the antisera with recombinant p64 (lane 3). That this was the case indicates that, despite the fact that the protein detected in the rat brain extract had a higher apparent molecular mass than the recombinant protein, they were in fact the same. This result reflects data previously described that indicated that native p64 was larger than the recombinant protein. TOAD-64 is known to be a phosphoprotein and it is possible that the apparent size differential between the native and recombinant p64 proteins may reflect differences in their phosphorylation
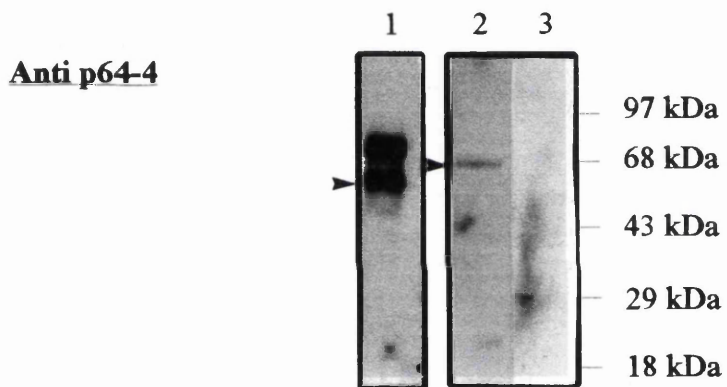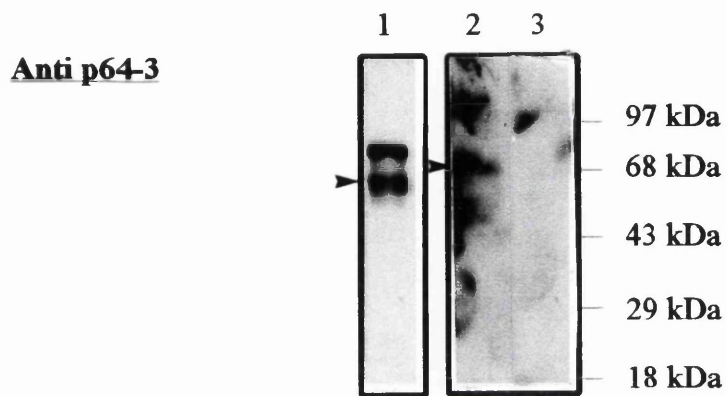
Figure 6.4: The Detection of Native TOAD-64

1μg samples of recombinant TOAD-64 (lane 1) and 10μl of rat brain extract (containing approximately 100μg total protein, lanes 2 and 3) were separated by SDS-PAGE, transferred to nitrocellulose filters and probed with either of the two anti-p64 antisera, anti p64-3 and anti p64-4, as indicated. The antisera were prepared following the sixth injection with antigen and were used at a dilution of 1/4000. The antiserum used to probe the samples in lane 3 had been pre-incubated with 5μg of the antigen. Arrowheads to the left of lane 1 indicate the detection of recombinant p64 and, to the left of lane 2, detection of native p64.

states.

6C <u>Summary</u>

Rabbit antisera were raised against B13 and TOAD-64, two potential target proteins of the chimaerin SH2 domain, using the recombinant proteins as antigens. Two antisera were raised to both p13 and p64 and Western blotting indicated that each could specifically detect the appropriate antigen. The p13 antisera could detect as little as 25ng but the p64 antisera were less sensitive. One of the p13 antisera (p13-2) was found to detect native p13 both in rat brain extracts and in subcellular fractions containing purified mitochondria. Both the p64 antisera detected a single protein in rat brain extract whose apparent molecular mass was around 68 kDa and this result could be blocked by pre-incubation of the antisera with recombinant p64. Further purification of the antisera may increase their sensitivity and reduce non-specific signals.

CHAPTER SEVEN

DISCUSSION

7A The Use of Affinity Chromatography

Affinity chromatography enables the isolation of a protein solely on the basis of its affinity for its binding partner and is therefore a powerful technique but a primary disadvantage is that purified proteins may not interact directly with the protein of interest. Whether this is so can be determined by *in vitro* assays following the purification. The use of multiple chromatographic steps during purification may lead to a loss of the protein determinants required for interaction with its target, for example phosphorylation or critical structural characteristics, such that interaction is lost. If detection of such an interaction rather than the bulk purification of protein is the ultimate goal, the relative speed and specificity of an affinity purification can make it a suitable approach. The literature contains many examples of the use of this technique to identify protein-protein interactions, including the isolation of a GAP for R-Ras directly from bovine brain extract (Yamamoto et al, 1995) and the identification of WASP as a target of the SH3 domains of Src family kinases (Banin et al, 1996). There is no constraint on the moiety that can be used to prepare an affinity column, allowing investigation of the interactions of specific regions of a protein or even co-factors with different proteins. This latter point is well-illustrated by the routine use of affinity chromatography to purify recombinant proteins, for example GST fusions are purified on glutathione agarose columns, polyhistidine-tagged proteins on nickel.

Brain extract was used as a source of protein targets of the RacGAP $\alpha2$ chimaerin. This protein is neuronally expressed and may have a distinct role in the regulation of neuronal morphology. Earlier work using small-scale chimaerin SH2 domain affinity columns indicated that the $\alpha2$ chimaerin SH2 domain could bind phosphoproteins present in PC12 cell extracts and, moreover, that the phosphorylation of these proteins was up-regulated in response to growth factors, implicating $\alpha2$ chimaerin in signalling pathways downstream of activated receptor tyrosine kinase receptor (Hall et al, 1993). Peptides derived from proteins bound to affinity columns could be sequenced and this information might then permit identification of the protein peptide sequencing, potentially placing $\alpha2$ chimaerin within an established signalling pathway. The preliminary experiments described in Figures 3.2 and 3.3 indicated that a large-scale $\alpha2$ chimaerin affinity columns can specifically bind proteins from the complex mixture present in rat brain extract; the distinct set of proteins selected included tyrosine-phosphorylated proteins. The pattern of protein staining seen in the SDS-PAGE

analysis of the proteins eluted from the affinity column did not mirror that seen in the anti-phosphotyrosine blots of the same samples, suggesting that a non-phosphotyrosine-dependent SH2 domain/target interaction was a possibility that could not be excluded at this stage. However, there may have been a loss of protein phosphorylation during sample handling. A further consideration is that since the cellular targets of an SH2 domain-containing protein may contain different protein modules, the likelihood is that they will also be able to form phosphotyrosine-independent interactions. The chimaerin affinity columns bound only small quantities of protein but the results obtained suggested that, if the methodology were adjusted to improve recovery of binding proteins, it might be possible to isolate proteins that interact specifically with the α2 chimaerin SH2 domain using this technique.

7B The Isolation and Identification of Potential Target Proteins of the α2 Chimaerin SH2 Domain by Affinity Chromatography

At least five different proteins have been isolated as potential binding partners of the chimaerin SH2 domain and of these three have been identified as actin, tubulin and glyoxalase II, the first two by immunoblotting and the latter on the basis of peptide sequence information (this work was described in Chapter 3). The two remaining proteins are presumed to be novel.

7B.1 Glyoxalase II as a Target of the α2 Chimaerin SH2 Domain

This enzyme is the second component of the glyoxalase system: glyoxalase I catalyses the conversion of the adduct formed spontaneously from methylglyoxal and glutathione to S-D-lactoylglutathione which is hydrolysed by glyoxalase II to produce D-lactic acid and release glutathione (reviewed by Thornalley, 1993). This enzyme system has been found in both eukaryotes and prokaryotes and is involved in the detoxification of 2-oxoaldehydes such as methylglyoxal (Mannervik, 1990). It is present in most mammalian tissues. It has been suggested that the glyoxalase I/ glyoxalase II ratio is an indicator of cellular proliferative status; a high ratio assures a good scavenging action against the potentially cytotoxic effects of methylglyoxal (Amicarelli et al, 1998). The intermediate in the system, lactoylglutatione, has been reported to mediate diverse cellular effects, including the potentiation of

microtubule assembly *in vitro* (Clelland & Thornalley, 1993) and the inhibition of growth of the HL 60 leukaemic cell line (Edwards et al, 1993). Expression of the Glyoxalase I gene in yeast is regulated by osmotic stress and induction of transcription is mediated by the Hog1 MAP Kinase pathway (Inoue et al, 1998).

A 30 kDa protein bound an α2 chimaerin SH2 domain column (see Figure 3.5, lane 3). The protein sample was resolved into two separate spots when analysed by two-dimensional SDS-PAGE (Figure 3.7). These were pooled and tryptic peptides derived from the protein sequenced. All five of the peptide sequences obtained could be matched with between 75% and 100% identity to a human liver glyoxalase II sequence (Figure 3.8). The existing protein database does not contain a rat brain glyoxalase II sequence. The high level of identity between the human liver sequence and the rat brain-derived peptides indicates a high degree of conservation between species. It is possible two different isoforms of the same protein were sequenced or that rat brain glyoxalase II occurs in differently-phosphorylated forms. Rat brain glyoxalase II was eluted from the chimaerin column in a single step with 1M NaCl and therefore it is not possible to comment on the resistance of the α2 chimaerin-glyoxalase II interaction to salt (Figure 3.5).

The suggestion of a connection between Rac-mediated signalling pathways and the glyoxalase cycle is of interest. The activities of the enzyme components of this metabolic detoxification pathway are developmentally regulated (Amicarelli et al, 1998) and it can be speculated that chimaerin could act as a regulator of glyoxalase II activity during development. High expression levels of a glyoxalase II, RSP29, that is secreted by rat spermatids, have been reported, and this has been suggested to be of importance in the regulation of spermatogenesis (Ji et al, 1997). α2 chimaerin is not expressed in spermatids but β2-chimaerin, which has a highly related SH2 domain is and possibly these two chimaerins have equivalent functions towards glyoxalase II. Major changes in cell morphology and the actin cytoskeleton occur during spermatogenesis and, since Rho family proteins are central mediators of such changes it may be speculated that chimaerin links aspects of development with the required cytoskeletal alterations.

A number of different approaches were used to try to confirm independently whether or not the α2 chimaerin SH2 domain could interact with glyoxalase II but none was successful. The quantity of the affinity-purified protein available was extremely low and

contained a mixture of proteins, but commercially available partly-purified glyoxalase II (obtained from bovine liver) failed to interact with the $\alpha2$ chimaerin SH2 domain *in vitro* (data not shown). Since the source differed from that of the brain glyoxalase II that bound a chimaerin column this may not be informative. Recombinant glyoxalase II was cloned into a bacterial expression vector by C.Monfries but the expressed protein was highly unstable and no reproducible results were obtained when it was tested.

An attempt to repeat the purification of glyoxalase II using an MBP-$\alpha2$ chimaerin SH2 domain were unsuccessful (data not shown) suggesting that its purification on a GST-$\alpha2$ chimaerin SH2 domain column could be an experimental artefact associated specifically with the use of a GST fusion protein. While GST control columns did not appear to bind a 30 kDa protein in the 1M NaCl fraction it is possible that this is misleading. It is relevant that native glyoxalase II can be affinity purified via glutathione (e.g. Ridderström et al, 1996). The GST and GST-$\alpha2$ chimaerin SH2 domain preparations used to produce covalently linked affinity columns contained low levels of residual glutathione, even after dialysis, which can bind Affigel. If the protein sample had been eluted in 10ml of 10mM glutathione, and was then dialysed against three changes of two litres of buffer, the final concentration of glutathione would be approximately (0.01 litre x 0.01 M)/ (3x2) litre, or 3.7μM. The yields of GST-$\alpha2$ chimaerin SH2 preparations were lower than those of GST preparations, sometimes by more than a factor of ten. A consequence of the use of a greater volume of chimaerin preparations than GST to produce affinity columns with equivalent levels of protein could be greater contamination of the column with glutathione. Therefore it is possible that glyoxalase II bound both the chimaerin and control columns, but only in detectable quantities in the case of the former. When chimaerin affinity columns were prepared using glutathione agarose rather than Affigel it would be predicted that any glyoxalase II present would bind to the column on the basis of its affinity for glutathione. However, no 30 kDa protein was eluted when stepwise elutions were performed from such columns (Figure 3.10), although it may be that greater than 1M NaCl would be required to release bound proteins. Several proteins of circa 30kDa, which may include glyoxalase II were detected when such columns were stripped with SDS (Figure 3.10) although these may be bacterial contaminants because proteins of similar size were present in the fusion protein sample.

At the present time the possibility of an interaction between the α2 chimaerin SH2 domain and glyoxalase II has not been excluded. However, attempts to verify this interaction have not been successful to date and, since it is possible that the purification of glyoxalase II represented an experimental artefact, this work has not been pursued.

7B.2 Tubulin and Actin as Targets of the α2 Chimaerin SH2 Domain

When rat cerebellum extract proteins eluted from a chimaerin SH2 domain affinity column were separated by two dimensional SDS-PAGE actin and both α and β tubulin could be identified on the basis of their distinctive patterns of electrophoretic mobility (Figure 3.7). Actin and tubulin were routinely present only in the samples bound to the chimaerin column (Figures 3.12 and 3.13 respectively). Whether these proteins interact directly with α2 chimaerin has not been definitively established. However, a chimaerin SH2 domain probe bound a protein of approximately 55 kDa in the 0.4 M NaCl fraction of rat brain proteins fractionated on Q Sepharose. This fraction also contained the highest levels of β-tubulin (Figure 3.16). Similarly, when proteins were separated on S Sepharose, there was a correlation between the samples in which tubulin was present and the detection of a 55 kDa protein in overlay binding assays. This suggests a direct interaction between α2 chimaerin and tubulin. There is less evidence to suggest that actin binds the chimaerin SH2 domain directly. It has been detected amongst the proteins eluted from an α2 chimaerin SH2 domain affinity column and a chimaerin SH2 domain probe detects a protein of the appropriate molecular weight in rat brain proteins fractionated on Q Sepharose (Figure 3.16B, lanes 2 and 3) but no Western blot analysis was performed to confirm this as actin.

A 55kDa protein is detected in brain extracts prepared from rats at different times between birth and adulthood (Figure 3.14). This is presumed to be tubulin. There did not appear to be a significant variation in the detection of this protein by a chimaerin probe during this period of development. Interestingly, no binding proteins were detected when intestine extract was probed.

The RasGAP neurofibromin (NF) associates with microtubules and the interaction with tubulin down-regulates NF-1 GAP activity (Bollag et al, 1993) which suggests that the interaction between tubulin and a p21-GAP may have physiological relevance. The amino acid residues of NF-1 that mediate tubulin binding are found within the GAP domain

(Gregory et al, 1993), whereas the putative tubulin/α2 chimaerin interaction must be mediated by residues present within the protein used to prepare the affinity column. i.e. with the SH2 domain itself plus the ten amino acid residues to the amino terminal or the thirty-amino acid residues to the carboxyl terminal. It has recently been reported that Ki-Ras binds tubulin in a prenylation-dependent fashion (Thissen et al, 1997). Furthermore, treatment of cells with taxol, an agent that disrupts microtubules, causes an accumulation of Ki-Ras at intracellular locations; Ha-Ras was targeted correctly to the plasma membrane. There may be a physiological connection between the binding of both a p21 GTPase and its GAP to microtubules and it is interesting that Rac has also been shown to bind tubulin (Best et al, 1996). Tubulin is a target for tyrosine phosphorylation, for example by Src (Maness & Matten, 1990), and can interact with the SH2 domains of different signalling molecules including Grb2 and PLC-γ1; binding to tubulin did not preclude binding to phosphotyrosine in these cases (Itoh et al, 1996). This suggests that the α2 chimaerin SH2 domain could interact with tubulin in either a phosphotyrosine-dependent or phosphotyrosine-independent manner. This may be a mechanism for localising α2 chimaerin to a site of Rac activity and possibly promote the formation of multimeric protein complexes. It would be interesting to investigate whether α2 chimaerin preferentially interacts with microtubules or isolated tubulin monomers and whether this association modulates microtubule dynamics.

The carboxyl terminal SH2 domain of PL-Cγ has been reported to interact with actin; as with the SH2 domain/tubulin interactions, the interaction between this SH2 domain and actin did not require the phosphotyrosine-binding pocket (Pei et al, 1996). In addition, interactions between actin binding proteins and SH2 domain proteins have been demonstrated, for example actin-filament-associated protein 110 (AFAP 10) binds the src SH2 domain in a conventional phosphotyrosine-dependent fashion (Guappone et al, 1998). Thus there are precedents for both direct and indirect associations between actin and SH2 domain-containing proteins.

The interactions of tubulin and actin with a chimaerin SH2 domain affinity column is consistent with the α2 chimaerin SH2 domain binding either the cytoskeleton or cytoskeletally-associated proteins. This correlates well with its action in regulating the activity of p21-Rac, a key regulator of the actin cytoskeleton. A connection with tubulin may lie in the putative interaction of α2 chimaerin with glyoxalase II whose substrate has been

reported to regulate microtubule dynamics (Clelland & Thornalley, 1993). Interactions with actin and tubulin may allow localisation of α2 chimaerin to a site of activity or may lead to regulation of its GAP activity.

7C Isolation of Two Novel Protein Targets of the α2 Chimaerin SH2 Domain

Two chimaerin target proteins of approximately 100 kDa and 200 kDa were purified from rat brain extract and peptide sequence information was obtained for each. The p200 peptide could be matched perfectly to a sequence present in cytosolic thymidine kinase (Figure 3.11B). However, this protein exists either as a tetramer or dimer of 24 kDa subunits and so would have a maximum molecular weight of 96 kDa (Munch-Peterson et al, 1993). The best match to a protein of an appropriate molecular weight was to a Rho-associated kinase (ROK) α sequence (93.5% identity). Interestingly, five consecutive amino acid residues of the peptide matched to the ROK sequence and the sixth was a conservative substitution. However, over 50 different database entries contained sequences with this level of similarity to the p200 peptide. The peptide match covered only six amino acid residues and any protein sequences to which the peptides could be matched are candidates but more sequence information would be required to allow a positive identification of the p200 protein.

More sequence data was obtained for the putative 100 kDa target of α2 chimaerin but no protein sequences were identified that matched the peptide sequences 100% and therefore the p100 is presumed to be a novel protein (Figure 3.11B). The best matches found in the database were to inositol 1,4,5-triphosphate receptors (IP_3). These proteins are calcium channels that mediate the release of intracellular calcium in response to IP_3; they contain around 2700 amino acid residues and have a molecular weight of over 300 kDa (e.g. Mikoshiba et al, 1992). The dynein heavy chain, the largest subunit of this microtubule-dependent motor protein, contained sequences with more than 50% identity to three of the four p100 peptides, one of the best matches identified by searching the protein database. Similar levels of sequence similarity were found to titin, a connective protein of around 3000kDa present in striated muscle (Maruyama, 1994). Titin is an extremely large protein and hence the similarity to the p100 peptides may a chance occurrence. The dynein heavy chain and IP_3 receptors are also too large to correspond to the p100 purified but it may be that they are related to the p100 in terms of function, for example inositol phospholipid or

tubulin binding, structure, gene family or some other feature.

The quantities of the two proteins purified was very low. Proteins binding control columns were concentrated and analysed in the same manner and comparison of the two suggested that the 100 kDa and 200 kDa proteins were specifically bound to the chimaerin column. However it is still necessary to determine whether they can interact with α2 chimaerin directly. There is sufficient peptide information from the p100 to allow the design of degenerate PCR primers and the appropriate cDNA could be isolated. Recombinant p100 could then be constructed and its interaction with α2 chimaerin tested *in vitro* but this work has not been within the scope of this project.

7D The Functional Implications of the Atypical α2 Chimaerin SH2 Domain Sequence

The quantities of proteins recovered in affinity purifications were low, suggesting that targets of the α2 chimaerin SH2 domain may be of low affinity or present in brain extract in low abundance. Evidence supporting this was obtained using a phosphopeptide library to identify tyrosine phosphorylated peptide sequences that specifically bound the chimaerin SH2 domains. When tested, no high affinity chimaerin target sequence was present in this library (Z. Songyang, Personal Communication). Another possible cause for low yields of protein targets is that the interaction between the chimaerin SH2 domain requires the presence of additional co-factors or even of other parts of the chimaerin molecule. The central cysteine rich domain binds phorbol esters in a phospholipid-dependent manner and this may itself modulate interaction of the SH2 domain with its target. The site of phospholipid binding to chimaerin has not been identified, although the CRD is known to be required for the modulatory effects of phospholipid on GAP activity. Direct phospholipid-SH2 domain interactions have been demonstrated for the PI-3-K and the Src SH2 domains and these can effectively block alternative interactions with tyrosine-phosphorylated substrates (Rameh et al, 1995). Conversely, it is conceivable that a chimaerin SH2 domain-lipid interaction could promote binding of the physiological target. Alternatively, the target itself may associate with lipid or other co-factors to promote binding.

There is evidence to suggest that regions outside SH2 domains can modulate SH2 domain-target interactions. For example, when a tryptophan to glutamate substitution is made at the βA1 residue in Src, binding to phosphotyrosine is abolished. If the SH2 domain

is associated with its SH3 domain, the effect of the mutation on the interaction with the target is ameliorated and binding at up to 30% of the wild-type levels is observed (Bibbins et al, 1993). The structural basis of this result is not known but resolution of the crystal structure of the ZAP 70 tyrosine kinase indicates a possible mechanism. This protein has tandem SH2 domains, the more amino terminal of which has a phenylalanine at the βA1 position rather than the conventional tryptophan. Sequences from the more carboxyl terminal SH2 domain contribute to the formation of a complete phosphotyrosine binding pocket (Hatada et al, 1995). Since the chimaerin SH2 domain has a glutamate at the βA1 position, a rather less conservative substitution than tryptophan to phenylalanine, it can be speculated that one consequence of this anomaly may be a novel mechanism of target interaction, quite possibly involving non-SH2 domain residues. In all the experiments performed the chimaerin SH2 domain construct contained either 10 or 48 amino acid residues amino terminal to the SH2 domain and 30 amino acid residues at its carboxyl terminal. These regions may be involved in binding any of the proteins that have been described as potential targets of the chimaerin SH2 domain, either by assisting in formation of the correct SH2 domain-ligand binding site or by themselves binding the protein directly. Constructs containing the SH2 domain and the CRD have been prepared to allow further investigation of the possible role that sequences outside the SH2 domain might play in regulating the interaction of chimaerin and its targets.

7E α2 Chimaerin Binds Kinases

Monitoring the enzymatic activity of a protein throughout different stages of purification can greatly facilitate its isolation. Enzymatic studies of the proteins bound to an α2 chimaerin SH2 domain column indicated the presence of kinases (Figure 4.1). The different pattern of signals seen when proteins binding an Abl SH2 domain column were tested indicated that these samples had a different kinase/substrate composition to those binding the chimaerin column, adding weight to the conclusion that these affinity columns were specifically selecting different sets of rat brain proteins. At least one kinase bound the α2 chimaerin SH2 domain. It may be either a serine/threonine kinase or a tyrosine kinase and could phosphorylate full length α2 chimaerin.

7F Analysis of the Different Kinases Binding the Chimaerin SH2 Domain

In-gel kinase assays involve the electrophoretic separation of proteins prior to the kinase assay step. The advantage of this technique is that it is possible to determine the apparent molecular weight of the kinase but kinases that are dependent upon the interaction of multiple subunits for activity, for example receptor tyrosine kinases, will not be active under the conditions used. In general, such assays have been used to investigate the activity of serine/threonine kinases. In the absence of a suitable substrate kinase autophosphorylation is detected but exogenous protein substrate can also be added to the gel. When such assays were performed it was found that the total kinase activity detected was low relative to the *in vitro* assays. This may reflect the limited number of kinases that are active 'in-gel'.

Initial results indicated that the α2 chimaerin SH2 domain bound more than one kinase. Since kinases may bind other kinases it is possible that some of these were purified on the basis of their affinity for a kinase that bound the chimaerin SH2 domain. Multiple bands were detected in the 90-100 kDa region when α2 chimaerin was present as a substrate; these may indicate differently phosphorylated states of a single kinase although only a single band was seen when MBP was used as a substrate, suggesting that there was more than one kinase present and that their substrate specificities were not identical (Figure 4.2, lanes 9-12). A kinase activity of higher molecular mass, circa 150 kDa, was also observed (B, lane 11) when the chimaerin substrate was present. The level of kinase activity detected during these experiments in the absence of exogenous substrate was negligible (C, lanes 9-12).

When glutathione agarose-α2 chimaerin SH2 domain columns (rather than covalently-linked Affigel columns) were used to purify proteins slightly different results were obtained, particularly regarding the level of kinase seen in the absence of protein substrate, which was much greater (Figure 4.3). The predominant kinase activity detected was in the 90-100 kDa region as previously but the signal strength was much greater than in the earlier experiments. This obscured any weaker signals in this region and hence it could not be determined whether one or several kinases was present. It could not be determined whether this was the same kinase as that detected in earlier experiments. A kinase of around 150 kDa was also observed, even in the absence of substrate which was not the case when binding proteins had been purified on covalent Affigel affinity columns. A 43 kDa kinase activity that

had not been detected previously bound the α2 chimaerin column and phosphorylated full length α2 chimaerin.

The interaction of the 43 kDa kinase with the α2 chimaerin SH2 domain appeared to be specific: a kinase of this size was not detected binding to a control GST column in any of the experiments described in Chapter 4. Whilst its detection appeared to be dependent on the presence of chimaerin, the signals detected were relatively weak and it may be that the assay was not sufficiently sensitive to detect autophosphorylation of this kinase. The use of testis rather than brain extract as the starting material for the purification of the kinase did not significantly improve the yield of any of the kinases of interest, but did contain a 43 kDa kinase that bound specifically to the chimaerin SH2 domain column and phosphorylated full-length α2 chimaerin (Figure 4.7).

Proteins corresponding to the kinases detected could not be visualised by Coomassie Blue staining, suggesting that they were present at very low concentrations (see Figure 3.9). Attempts to enrich for the protein prior to the affinity chromatography step by fraction of rat brain extract proteins was not successful (Figures 4.4 and 4.5).

7G Potential Regulation of α2 Chimaerin by Phosphorylation

Interaction between the chimaerin SH2 domain and kinases implies that the activity of full length α2 chimaerin may be regulated by phosphorylation. It could be speculated, for example, that a possible explanation for the high *in vitro* GAP activity of α2 chimaerin relative to α1 chimaerin (Hall et al, 1993) is that recombinant proteins lack their correct phosphorylation pattern, which in the case of α2 chimaerin could potentiate an intramolecular interaction that could effectively inhibit its GAP activity. The interaction between p190RhoGAP and p120 RasGAP is dependent upon tyrosine phosphorylation of p190 (Bryant et al, 1995). Two phosphotyrosine-containing peptides in p190 Rho GAP bind the tandem amino terminal SH2 domains of p120-Ras GAP. It has been reported that this interaction induces a conformation al change in p120, increasing the accessibility of its SH3 domain to potential target proteins (Hu & Settleman, 1997). Analogously, it is possible that phosphorylation of α2 chimaerin might allow it to interact with a target protein resulting in a conformational change within chimaerin that affects some aspect of its function, the interactions of the SH2 domain, the ability to bind phospholipid or the GAP activity. The

levels of p190 phosphorylation have been shown to be reduced in cells lacking p120-GAP which suggests that phosphorylation of this RhoGAP can be modulated by its binding partner (van der Geer et al, 1997). An alternative suggestion is that phosphorylation may regulate α2 chimaerin directly: the activity of vav, a nucleotide exchange factor for Rho family proteins, is stimulated by tyrosine phosphorylation (Crespo et al, 1997). Interestingly, vav also contains an SH2 domain. A different RhoGAP, graf, has been reported to be a substrate for serine phosphorylation by MAP kinase (Taylor et al, 1998), suggesting that phosphorylation may be a general mechanism by which GAPs for Rho family proteins are regulated.

7H   The Identification of Kinases Binding α2 Chimaerin SH2 Domain Affinity Columns

It is possible to speculate on the identity of the kinases that bind the α2 chimaerin SH2 domain on the basis of their apparent molecular masses. MAP kinases are candidates for the 43 kDa kinase activity. Members of this family of serine/threonine kinases range in molecular weight from around 35 to 55 kDa. The kinase detected binding to the chimaerin column was consistently found to co-migrate with the 43 kDa molecular weight marker and, of the MAP Kinases, this would suggest that it may be one of the ERKs (ERK 1 is a 42 kDa protein and ERK a 44 kDa protein) or JNKs (JNK 1 is 46 kDa, JNK 2 44kDa). Furthermore, MAP kinases have previously been shown to be active under the conditions used. Previous work indicated that the chimaerin SH2 domain could bind a phosphoprotein of approximately 38 kDa in PC12 cells (Sin, Ph.D. Thesis, 1994). Immunoblotting indicated that this was neither ERK1 or ERK2 but the methodolology used differed from that described in this thesis and it not possible to exclude the ERKs as candidates for the 43 kDa kinase binding the α2 chimaerin SH2 domain affinity column. Samples of the proteins binding the column were probed with anti-JNK 1 but no protein of the appropriate size was detected (data not shown) although it is possible that the antibody used was not sufficiently sensitive to detect the very low levels of protein present. MAP kinases phosphorylate a variety of cellular substrates upon activation and it is possible that, even if the 43 kDa kinase does not bind the α2 chimaerin SH2 domain directly, that its ability to phosphorylate it is of physiological significance, as discussed previously. Interestingly, Rac1 can stimulate JNK activity and if,

in its turn JNK could phosphorylate chimaerin and modulate its GAP activity this may then feedback and regulate Rac activity.

Rho-associated kinase is a serine/threonine kinase with an apparent molecular weight of 160 kDa and is therefore a candidate for the higher molecular weight kinase that bound chimaerin affinity columns. A Western blot was performed using anti-ROK β but no protein of the appropriate size was detected (data not shown). Again, this did not positively exclude this kinase as a chimaerin target because the quantity of protein present may have been too small to detect. There are also other related kinases of this size and hence it would have been necessary to use several different antisera.

Two novel proteins, p100 and p200, were identified amongst those binding α2 chimaerin SH2 domain affinity columns. It is possible that p200 may correspond to a kinase whose molecular mass is closer to 160 kDa: the gels used would not resolve proteins of these molecular masses sufficiently to allow their sizes to be described with any accuracy. Interestingly, the p200 peptide had 93.5% similarity to a sequence present in ROKα. The p100 peptide sequences could not be matched to the sequence of a known kinase but it is interesting that the chimaerin SH2 domain did bind at least one kinase of circa 90-100 kDa.

7I Investigation of the Molecular Basis for the Interaction of the α2 Chimaerin SH2 Domain with its Targets

The α2 chimaerin SH2 domain was mutated at four sites known to affect the interaction with phosphotyrosine peptide substrate in the Src SH2 domain (Bibbins et al, 1993). Proteins containing these amino acid residue substitutions were tested for effects on phosphotyrosine binding using affinity chromatography on a phosphotyrosine column. The interaction between an SH2 domain and phosphotyrosine may be up to a thousand-fold less avid than the interaction between the SH2 domain and its specific phosphopeptide target but Koegl et al (1994) demonstrated that phosphotyrosine agarose columns could be used to purify SH2 domains from cell extracts and this is a convenient assay system.

Preliminary experiments indicated that purified GST-α2 chimaerin SH2 domain could bind phosphotyrosine agarose and glutathione agarose equally effectively (Figure 5.4). This contrasted with results obtained using the full length protein was tested: only around 50% of the available protein bound phosphotyrosine. This suggests the possibility of an

intramolecular interaction which could reduce the availability of the SH2 domain for its phosphotyrosine-containing targets. This could potentially act as a mechanism for regulating the localisation or GAP activity of the full length protein. An intramolecular interaction between the Src SH2 domain and a phosphorylated tyrosine residue at the carboxyl terminus is known to inhibit the kinase activity of this protein (Xu et al, 1997).

A wild-type GST-α2 SH2 domain protein containing forty eight amino acid residues of chimaerin sequence amino terminal to the SH2 domain, unlike that in the previous experiment which had only ten amino acid residues amino terminal to the SH2 domain with phosphotyrosine, did not bind phosphotyrosine as effectively as glutathione. Only around 20% of the available protein was retained (Figure 5.5A). This may simply reflect a methodological difference since in the previous case a purified fusion protein was tested for binding to the column. However, since full length α2 chimaerin also appeared to have a reduced ability to bind phosphotyrosine relative to the isolated SH2 domain, it is tempting to speculate that the extreme amino terminal region of α2 chimaerin is involved either in the formation of an intramolecular interaction reducing the availability of the SH2 or in causing a steric block with the same effect. Either constraint could potentially be removed by conformational alterations elsewhere in the protein in response to binding of a target protein or possibly phospholipid.

7J **The Chimaerin SH2 Domain has Distinct Phosphotyrosine-Binding Characteristics**
Analysis of the binding to a phosphotyrosine agarose column of α2 chimaerin SH2 domain proteins with point mutations indicated that alteration of both the invariant βB5 (R73L) and the highly conserved αA1 (R56L) arginine residues could abolish the interaction (Figures 5.5A and 5.5B). Solution of the crystal structure of the Src SH2 domain complexed with its phosphopeptide ligand revealed that the βB5 arginine co-ordinates with the phosphate of the target peptide (Waksman et al, 1992) and a point mutation at this position in the Src SH2 domain abolishes binding to phosphotyrosine (Bibbins et al, 1993). However, this was not the case when the Src αA1 arginine was mutated: this mutation reduced binding to phosphotyrosine by only 20% as opposed to the complete abolition of binding that resulted from an equivalent mutation in the chimaerin SH2 domain (R56L). These results suggest that the residues that are directly involved in co-ordinating the phosphate of the target

234

peptide may differ between the Src SH2 domain and the chimaerin SH2 domain and that the αA1 arginine has a more critical function in chimaerin than Src. Mutation at the βD4 position in the chimaerin SH2 domain (N94H) appeared to have little effect on phosphotyrosine binding. In Src SH2 domains this residue forms part of the mouth of the binding pocket and has been implicated in maintaining the geometry of the active site but a histidine (wild-type) to asparagine substitution has little effect on binding to phosphotyrosine (approximately 20% reduction). It is more probable that any effect would be related to the selection of a specific peptide substrate. Mutation of the chimaerin SH2 domain βD4 residue (N94H) may have reduced phosphotyrosine binding relative to the wild-type on a scale similar to that seen when this residue was mutated in Src, but it was not possible to quantify the data obtained precisely.

The chimaerin SH2 domain sequence is anomalous in that its first residue (the βA1 position, E49) is a glutamate rather than the conventional tryptophan that is found in all except three other SH2 domains (Leung et al, 1994, Hatada et al, 1995 and Thompson et al, 1996). This residue is critical to the structure of an SH2 domain whose discrete modular structure is dependent on the close apposition of the amino and carboxyl termini. A tryptophan to glutamate substitution in the Src SH2 domain abolishes binding to its tyrosine-phosphorylated peptide whereas a glutamate to tryptophan mutation at this position in the chimaerin SH2 domain (E49W) has no obvious effect on the interaction with a phosphotyrosine column. This suggests that this residue cannot be of such critical importance in the chimaerin SH2 domain, although it is possible that different results would be obtained if the binding of this mutant to a high affinity peptide ligand were investigated..

These results suggest that the overall tertiary structures of the chimaerin and other SH2 domains may differ. The more amino terminal of the ZAP-70 SH2 domains, which has a phenylalanine at the βA1 position, requires regions of the more carboxyl terminal SH2 domain to produce a complete phosphotyrosine binding site. By analogy, the chimaerin SH2 domain may interact with phosphotyrosine using a similar mechanism. If sequences outside the SH2 domain are required for the interaction with phosphotyrosine, it would appear unlikely that they are at the amino terminal since, when these sequences are present, for example in the full length protein and certain SH2 domain constructs, interaction with phosphotyrosine was reduced. The activity of the SH2 domain could also be regulated by

binding of other molecules to different sequences in full length α2 chimaerin.

7K Point mutation of its SH2 Domain Does not Affect α2 Chimaerin GAP Activity

Mutation of the chimaerin SH2 domain did not appear to affect the GAP activity of the full length protein (Figure 5.3) although it is possible that, in a more sensitive analysis, more subtle effects would be observed. This would suggest that the SH2 domain does not play a regulatory role in the activity of the full length protein but bacterially-expressed recombinant fusion proteins were used in these experiments and the SH2 domain may not be fully active or may lack post-translational modifications which could be critical for such activity. When a point mutation was introduced into its PH domain, the GAP activity of p120RasGAP was reduced in response to tyrosine phosphorylation by Src (Nakata & Watanabe, 1998). This suggests that GAP activity may be sensitive to changes in other regions of a multi-domain protein although perhaps only in the presence of an appropriate modulator.

7L.1 The Interaction of α2 Chimaerin and A Potential Target Protein, p64-TOAD

The α2 chimaerin SH2 domain binds a protein of approximately 60 kDa that is present in rat brain extracts (Teo, Ph.D Thesis, 1994). This was subsequently identified as the neuronally expressed phosphoprotein, TOAD-64, on the basis of peptide sequence information. Recombinant TOAD-64 (Figure 5.7), hereafter referred to simply as p64, has been expressed and used as a target substrate to study the interaction of the α2 chimaerin SH2 domain with a potential binding protein *in vitro* (Figure 5.14). It was confirmed that recombinant p64 could interact directly with radiolabelled full length or SH2 domain α2 chimaerin probes (Figure 5.9A). The presence of recombinant p64 did not affect the GAP activity of α2 chimaerin *in vitro* assays, arguing against a direct role in regulation of the function of this protein although the assay conditions may not have permitted detection of any modulatory effect. Alternatively, the interaction between α2 chimaerin and TOAD-64 may affect the localisation of α2 chimaerin, for example recruiting it to the site of Rac activation, whereupon it could down-regulate Rac activity. Partly-purified native TOAD-64 does not affect α2 chimaerin GAP activity (Teo, Ph.D. Thesis, 1994) and therefore the results obtained with the recombinant protein correlate well with earlier findings.

Bacteria do not contain tyrosine kinases although serine/threonine phosphorylation

can occur. Thus, these binding assays will only detect proteins whose binding is not phosphotyrosine-dependent. However, at least one potential α2 chimaerin SH2 domain target had been identified using a system in which mammalian tyrosine kinases are not present (the B13 subunit of the mitochondrial Complex I oxidoreductase). Furthermore, binding studies using mutated chimaerin SH2 domain proteins had suggested that the chimaerin SH2 domain interaction with phosphotyrosine may differ from that described in other cases, possibly indicating a novel mechanism of substrate binding that may or may not be dependent on phosphotyrosine. Phosphotyrosine-independent SH2 domain interactions have been described in the literature (references in Section 1J.5).

7L.1   The Interaction of α2 Chimaerin SH2 Domain Mutants with Recombinant TOAD-64

Mutation of the chimaerin SH2 domain at the βD4 position (N94H) could abolish the interaction chimaerin SH2 domain/p64 interaction *in vitro* (Figure 5.9B). This mutant could still bind a phosphotyrosine agarose column, suggesting that these two binding activities are distinct (Figure 5.5A). The predicted position of this N94 amino acid residue within the tertiary structure of the SH2 domain is at the mouth of the phosphotyrosine-binding pocket. The substitution of a residue with a bulky, basic side-chain for one with an uncharged polar sidechain may affect the availability of the binding site to its target or reduce the affinity of the interaction. Structural studies indicate that the sidechains of threonine and serine, when phosphorylated, cannot extend sufficiently into the binding pocket to co-ordinate with the invariant arginine (Lee et al, 1994). It can be speculated that if this were the case perhaps amino acid residue sidechains in other regions of the binding site play a role in the formation of a stable SH2 domain-target complex. Mutation of the βB5 arginine (R73L) would then not affect the interaction whereas mutations of residues elsewhere would. Native TOAD-p64 is known to be a phosphoprotein and therefore it is possible that its interaction with chimaerin is phosphorylation-dependent if not phosphotyrosine-dependent. *In vitro* kinase assays demonstrated that native and recombinant p64 were substrates for phosphorylation by the ERK1 MAP kinase but phosphorylation did not appear to alter the interaction with a chimaerin SH2 domain probe in overlay binding assays (Figures 5.12A and B).

237

7L.2 The Apparent Molecular Weight of Native TOAD-64 is Greater than that of Recombinant TOAD-64

When partly-purified native TOAD-64 samples (prepared by M.Teo) and recombinant p64 were immobilised on nitrocellulose and probed with radiolabelled $\alpha 2$ chimaerin probes they were found to differ slightly in their apparent molecular weight, the native protein being the larger of the two (Figure 5.11). It was also confirmed that full length $\alpha 2$ chimaerin could bind the native p64 as well as the recombinant, although both these interactions appeared weaker than those seen when a chimaerin SH2 domain probe was used. When antibodies were raised to recombinant p64 and used to probe brain extract proteins a single protein with a higher molecular mass than the recombinant p64 was detected, implying that the native protein truly does have a higher apparent molecular mass than the recombinant, possibly as a result of post-translational modification (Figure 6.1). However, TOAD-64 is a member of the CRMP family of proteins and the possibility cannot be excluded that the p64 cDNA clone from which recombinant p64 was produced actually encodes a different CRMP to that which is detected in brain extracts by $\alpha 2$ chimaerin probes in overlay assays.

7L.3 TOAD-64 as a Target of the $\alpha 2$ Chimaerin SH2 Domain

TOAD-64 is a neuronal phosphoprotein that is highly expressed in post-mitotic neuronal cells, decreasing to lower levels in the adult (Minturn et al, 1995a). This correlates well with the expression pattern of $\alpha 2$ chimaerin mRNA (Hall et al, 1993). The precise function of TOAD-64 is as yet unclear. However, a TOAD-64/Unc-33-related protein, CRMP (collapsin response mediator protein, Goshima et al, 1995) is believed to act downstream of receptors activated by collapsin, a member of the semaphorin family of neurotransmitters. Micro-injection of anti-CRMP antibodies into DRG neurons inhibits collapsin-induced growth cone collapse. Recently, Rac1 has been implicated in collapsin-mediated signalling pathways (Jin & Strittmatter, 1997). When triturated into DRG neurons, dominant-negative (N17) Rac1 inhibits collapsin-induced growth cone collapse whereas constitutively-activated (V12) Rac1 partially mimics the effects of collapsin. These effects appear to be specific to Rac1 since Cdc42 mutants have no detectable effects on collapsin-induced growth cone collapse. The involvement of Rho in collapsin initiated signalling pathways is more difficult

to determine as inhibition of Rho with C3 toxin induces complex effects on growth cone morphology in the absence of collapsin.

At the present time it is unclear whether CRMP acts upstream or downstream of Rac in the collapsin pathway and the relationship between CRMP family proteins, Rac1 and α2 chimaerin can only be speculated upon. Antiserum has been raised against TOAD-64 and it is hoped that this reagent will facilitate investigation of the relationship between TOAD-64, α2 chimaerin and Rac1. Trituration or microinjection into neuronal cells of constitutively active and dominant negative Rac1 in combination with anti-TOAD-64 should make it possible to obtain information regarding the relative positions of TOAD-64 and Rac1 within this signal transduction pathway. The anti-TOAD-64 antiserum may also be used in conjunction with anti-α2 chimaerin antiserum to establish whether these proteins co-localise *in vivo*.

7M The Interaction Between the α2 Chimaerin SH2 Domain and B13

The B13 subunit of the inner mitochondrial membrane NADH ubiquinone oxidoreductase, has been shown to bind the α2 chimaerin in absence of tyrosine phosphorylation using the yeast two hybrid system (C.Monfries). The interaction of this protein with the α2 chimaerin SH2 domain was studied *in vitro* using recombinant B13, hereafter referred to as GST-p13 and it was found that GST-p13 could directly bind both full-length α2 chimaerin and its isolated SH2 domain (Figure 5.8 A).

7M.1 Mutation of the Chimaerin SH2 Domain Affects its Interaction with Recombinant B13

Point mutation of the chimaerin SH2 domain drastically affected its *in vitro* interaction with GST-13 and substitutions of amino acid residues at either the βB5 or the βD4 positions (R73L and N94H respectively) reduced binding to negligible levels (Figure 5.8B). This contrasts with the chimaerin SH2 domain / GST-p64 interaction which was sensitive only to the N94H mutation, suggesting that the two proteins interact with the chimaerin SH2 domain at different sites or by different mechanisms. It is possible that the N94H substitution causes a gross distortion of the tertiary structure of the SH2 domain.

The effect of substitution of the invariant arginine (R73L) would be suggestive of

a phosphotyrosine-dependent interaction but this is not a possibility in this case. The interactions between the Grb10 SH2 domain and MEK1 and Raf1 are not dependent on tyrosine phosphorylation but can be abolished by mutation of the Grb10 βD5 arginine (Nantel et al, 1998). This indicates that this residue may also play a role in mediating SH2 domain/ target interactions by an unconventional mechanism. The R56L mutant chimaerin SH2 domain protein can still bind GST-p13 but was shown not to interact with phosphotyrosine in previous experiments (Figure 5.5A), further indicating that there may be no correlation between the ability to bind phosphotyrosine and the ability to bind target proteins. Whilst the βB5 residue (R73) is critical for the interaction of chimaerin with GST-p13 and with also phosphotyrosine, the R73L mutant protein may not be compromised in any other regard if the interaction of the chimaerin SH2 domain with its physiological ligand is phosphotyrosine-independent. That it can still bind another potential target protein, p64, argues against a significant alteration in protein conformation. When assayed for binding to GST-p64, the E49W substitution did not diminish the ability of the α2 chimaerin SH2 domain probe to bind GST-p13. This also suggests that this residue does not have a role in stabilising the overall structure of the chimaerin SH2 domain whereas it is predicted to do so in other SH2 domains. It is possible that the ability of the chimaerin SH2 domain to interact both with phosphotyrosine and with binding proteins in a phosphotyrosine-independent manner is a mechanism for increasing the substrate range of this protein module.

7M.2 The Physiological Significance of the α2 Chimaerin-B13 Interaction

The physiological significance of an interaction between α2 chimaerin and this mitochondrial protein is not known. Its presence did not appear to affect the *in vitro* GAP activity of α2 chimaerin (Figure 5.13). A Western immunoblot analysis of purified mitochondrial proteins indicated that α2 chimaerin was present (C.Hall, Personal Communication) and a study of the sub-cellular distribution of Rho family proteins in the kidney found that only Rac could be detected in the mitochondria (Boivin & Beliveau, 1995). Thus α2 chimaerin may modulate Rac activity within the mitochondria and its interaction with B13 may be relevant *in vivo*. NADH oxidoreductases also exist at the plasma membrane and possibly B13 was simply selected on the basis of its homology to chimaerin's true target. However, preliminary

work suggests that B13 is present in synaptic plasma membrane fractions; α2 chimaerin is also present in this fraction (C.Hall, Personal Communication) Interestingly, purification of oxidoreductase activity from synaptic plasma membranes indicated that the enzyme activity could be attributed to a tightly-associated complex of proteins, one of which was identified as TOAD-64 (Bulliard et al, 1997). This suggests that α2 chimaerin could link Rac-mediated signalling processes to an oxidoreductase activity that associates with TOAD-64 and B13 or B13-like proteins.

7N Multiple Targets of the α2 Chimaerin SH2 Domain

Affinity chromatography experiments led to the isolation of five different potential target proteins: tubulin, actin, glyoxalase and two novel proteins. Kinase assays suggested that there are more since there were two kinase activities of 43 and 150 kDa approximately that bound the chimaerin column but no proteins of this size were detected when samples binding the columns were analysed by SDS-PAGE. Furthermore two more binding proteins, p13 and p64, were identified independently. Whilst each of these may not interact directly with the chimaerin SH2 domain, this does suggest that α2 chimaerin may be a component of a multiprotein signalling complex. The indirect association of proteins with an affinity column may actually be an advantage rather than a disadvantage. Indeed, a case was recently reported in which this technique has been used with the specific intent of isolating a complex of proteins: affinity columns were used to identify complexes of mouse brain proteins that bound profilin I and profilin II (Witke et al, 1998). The expression of α2-chimaerin is developmentally regulated and it may therefore mediate its downstream effectors through different targets at different times. The interaction with any one protein may be dependent on other cellular molecules, either proteins or other co-factors, allowing precise regulation of α2 chimaerin-dependent processes.

Conclusions

α2 chimaerin SH2 domain is a neuronally expressed GTPase activating protein (GAP) for p21-Rac, implicating it in the regulation of Rac-mediated signalling pathways in the brain. It has an amino terminal SH2 domain derived by alternate splicing that is atypical in its sequence, suggesting that it may interact with its target proteins by a mechanism other than that which has been described for the binding of an SH2 domain to its phosphotyrosine peptide ligand in other cases. Attempts have been to isolate target proteins of the chimaerin SH2 domain and also to investigate its interactions with previously identified putative target proteins.

Affinity chromatography was used to isolate potential target proteins of the SH2 domain from rat brain extract. Initial experiments confirmed that such columns could specifically select proteins from a complex mixture and that some of these were phosphorylated on tyrosine residues. Three proteins that bound to chimaerin SH2 domain affinity columns were identified. A glutathione-dependent metabolic enzyme, glyoxalase II was identified on the basis of peptide sequences but it has not possible to independently confirm that it binds α2 chimaerin. Two other proteins, tubulin and actin, were identified by immunoblotting. α2 chimaerin probes detected proteins of the appropriate molecular weights in overlay binding assays, suggesting that these might be direct interactions. Two further proteins of 100 kDa and 200 kDa were isolated. Neither protein could be identified by searching the protein database with peptide sequences derived from these proteins. The p100 is presumed to be novel but only a single peptide was obtained from the p200 and this would not be sufficent to permit positive identification. Analysis of the kinase activities of proteins bound to an α2 chimaerin SH2 domain affinity column indicated that at least three kinases were present. A 43 kDa kinase that phosphorylated full length α2 chimaerin.

It was established that the α2 chimaerin SH2 domain could bind phosphotyrosine. and there was some evidence to suggest that chimaerin sequences amino terminal to the SH2 domain could impede this interaction. Studies using chimaerin SH2 domain proteins with a point mutation in their SH2 domains identified two residues that were critical for interaction with phosphotyrosine. Mutation at one, but not both of the equivalent sites in the Src SH2 domain gave the same result (Bibbins et al, 1993), suggesting that the molecular mechanism

of phosphotyrosine-binding by the chimaerin SH2 domain may be atypical. Two potential target proteins of α2 chimaerin, B13, a sub-unit of the mitochondrial Complex 1 oxidoreductase, and TOAD-64, a neuronally expressed phosphoprotein that can mediate growth cone collapse, were expressed as recombinant proteins and overlay binding assays were used to confirm that they could bind the α2 chimaerin SH2 domain. B13 and TOAD-64 were not observed to exert any modulatory effects on the GAP activity of the full length protein. These interactions responded differently to point mutation of the chimaerin SH2 domain at the R73L position but both were abolished by an amino acid residue substitution at the N94 position of the chimaerin SH2 domain. Both interactions were independent of phosphotyrosine. p64 is a substrate for phosphorylation by ERK1 but this does not modulate its interaction with α2 chimaerin. Thus, although the α2 chimaerin SH2 domain can bind phosphotyrosine this is not necessarily a critical determinant of its interactions with some target proteins. The possibility that phosphorylation of amino acid residues other than tyrosine residues is required has not been excluded and the evidence suggests that the targets of the α2 chimaerin SH2 domain may include both tyrosine- and phosphorylated and non-tyrosine-phosphorylated proteins.

REFERENCES

Ahmed, S., Kozma, R., Lee, J., Monfries, C., Harden, N. and Lim, L. (1991). The cysteine-rich domain of human proteins, neuronal chimaerin, protein kinase C and diacylglycerol kinase binds zinc. Evidence for the involvement of a zinc-dependent structure in phorbol ester binding. *Biochem J* **280,** 233-41.

Ahmed, S., Maruyama, I. N., Kozma, R., Lee, J., Brenner, S. and Lim, L. (1992). The Caenorhabditis-Elegans Unc-13 Gene-Product Is a Phospholipid- Dependent High-Affinity Phorbol Ester Receptor. *Biochemical Journal* **287,** 995-999.

Ahmed, S., Lee, J., Kozma, R., Best, A., Monfries, C. and Lim, L. (1993). A novel functional target for tumor-promoting phorbol esters and lysophosphatidic acid. The p21rac-GTPase activating protein n-chimaerin. *J Biol Chem* **268,** 10709-12.

Ahmed, S., Lee, J., Wen, L. P., Zhao, Z., Ho, J., Best, A., Kozma, R. and Lim, L. (1994). Breakpoint cluster region gene product-related domain of n-chimaerin. Discrimination between Rac-binding and GTPase-activating residues by mutational analysis. *J Biol Chem* **269,** 17642-8.

Ahn, S.J., Han, S.J., Mo, H.J., Chung, J.K., Hong, S.H., Park, T.K. and Kim, C.G. (1998). Interaction of phospholipase Cγ1 via its COOH-terminal Src homology 2 domain with synaptojanin. *Biochem Biophys Res Comm* **244,** 62-67.

Allen, W. E., Jones, G. E., Pollard, J. W. and Ridley, A. J. (1997). Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *J Cell Sci* **110,** 707-20.

Amicarelli, F., Sacchetta, P., Colafarina, S., Angelucci, S., Miranda, M. and Di Ilio, C. (1998). Glyoxalases activity during Bufo bufo embryo development. *Mech Ageing Dev* **100,** 261-7.

Amor, J. C., Harrison, D. H., Kahn, R. A. and Ringe, D. (1994). Structure of the human ADP-ribosylation factor 1 complexed with GDP. *Nature* **372,** 704-8.

Andra, K., Lassmann, H., Bittner, R., Shorny, S., Fassler, R., Propst, F. and Wiche, G. (1997). Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture. *Genes Dev* **11,** 3143-56.

Areces, L. B., Kazanietz, M. G. and Blumberg, P. M. (1994). Close similarity of baculovirus-expressed n-chimaerin and protein kinase C alpha as phorbol ester receptors. *J Biol Chem* **269**, 19553-8.

Arpin, M., Algrain, M. and Louvard, D. (1994). Membrane-actin microfilament connections: an increasing diversity of players related to band 4.1. *Curr Opin Cell Biol* **6**, 136-41.

Ayscough, K. R. (1998). In vivo functions of actin-binding proteins. *Curr Opin Cell Biol* **10**, 102-11.

Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J. and Cerione, R. A. (1995a). Identification of a mouse p21Cdc42/Rac activated kinase. *J Biol Chem* **270**, 22731-7.

Bagrodia, S., Derijard, B., Davis, R. J. and Cerione, R. A. (1995b). Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J Biol Chem* **270**, 27995-8.

Baker, J. P. and Titus, M. A. (1998). Myosins: matching functions with motors. *Curr Opin Cell Biol* **10**, 80-6.

Band, C. J., Posner, B. I., Dumas, V. and Contreres, J. O. (1997). Early signaling events triggered by peroxovanadium [bpV(phen)] are insulin receptor kinase (IRK)-dependent: specificity of inhibition of IRK-associated protein tyrosine phosphatase(s) by bpV(phen). *Mol Endocrinol* **11**, 1899-910.

Banin, S., Truong, O., Katz, D. R., Waterfield, M. D., Brickell, P. M. and Gout, I. (1996). Wiskott-Aldrich syndrome protein (WASp) is a binding partner for c-Src family protein-tyrosine kinases. *Curr Biol* **6**, 981-8.

Bar-Sagi, D., Rotin, D., Mandiyan, V. and Schlessinger, J. (1993). SH3 domains direct cellular localisation of signalling molecules. *Cell* **74**, 83-91.

Barbacid, M. (1987). Ras Genes. *Annual Review of Biochemistry* **56**, 779-827.

Barfod, E. T., Zheng, Y., Kuang, W. J., Hart, M. J., Evans, T., Cerione, R. A. and Ashkenazi, A. (1993). Cloning and expression of a human CDC42 GTPase-activating protein reveals a functional SH3-binding domain. *J Biol Chem* **268,** 26059-62.

Barrett, T., Xiao, B., Dodson, E.J., Dodson, G., Ludbrook, S.B., Nurmahomed, K., Gamblin, S.J., Musacchio, A., Smerdon, S.J. and Eccleston, J.F. (1997). The structure of the GTPase-activating domain from p50rhoGAP. *Nature* **385,** 458-61.

Bashour, A. M., Fullerton, A. T., Hart, M. J. and Bloom, G. S. (1997). IQGAP1, a Rac- and Cdc42-binding protein, directly binds and cross-links microfilaments. *J Cell Biol* **137,** 1555-66.

Batty, I. H., Hickinson, D. M. and Downes, C. P. (1997). Cross-talk between phospholipase C and phosphoinositide 3-kinase signalling pathways. *Biochem Soc Trans* **25,** 1132-7.

Bax, B. (1998). Domains of rasGAP and rhoGAP are related [letter]. *Nature* **392,** 447-8.

Bellanger, J. M., Lazaro, J. B., Diriong, S., Fernandez, A., Lamb, N. and Debant, A. (1998). The two guanine nucleotide exchange factor domains of Trio link the Rac1 and the RhoA pathways in vivo. *Oncogene* **16,** 147-152.

Bennett, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T. and Neel, B. G. (1994). Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor beta to Ras. *Proc Natl Acad Sci U S A* **91,** 7335-9.

Bentley, D. and Toroian Raymond, A. (1986). Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. *Nature* **323,** 712-5.

Bentley, D. and O'Connor T.P. (1994). Cytoskeletal events in growth cone steering. *Curr Opin Neurobiol* **4,** 43-8.

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361,** 315-25.

Best, A., Ahmed, S., Kozma, R. and Lim, L. (1996). The Ras-Related Gtpase Rac1 Binds Tubulin. *Journal Of Biological Chemistry* **271**, 3756-3762.

Bhatnagar, R. S. and Gordon, J. I. (1995). Thermodynamic studies of myristoyl-CoA: protein N-myristoyltransferase using isothermal titration calorimetry. *Methods Enzymol* **250**, 467-86.

Bibbins, K. B., Boeuf, H. and Varmus, H. E. (1993). Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides. *Mol Cell Biol* **13**, 7278-87.

Billuart, P., Bienvenu, T., Ronce, N., des Portes, V., Vinet, M. C., Zemni, R., Crollius, H. R., Carrie, A., Fauchereau, F., Cherry, M., Briault, S., Hamel, B., Fryns, J. P., Beldjord, C., Kahn, A., Moraine, C. and Chelly, J. (1998). Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature* **392**, 923-6.

Blenis, J. (1993). Signal transduction via the MAP kinases: proceed at your own RSK. *Proc Natl Acad Sci U S A* **90**, 5889-92.

Boccaccio, C., Ando, M., Tamagnone, L., Bardelli, A., Michieli, P., Battistini, C. and Comoglio, P. M. (1998). Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature* **391**, 285-8.

Boguski, M. S. and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature* **366**, 643-54.

Boivin, D. and Beliveau, R. (1995). Subcellular distribution and membrane association of Rho-related small GTP-binding proteins in kidney cortex. *Am J Physiol* **269**, F180-9.

Bokoch, G. M. (1995). Regulation Of the Phagocyte Respiratory Burst By Small Gtp-Binding Proteins. *Trends In Cell Biology* **5**, 109-113.

Bokoch, G. M., Vlahos, C. J., Wang, Y., Knaus, U. G. and Traynor Kaplan, A. E. (1996). Rac GTPase interacts specifically with phosphatidylinositol 3-kinase. *Biochem J* **315**, 775-9.

Bollag, G. and McCormick, F. (1991). Regulators and effectors of ras proteins. *Annu Rev Cell Biol* 7, 601-32.

Bollag, G., McCormick, F. and Clark, R. (1993). Characterization of full-length neurofibromin: tubulin inhibits Ras GAP activity. *Embo J* 12, 1923-7.

Bork, P. and Margolis, B. (1995). A phosphotyrosine interaction domain [letter]. *Cell* 80, 693-4.

Bos, J. L. (1989). ras oncogenes in human cancer: a review [published erratum appears in Cancer Res 1990 Feb 15;50(4):1352]. *Cancer Res* 49, 4682-9.

Boschelli, F., Uptain, S. M. and Lightbody, J. J. (1993). The lethality of p60v-src in Saccharomyces cerevisiae and the activation of p34CDC28 kinase are dependent on the integrity of the SH2 domain. *J Cell Sci* 105, 519-28.

Boulton, T. G., Stahl, N. and Yancopoulos, G. D. (1994). Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. *J Biol Chem* 269, 11648-55.

Bourne, H. R., Sanders, D. A. and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* 348, 125-32.

Bourne, H. R., Sanders, D. A. and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 349, 117-27.

Bradshaw, J. M., Grucza, R. A., Ladbury, J. E. and Waksman, G. (1998). Probing the "two-pronged plug two-holed socket" model for the mechanism of binding of the Src SH2 domain to phosphotyrosyl peptides: a thermodynamic study. *Biochemistry* 37, 9083-90.

Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. and Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. *Science* 259, 1760-3.

Brill, S., Li, S., Lyman, C. W., Church, D. M., Wasmuth, J. J., Weissbach, L., Bernards, A. and Snijders, A. J. (1996). The Ras GTPase-activating-protein-related human protein

IQGAP2 harbors a potential actin binding domain and interacts with calmodulin and Rho family GTPases. *Mol Cell Biol* **16,** 4869-78.

Brinkley, W. (1997). Microtubules: a brief historical perspective. *J Struct Biol* **118,** 84-6.

Brown, H. A., Gutowski, S., Kahn, R. A. and Sternweis, P. C. (1995). Partial purification and characterization of Arf-sensitive phospholipase D from porcine brain. *J Biol Chem* **270,** 14935-43.

Bryant, S. S., Briggs, S., Smithgall, T. E., Martin, G. A., McCormick, F., Chang, J. H., Parsons, S. J. and Jove, R. (1995). Two SH2 domains of p120 Ras GTPase-activating protein bind synergistically to tyrosine phosphorylated p190 Rho GTPase-activating protein. *J Biol Chem* **270,** 17947-52.

Bulliard, C., Zurbriggen, R., Tornare, J., Faty, M., Dastoor, Z. and Dreyer, J. L. (1997). Purification of a dichlorophenol-indophenol oxidoreductase from rat and bovine synaptic membranes: tight complex association of a glyceraldehyde-3-phosphate dehydrogenase isoform, TOAD64, enolase-gamma and aldolase C. *Biochem J* **324,** 555-63.

Caloca, M. J., Fernandez, N., Lewin, N. E., Ching, D., Modali, R., Blumberg, P. M. and Kazanietz, M. G. (1997). Beta2-chimaerin is a high affinity receptor for the phorbol ester tumor promoters. *J Biol Chem* **272,** 26488-96.

Cantley, L. C. and Zhou, S. Y. (1994). Specificity In Recognition Of Phosphopeptides By Src-Homology-2 Domains. *Journal Of Cell Science* , 121-126.

Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S. and Cantley, L. C. (1990). Purification and characterisation of phosphoinositide 3-kinase from rat liver. *J Biol Chem* **265,** 19704-11.

Carpenter, C. L. and Cantley, L. C. (1996). Phosphoinositide kinases. *Curr Opin Cell Biol* **8,** 153-8.

Cerione, R. A. and Zheng, Y. (1996). The Dbl family of oncogenes. *Curr Opin Cell Biol* **8,** 216-22.

Chang, J. S., Kobayashi, M., Wang, D. Z., Maruta, H. and Iwashita, S. (1995). Two regions with differential growth-modulating activity in the N-terminal domain of ras GTPase-activating protein (p120GAP) src homology and Gly-Ala-Pro-rich regions. *Eur J Biochem* **232,** 691-9.

Chen, W. N., Blanc, J. and Lim, L. (1994). Characterization Of a Promiscuous Gtpase-Activating Protein That Has a Bcr-Related Domain From Caenorhabditis-Elegans. *Journal Of Biological Chemistry* **269,** 820-823.

Chen, J., Cohn, J. A. and Mandel, L. J. (1995). Dephosphorylation of ezrin as an early event in renal microvillar breakdown and anoxic injury. *Proc Natl Acad Sci U S A* **92,** 7495-9.

Chen, W., Chen, S., Yap, S. F. and Lim, L. (1996). The Caenorhabditis elegans p21-activated kinase (CePAK) colocalizes with CeRac1 and CDC42Ce at hypodermal cell boundaries during embryo elongation. *J Biol Chem* **271,** 26362-8.

Chien, C. T., Bartel, P. L., Sternglanz, R. and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A* **88,** 9578-82.

Chong, L. D., Traynorkaplan, A., Bokoch, G. M. and Schwartz, M. A. (1994). The Small Gtp-Binding Protein-Rho Regulates a Phosphatidylinositol 4- Phosphate 5-Kinase In Mammalian-Cells. *Cell* **79,** 507-513.

Chou, M. M. and Blenis, J. (1996). The 70 kDa S6 kinase complexes with and is activated by the Rho family G proteins Cdc42 and Rac1. *Cell* **85,** 573-83.

Chou, Y. H., Skalli, O. and Goldman, R. D. (1997). Intermediate filaments and cytoplasmic networking: new connections and more functions. *Curr Opin Cell Biol* **9,** 49-53.

Clarke, S. (1992). Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu Rev Biochem* **61,** 355-86.

Cleghon, V. and Morrison, D. K. (1994). Raf-1 interacts with Fyn and Src in a non-phosphotyrosine-dependent manner. *J Biol Chem* **269,** 17749-55.

Clelland, J. D. and Thornalley, P. J. (1993). The potentiation of GTP-dependent assembly of microtubules by S-D-lactoylglutathione. *Biochem Soc Trans* **21**, 160s.

Cohen, G. B., Ren, R. B. and Baltimore, D. (1995). Modular Binding Domains In Signal-Transduction Proteins. *Cell* **80**, 237-248.

Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J. S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137-46.

Craig, S. W. and Johnson, R. P. (1996). Assembly Of Focal Adhesions - Progress, Paradigms, and Portents. *Current Opinion In Cell Biology* **8**, 74-85.

Cremona, O. and De Camilli, P. (1997). Synaptic vesicle endocytosis. *Curr Opin Neurobiol* **7**, 323-30.

Crespo, P., Schuebel, K. E., Ostrom, A. A., Gutkind, J. S. and Bustelo, X. R. (1997). Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. *Nature* **385**, 169-172.

Dallery, E., Galiegue Zouitina, S., Collyn d'Hooghe, M., Quief, S., Denis, C., Hildebrand, M. P., Lantoine, D., Deweindt, C., Tilly, H., Bastard, C. and et al. (1995). TTF, a gene encoding a novel small G protein, fuses to the lymphoma-associated LAZ3 gene by t(3;4) chromosomal translocation. *Oncogene* **10**, 2171-8.

Dallery, E., Quief, S., Ben Jilany, K. E., Kerckaert, J. P. and Hagege, D. (1996). Molecular cloning and structural analysis of cDNAs that encode 3 small GTP-binding proteins from sugar beet. *C R Acad Sci III* **319**, 91-7.

Daum, G., Eisenmann Tappe, I., Fries, H. W., Troppmair, J. and Rapp, U. R. (1994). The ins and outs of Raf kinases. *Trends Biochem Sci* **19**, 474-80.

Davis, S., Gale, N. W., Aldrich, T. H., Maisonpierre, P. C., Lhotak, V., Pawson, T., Goldfarb, M. and Yancopoulos, G. D. (1994). Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science* **266**, 816-9.

de Vos, A. M., Ultsch, M. and Kossiakoff, A. A. (1992). Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**, 306-12.

Diamond, L. (1984). Tumor promoters and cell transformation. *Pharmacol Ther* **26**, 89-145.

Didsbury, J., Weber, R. F., Bokoch, G. M., Evans, T. and Snyderman, R. (1989). rac, a novel ras-related family of proteins that are botulinum toxin substrates. *J Biol Chem* **264**, 16378-82.

Diekmann, D., Brill, S., Garrett, M. D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L. and Hall, A. (1991). Bcr encodes a GTPase-activating protein for p21rac. *Nature* **351**, 400-2.

Diekmann, D., Abo, A., Johnston, C., Segal, A. W. and Hall, A. (1994). Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. *Science* **265**, 531-3.

DiNubile, M. J. and Huang, S. (1997). Capping of the barbed ends of actin filaments by a high-affinity profilin-actin complex. *Cell Motil Cytoskeleton* **37**, 211-25.

Dong, J.M., Smith, P., Hall, C. and Lim, L. (1995). Promoter region of the transcriptional unit for α1-chimaerin, a neuron-specific GTPase-activating protein for p21rac. *Eur J Biochem* **227**, 636-46.

Dong, J.M. and Lim, L. (1996). Selective up-regulation of α1-chimaerin mRNA in SK-N-SH neuroblastoma cells by K^+-induced depolarisation. *Eur J Biochem* **236**, 820-26.

Doupnik, C. A., Davidson, N., Lester, H. A. and Kofuji, P. (1997). RGS proteins reconstitute the rapid gating kinetics of gbetagamma-activated inwardly rectifying K+ channels. *Proc Natl Acad Sci U S A* **94**, 10461-6.

Downing, K. H. and Nogales, E. (1998). Tubulin and microtubule structure. *Curr Opin Cell Biol* **10**, 16-22.

Downward, J. (1998a). Mechanisms and consequences of activation of protein kinase B/Akt. *Curr Opin Cell Biol* **10**, 262-7.

Downward, J. (1998b). Ras signalling and apoptosis. *Current Opinion In Genetics & Development* **8**, 49-54.

Duchesne, M., Schweighoffer, F., Parker, F., Clerc, F., Frobert, Y., Thang, M. N. and Tocque, B. (1993). Identification of the SH3 domain of GAP as an essential sequence for Ras-GAP-mediated signaling. *Science* **259**, 525-8.

Eaton, S., Auvinen, P., Luo, L., Jan, Y. N. and Simons, K. (1995). CDC42 and Rac1 control different actin-dependent processes in the Drosophila wing disc epithelium. *J Cell Biol* **131**, 151-64.

Eck, M. J., Shoelson, S. E. and Harrison, S. C. (1993). Recognition of a high-affinity phosphotyrosyl peptide by the Src homology-2 domain of p56lck. *Nature* **362**, 87-91.

Edwards, L. G., Clelland, J. D. and Thornalley, P. J. (1993). Mechanism of the inhibition of growth of human leukaemia 60 cells by S-D-lactoylglutathione. *Biochem Soc Trans* **21**, 166s.

Emala, C. W., Schwindinger, W. F., Wand, G. S. and Levine, M. A. (1994). Signal-transducing G proteins: basic and clinical implications. *Prog Nucleic Acid Res Mol Biol* **47**, 81-111.

Fan, W. T., Koch, C. A., deHoog, C. L., Fam, N. P. and Moran, M. F. (1998). The exchange factor Ras-GRF2 activates Ras-dependent and Rac- dependent mitogen-activated protein kinase pathways. *Current Biology* **8**, 935-938.

Fanger, G. R., Gerwins, P., Widmann, C., Jarpe, M. B. and Johnson, G. L. (1997). MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr Opin Genet Dev* **7**, 67-74.

Feinstein, E., Kimchi, A., Wallach, D., Boldin, M. and Varfolomeev, E. (1995). The death domain: a module shared by proteins with diverse cellular functions [letter]. *Trends Biochem Sci* **20**, 342-4.

Feng, S., Chen, J. K., Yu, H., Simon, J. A. and Schreiber, S. L. (1994). Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. *Science* **266**, 1241-7.

Fernandez Sarabia, M. J. and Bischoff, J. R. (1993). Bcl-2 associates with the ras-related protein R-ras p23. *Nature* **366**, 274-5.

Foster, D. A. (1993). Intracellular signalling mediated by protein-tyrosine kinases: networking through phospholipid metabolism. *Cell Signal* **5**, 389-99.

Foster, R., Hu, K. Q., Lu, Y., Nolan, K. M., Thissen, J. and Settleman, J. (1996). Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation. *Mol Cell Biol* **16**, 2689-99.

Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. B. and Yamamoto, K. R. (1988). The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature* **334**, 543-6.

Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P. E. and Cobb, M. H. (1997). Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *Embo Journal* **16**, 6426-6438.

Fuchs, E. and Cleveland, D. W. (1998). A structural scaffolding of intermediate filaments in health and disease. *Science* **279**, 514-9.

Fukasawa, K., Rulong, S., Resau, J., Pinto da Silva, P. and Woude, G. F. (1995). Overexpression of mos oncogene product in Swiss 3T3 cells induces apoptosis preferentially during S-phase. *Oncogene* **10**, 1-8.

Fukuda, M. and Mikoshiba, K. (1996). Structure-function relationships of the mouse Gap1m. Determination of the inositol 1,3,4,5-tetrakisphosphate-binding domain. *J Biol Chem* **271**, 18838-42.

Gadella, T. W., Jr. and Jovin, T. M. (1995). Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J Cell Biol* **129**, 1543-58.

Galisteo, M. L., Chernoff, J., Su, Y. C., Skolnik, E. Y. and Schlessinger, J. (1996). The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. *J Biol Chem* **271,** 20997-1000.

Garrett, M.D., Self, A.J., van Oers, C. and Hall, A. (1989). Identification of distinct cytoplasmic targets for ras/R-ras and rho regulatory proteins. *J Biol Chem* **264**, 10-13.

Gilmore, A. P. and Burridge, K. (1996). Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. *Nature* **381,** 531-5.

Goldman, R. D., Khuon, S., Chou, Y. H., Opal, P. and Steinert, P. M. (1996). The function of intermediate filaments in cell shape and cytoskeletal integrity. *J Cell Biol* **134,** 971-83.

Gordon, J. A. (1991). Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol* **201,** 477-82.

Gorvel, J. P., Chang, T. C., Boretto, J., Azuma, T. and Chavrier, P. (1998). Differential properties of D4/LyGDI versus RhoGDI: phosphorylation and rho GTPase selectivity. *FEBS Lett* **422,** 269-73.

Goshima, Y., Nakamura, F., Strittmatter, P. and Strittmatter, S. M. (1995). Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. *Nature* **376,** 509-14.

Greene, L. A and Tischler, A.S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* **73**, 2424-8

Gregory, P. E., Gutmann, D. H., Mitchell, A., Park, S., Boguski, M., Jacks, T., Wood, D. L., Jove, R. and Collins, F. S. (1993). Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. *Somat Cell Mol Genet* **19,** 265-74.

Grundke-Iqbal, I. and Iqbal, K. (1989). Neuronal cytoskeleton in the biology of Alzheimer disease. *Prog Clin Biol Res* **317,** 745-53.

Guappone, A. C., Weimer, T. and Flynn, D. C. (1998). Formation of a stable src-AFAP-110 complex through either an amino-terminal or a carboxy-terminal SH2-binding motif. *Mol Carcinog* **22,** 110-9.

Habets, G. G., Scholtes, E. H., Zuydgeest, D., van der Kammen, R. A., Stam, J. C., Berns, A. and Collard, J. G. (1994). Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. *Cell* **77,** 537-49.

Hall, C., Monfries, C., Smith, P., Lim, H. H., Kozma, R., Ahmed, S., Vanniasingham, V., Leung, T. and Lim, L. (1990). Novel human brain cDNA encoding a 34,000 Mr protein n-chimaerin, related to both the regulatory domain of protein kinase C and BCR, the product of the breakpoint cluster region gene. *J Mol Biol* **211,** 11-6.

Hall, C., Sin, W. C., Teo, M., Michael, G. J., Smith, P., Dong, J. M., Lim, H. H., Manser, E., Spurr, N. K., Jones, T. A. and et al. (1993). Alpha 2-chimerin, an SH2-containing GTPase-activating protein for the ras-related protein p21rac derived by alternate splicing of the human n-chimerin gene, is selectively expressed in brain regions and testes. *Mol Cell Biol* **13,** 4986-98.

Hall, A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu Rev Cell Biol* **10,** 31-54.

Hamajima, N., Matsuda, K., Sakata, S., Tamaki, N., Sasaki, M. and Nonaka, M. (1996). A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution. *Gene* **180,** 157-63.

Hamm, H. E. and Gilchrist, A. (1996). Heterotrimeric G proteins. *Current Opinion In Cell Biology* **8,** 189-196.

Hansen, H., Svensson, U., Zhu, J., Laviola, L., Giorgino, F., Wolf, G., Smith, R. J. and Riedel, H. (1996). Interaction between the Grb10 SH2 domain and the insulin receptor carboxyl terminus. *J Biol Chem* **271,** 8882-6.

Harden, N., Lee, J., Loh, H. Y., Ong, Y. M., Tan, I., Leung, T., Manser, E. and Lim, L. (1996). A Drosophila homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. *Mol Cell Biol* **16,** 1896-908.

257

Harhammer, R., Gohla, A. and Schultz, G. (1996). Interaction of G protein Gbetagamma dimers with small GTP-binding proteins of the Rho family. *FEBS Lett* **399,** 211-4.

Hart, M. J., Eva, A., Zangrilli, D., Aaronson, S. A., Evans, T., Cerione, R. A. and Zheng, Y. (1994). Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. *J Biol Chem* **269,** 62-5.

Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A. and Stossel, T. P. (1995). Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* **82,** 643-53.

Haslam, R. J., Koide, H. B. and Hemmings, B. A. (1993). Pleckstrin domain homology [letter]. *Nature* **363,** 309-10.

Hatada, M. H., Lu, X., Laird, E. R., Green, J., Morgenstern, J. P., Lou, M., Marr, C. S., Phillips, T. B., Ram, M. K., Theriault, K. and et al. (1995). Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor [see comments]. *Nature* **377,** 32-8.

Hawkins, P. T., Eguinoa, A., Qiu, R. G., Stokoe, D., Cooke, F. T., Walters, R., Wennstrom, S., Claesson Welsh, L., Evans, T., Symons, M. and et al. (1995). PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr Biol* **5,** 393-403.

He, W., Rose, D. W., Olefsky, J. M. and Gustafson, T. A. (1998). Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the pleckstrin homology and SH2 domains. *J Biol Chem* **273,** 6860-7.

Heisterkamp, N., Stam, K., Groffen, J., de Klein, A. and Grosveld, G. (1985). Structural organization of the bcr gene and its role in the Ph' translocation. *Nature* **315,** 758-61.

Heldin, C. H. and Westermark, B. (1989). Platelet-derived growth factor: three isoforms and two receptor types. *Trends Genet* **5,** 108-11.

Heldin, C. H. (1995). Dimerization of cell surface receptors in signal transduction. *Cell* **80,** 213-23.

Heldin, C. H. (1997). Simultaneous induction of stimulatory and inhibitory signals by PDGF. *Febs Letters* **410,** 17-21.

Herskowitz, I. (1995). MAP kinase pathways in yeast: for mating and more. *Cell* **80,** 187-97.

Higley, S. and Way, M. (1997). Actin and cell pathogenesis. *Curr Opin Cell Biol* **9,** 62-9.

Hill, C. S., Wynne, J. and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* **81,** 1159-70.

Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y., Tsukita, S. and Tsukita, S. (1996). Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J Cell Biol* **135,** 37-51.

Hirokawa, N. (1994). Microtubule organization and dynamics dependent on microtubule-associated proteins. *Curr Opin Cell Biol* **6,** 74-81.

Hirokawa, N., Noda, Y. and Okada, Y. (1998). Kinesin and dynein superfamily proteins in organelle transport and cell division. *Curr Opin Cell Biol* **10,** 60-73.

Hirshberg, M., Stockley, R. W., Dodson, G. and Webb, M. R. (1997). The crystal structure of human rac1, a member of the rho-family complexed with a GTP analogue. *Nat Struct Biol* **4,** 147-52.

Holland, S. J., Gale, N. W., Gish, G. D., Roth, R. A., Zhou, S. Y., Cantley, L. C., Henkemeyer, M., Yancopoulos, G. D. and Pawson, T. (1997). Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *Embo Journal* **16,** 3877-3888.

Homma, Y. and Emori, Y. (1995). A dual functional signal mediator showing RhoGAP and phospholipase C-delta stimulating activities. *Embo J* **14**, 286-91.

Houseweart, M. K. and Cleveland, D. W. (1998). Intermediate filaments and their associated proteins: multiple dynamic personalities. *Curr Opin Cell Biol* **10**, 93-101.

Hu, C. D., Kariya, K., Tamada, M., Akasaka, K., Shirouzu, M., Yokoyama, S. and Kataoka, T. (1995). Cysteine-rich region of Raf-1 interacts with activator domain of post-translationally modified Ha-Ras. *J Biol Chem* **270**, 30274-7.

Hu, K. Q. and Settleman, J. (1997). Tandem SH2 binding sites mediate the RasGAP-RhoGAP interaction: A conformational mechanism for SH3 domain regulation. *Embo Journal* **16**, 473-483.

Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225-36.

Huyer, G. and Ramachandran, C. (1998). The specificity of the N-terminal SH2 domain of SHP-2 is modified by a single point mutation. *Biochemistry* **37**, 2741-2747.

Igarashi, K., Isohara, T., Kato, T., Shigeta, K., Yamano, T. and Uno, I. (1998). Tyrosine 1213 of Flt-1 is a major binding site of Nck and SHP-2. *Biochem Biophys Res Commun* **246**, 95-9.

Ihara, K., Muraguchi, S., Kato, M., Shimizu, T., Shirakawa, M., Kuroda, S., Kaibuchi, K. and Hakoshima, T. (1998). Crystal structure of human RhoA in a dominantly active form complexed with a GTP analogue. *J Biol Chem* **273**, 9656-66.

Inoue, Y., Tsujimoto, Y. and Kimura, A. (1998). Expression of the glyoxalase I gene of Saccharomyces cerevisiae is regulated by high osmolarity glycerol mitogen-activated protein kinase pathway in osmotic stress response. *J Biol Chem* **273**, 2977-83.

Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N. and Narumiya, S. (1996). The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *Embo Journal* **15**, 1885-1893.

Itoh, T., Miura, K., Miki, H. and Takenawa, T. (1996). Beta-tubulin binds Src homology 2 domains through a region different from the tyrosine-phosphorylated protein-recognizing site. *J Biol Chem* **271,** 27931-5.

Jefferson, A. B., Klippel, A. and Williams, L. T. (1998). Inhibition of mSOS-activity by binding of phosphatidylinositol 4,5-P2 to the mSOS pleckstrin homology domain. *Oncogene* **16,** 2303-10.

Ji, X., Moore, H. D., Russell, R. G. and Watts, D. J. (1997). cDna cloning and characterization of a rat spermatogenesis-associated protein Rsp29. *Biochem Biophys Res Commun* **241,** 714-9.

Jiang, H., Lu, Z., Luo, J. Q., Wolfman, A. and Foster, D. A. (1995a). Ras mediates the activation of phospholipase D by v-Src. *J Biol Chem* **270,** 6006-9.

Jiang, H., Luo, J. Q., Urano, T., Frankel, P., Lu, Z., Foster, D. A. and Feig, L. A. (1995b). Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature* **378,** 409-12.

Jin, Z. and Strittmatter, S. M. (1997). Rac1 mediates collapsin-1-induced growth cone collapse. *J Neurosci* **17,** 6256-63.

Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J. C., Hu, S., Altrock, B. W. and Fox, G. M. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **85,** 1113-24.

Josso, N. and di Clemente, N. (1997). Serine/threonine kinase receptors and ligands. *Curr Opin Genet Dev* **7,** 371-7.

Kahn, R. A., Der, C. J. and Bokoch, G. M. (1992). The ras superfamily of GTP-binding proteins: guidelines on nomenclature [news]. *Faseb J* **6,** 2512-3.

Katoh, H., Aoki, J., Ichikawa, A. and Negishi, M. (1998). p160 Rhoa-binding kinase Rokalpha induces neurite retraction. *J Biol Chem* **273,** 2489-92.

Kauffmann Zeh, A., Thomas, G. M., Ball, A., Prosser, S., Cunningham, E., Cockcroft, S. and Hsuan, J. J. (1995). Requirement for phosphatidylinositol transfer protein in epidermal growth factor signaling. *Science* **268**, 1188-90.

Kavanaugh, W. M., Turck, C. W., Klippel, A. and Williams, L. T. (1994). Tyrosine 508 of the 85-kilodalton subunit of phosphatidylinositol 3-kinase is phosphorylated by the platelet-derived growth factor receptor. *Biochemistry* **33**, 11046-50.

Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1995). PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science* **268**, 1177-9.

Kayne, P. S. and Sternberg, P. W. (1995). Ras pathways in Caenorhabditis elegans. *Curr Opin Genet Dev* **5**, 38-43.

Kazanietz, M. G., Lewin, N. E., Bruns, J. D. and Blumberg, P. M. (1995). Characterization of the cysteine-rich region of the Caenorhabditis elegans protein Unc-13 as a high affinity phorbol ester receptor. Analysis of ligand-binding interactions, lipid cofactor requirements, and inhibitor sensitivity. *J Biol Chem* **270**, 10777-83.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J. H., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-Associated kinase (Rho-kinase). *Science* **273**, 245-248.

Koegl, M., Kypta, R. M., Bergman, M., Alitalo, K. and Courtneidge, S. A. (1994). Rapid and efficient purification of Src homology 2 domain-containing proteins: Fyn, Csk and phosphatidylinositol 3-kinase p85. *Biochem J* **302**, 737-44.

Koslowski, R., Knoch, K. P. and Wenzel, K. W. (1998). Proteinases and proteinase inhibitors during the development of pulmonary fibrosis in rat. *Clin Chim Acta* **271**, 45-56.

Kozma, R., Ahmed, S., Best, A. and Lim, L. (1995). The Ras-Related Protein Cdc42hs and Bradykinin Promote Formation Of Peripheral Actin Microspikes and Filopodia In Swiss 3t3 Fibroblasts. *Molecular and Cellular Biology* **15**, 1942-1952.

Kozma, R., Ahmed, S., Best, A. and Lim, L. (1996). The Gtpase-Activating Protein N-Chimaerin Cooperates With Rac1 and Cdc42hs to Induce the Formation Of Lamellipodia and Filopodia. *Molecular and Cellular Biology* **16,** 5069-5080.

Kozma, R., Sarner, S., Ahmed, S. and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodelling: Relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Molecular and Cellular Biology* **17,** 1201-1211.

Krishnan, K., Singh, B. and Krolewski, J. J. (1998). Identification of amino acid residues critical for the Src-homology 2 domain-dependent docking of Stat2 to the interferon alpha receptor. *J Biol Chem* **273,** 19495-501.

Kuroda, S., Fukata, M., Kobayashi, K., Nakafuku, M., Nomura, N., Iwamatsu, A. and Kaibuchi, K. (1996). Identification of IQGAP as a putative target for the small GTPases, Cdc42 and Rac1. *J Biol Chem* **271,** 23363-7.

Kurosu, H., Maehama, T., Okada, T., Yamamoto, T., Hoshino, S., Fukui, Y., Ui, M., Hazeki, O. and Katada, T. (1997). Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110beta is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. *J Biol Chem* **272,** 24252-6.

Lai, C. C., Boguski, M., Broek, D. and Powers, S. (1993). Influence of guanine nucleotides on complex formation between Ras and CDC25 proteins. *Mol Cell Biol* **13,** 1345-52.

Lancaster, C. A., Taylor Harris, P. M., Self, A. J., Brill, S., van Erp, H. E. and Hall, A. (1994). Characterization of rhoGAP. A GTPase-activating protein for rho-related small GTPases. *J Biol Chem* **269,** 1137-42.

Leblanc, V., Tocque, B. and Delumeau, I. (1998). Ras-GAP controls Rho-mediated cytoskeletal reorganization through its SH3 domain. *Mol Cell Biol* **18,** 5567-78.

Lee, C. H., Kominos, D., Jacques, S., Margolis, B., Schlessinger, J., Shoelson, S. E. and Kuriyan, J. (1994). Crystal structures of peptide complexes of the amino-terminal SH2 domain of the Syp tyrosine phosphatase. *Structure* **2,** 423-38.

Lee, S. B. and Rhee, S. G. (1995). Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr Opin Cell Biol* **7**, 183-9.

Leeuwen, F. N., Kain, H. E., Kammen, R. A., Michiels, F., Kranenburg, O. W. and Collard, J. G. (1997). The guanine nucleotide exchange factor Tiam1 affects neuronal morphology; opposing roles for the small GTPases Rac and Rho. *J Cell Biol* **139**, 797-807.

Lemmon, M. A. and Schlessinger, J. (1994). Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem Sci* **19**, 459-63.

Lemmon, M. A., Ferguson, K. M. and Schlessinger, J. (1996). PH domains: Diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* **85**, 621-624.

Leung, T., How, B. E., Manser, E. and Lim, L. (1993). Germ cell beta-chimaerin, a new GTPase-activating protein for p21rac, is specifically expressed during the acrosomal assembly stage in rat testis. *J Biol Chem* **268**, 3813-6.

Leung, T., How, B. E., Manser, E. and Lim, L. (1994). Cerebellar β2-chimaerin, a GTPase-activating protein for p21 Ras-related Rac is specifically expressed in granule cells and has a unique N-terminal SH2 domain. *J Biol Chem* **269**, 12888-92.

Leung, T., Manser, E., Tan, L. and Lim, L. (1995). A Novel Serine/Threonine Kinase Binding the Ras-Related Rhoa GTPase Which Translocates the Kinase to Peripheral Membranes. *J Biol Chem* **270**, 29051-29054.

Leung, T., Chen, X. Q., Manser, E. and Lim, L. (1996). The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol Cell Biol* **16**, 5313-27.

Leung, T., Chen, X. Q., Tan, I., Manser, E. and Lim, L. (1998). Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Molecular and Cellular Biology* **18**, 130-140.

Li, S. C., Songyang, Z., Vincent, S. J., Zwahlen, C., Wiley, S., Cantley, L., Kay, L. E., Forman Kay, J. and Pawson, T. (1997). High-affinity binding of the Drosophila Numb phosphotyrosine-binding domain to peptides containing a Gly-Pro-(p)Tyr motif. *Proc Natl Acad Sci U S A* **94**, 7204-9.

Li, R. and Zheng, Y. (1997). Residues of the Rho family GTPases Rho and Cdc42 that specify sensitivity to Dbl-like guanine nucleotide exchange factors. *J Biol Chem* **272**, 4671-9.

Lim, H. H., Michael, G. J., Smith, P., Lim, L. and Hall, C. (1992). Developmental regulation and neuronal expression of the mRNA of rat n-chimaerin, a p21rac GAP:cDNA sequence. *Biochem J* **287**, 415-22.

Lim, L., Manser, E., Leung, T. and Hall, C. (1996). Regulation of phosphorylation pathways by p21 GTPases. *Eur J Biochem* **242**, 171-85.

Lim, W. A., Richards, F. M. and Fox, R. O. (1994). Structural Determinants Of Peptide-Binding Orientation and Of Sequence Specificity In Sh3 Domains. *Nature* **372**, 375-379.

Lo, Y. Y. and Cruz, T. F. (1995). Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J Biol Chem* **270**, 11727-30.

Lohia, A. and Samuelson, J. (1996). Heterogeneity of Entamoeba histolytica rac genes encoding p21rac homologues. *Gene* **173**, 205-8.

Lopez Ilasaca, M., Gutkind, J. S. and Wetzker, R. (1998). Phosphoinositide 3-kinase gamma is a mediator of Gbetagamma-dependent Jun kinase activation. *J Biol Chem* **273**, 2505-8.

Ludbrook, S. B., Eccleston, J. F. and Strom, M. (1997). Cloning and characterization of a rhoGAP homolog from Dictyostelium discoideum. *J Biol Chem* **272**, 15682-6.

Luduena, R. F. (1998). Multiple forms of tubulin: different gene products and covalent modifications. *Int Rev Cytol* **178**, 207-75.

Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev* **8,** 1787-802.

Luo, L., Hensch, T. K., Ackerman, L., Barbel, S., Jan, L. Y. and Jan, Y. N. (1996). Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. *Nature* **379,** 837-40.

Lupher, M. L., Jr., Songyang, Z., Shoelson, S. E., Cantley, L. C. and Band, H. (1997). The Cbl phosphotyrosine-binding domain selects a D(N/D)XpY motif and binds to the Tyr292 negative regulatory phosphorylation site of ZAP-70. *J Biol Chem* **272,** 33140-4.

Maccioni, R. B. and Cambiazo, V. (1995). Role of microtubule-associated proteins in the control of microtubule assembly. *Physiol Rev* **75,** 835-64.

Machesky, L. M. and Hall, A. (1996). Rho - a Connection Between Membrane-Receptor Signaling and the Cytoskeleton. *Trends In Cell Biology* **6,** 304-310.

Maclennan, A. J. and Shaw, G. (1993). A yeast SH2 domain [letter]. *Trends Biochem Sci* **18,** 464-5.

Madaule, P. and Axel, R. (1985). A novel ras-related gene family. *Cell* **41,** 31-40.

Maness, P. F. and Matten, W. T. (1990). Tyrosine phosphorylation of membrane-associated tubulin in nerve growth cones enriched in pp60c-src. *Ciba Found Symp* **150,** 57-69;.

Mannervik, B., Berhane, K., Castro, V. M., Olin, B., Ridderstrom, M., Vignani, R., Kozarich, J. W. and Ringborg, U. (1990). Glutathione-linked enzymes in normal and tumor cells and their role in resistance against genotoxic agents. *Princess Takamatsu Symp* **21,** 253-62.

Manser, E., Leung, T., Monfries, C., Teo, M., Hall, C. and Lim, L. (1992). Diversity and versatility of GTPase activating proteins for the p21rho subfamily of ras G proteins detected by a novel overlay assay. *J Biol Chem* **267,** 16025-8.

Manser, E., Leung, T., Salihuddin, H., Tan, L. and Lim, L. (1993). A non-receptor tyrosine kinase that inhibits the GTPase activity of p21cdc42. *Nature* **363**, 364-7.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S. and Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367**, 40-6.

Manser, E., Chong, C., Zhao, Z. S., Leung, T., Michael, G., Hall, C. and Lim, L. (1995). Molecular-Cloning Of a New Member Of the p21-Cdc42/Rac-Activated Kinase (Pak) Family. *Journal Of Biological Chemistry* **270**, 25070-25078.

Manser, E., Loo, T. H., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan, I., Leung, T. and Lim, L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Molecular Cell* **1**, 183-192.

Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F. and Ahn, N. G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265**, 966-70.

Marengere, L. E., Songyang, Z., Gish, G. D., Schaller, M. D., Parsons, J. T., Stern, M. J., Cantley, L. C. and Pawson, T. (1994). SH2 domain specificity and activity modified by a single residue. *Nature* **369**, 502-5.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-85.

Marte, B. M. and Downward, J. (1997). PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends In Biochemical Sciences* **22**, 355-358.

Martin, G. A., Yatani, A., Clark, R., Conroy, L., Polakis, P., Brown, A. M. and McCormick, F. (1992). GAP domains responsible for ras p21-dependent inhibition of muscarinic atrial K+ channel currents [see comments]. *Science* **255**, 192-4.

Maruyama, K. (1994). Connectin, an elastic protein of striated muscle. *Biophys Chem* **50**, 73-85.

Matsudaira, P. (1994). Actin crosslinking proteins at the leading edge. *Semin Cell Biol* **5**, 165-74.

Matus, A. (1988). Microtubule-associated proteins: their potential role in determining neuronal morphology. *Annu Rev Neurosci* **11,** 29-44.

Mayer, B. J. and Baltimore, D. (1994). Mutagenic analysis of the roles of SH2 and SH3 domains in regulation of the Abl tyrosine kinase. *Mol Cell Biol* **14,** 2883-94.

McCallum, S. J., Wu, W. J. and Cerione, R. A. (1996). Identification of a putative effector for Cdc42Hs with high sequence similarity to the RasGAP-related protein IQGAP1 and a Cdc42Hs binding partner with similarity to IQGAP2. *J Biol Chem* **271,** 21732-7.

McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, S., Rozakis Adcock, M., Chen, L. B. and Pawson, T. (1993). The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. *Embo J* **12,** 3073-81.

McNally, F. J. (1996). Modulation of microtubule dynamics during the cell cycle. *Curr Opin Cell Biol* **8,** 23-9.

Mermall, V., Post, P. L. and Mooseker, M. S. (1998). Unconventional myosins in cell movement, membrane traffic, and signal transduction. *Science* **279,** 527-33.

Mikita, T., Daniel, C., Wu, P. and Schindler, U. (1998). Mutational analysis of the STAT6 SH2 domain. *J Biol Chem* **273**, 17634-42

Mikoshiba, K., Furuichi, T., Miyawaki, A., Yoshikawa, S., Maeda, N., Niinobe, M., Nakade, S., Nakagawa, T., Okano, H. and Aruga, J. (1992). The inositol 1,4,5-trisphosphate receptor. *Ciba Found Symp* **164,** 17-29;.

Milburn, M. V., Tong, L., deVos, A. M., Brunger, A., Yamaizumi, Z., Nishimura, S. and Kim, S. H. (1990). Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. *Science* **247,** 939-45.

Minden, A., Lin, A., Claret, F. X., Abo, A. and Karin, M. (1995). Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81,** 1147-57.

Mineo, C., Anderson, R. G. and White, M. A. (1997). Physical association with ras enhances activation of membrane-bound raf (RafCAAX). *J Biol Chem* **272**, 10345-8.

Minturn, J. E., Fryer, H. J., Geschwind, D. H. and Hockfield, S. (1995a). TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth. *J Neurosci* **15**, 6757-66.

Minturn, J. E., Geschwind, D. H., Fryer, H. J. and Hockfield, S. (1995b). Early postmitotic neurons transiently express TOAD-64, a neural specific protein. *J Comp Neurol* **355**, 369-79.

Moodie, S. A., Paris, M., Villafranca, E., Kirshmeier, P., Willumsen, B. M. and Wolfman, A. (1995). Different structural requirements within the switch II region of the Ras protein for interactions with specific downstream targets. *Oncogene* **11**, 447-54.

Muller, B. K., Bonhoeffer, F. and Drescher, U. (1996). Novel gene families involved in neural pathfinding. *Curr Opin Genet Dev* **6**, 469-74.

Muller, R. T., Honnert, U., Reinhard, J. and Bahler, M. (1997). The rat myosin myr 5 is a GTPase-activating protein for Rho in vivo: essential role of arginine 1695. *Mol Biol Cell* **8**, 2039-53.

Munch Petersen, B., Tyrsted, G. and Cloos, L. (1993). Reversible ATP-dependent transition between two forms of human cytosolic thymidine kinase with different enzymatic properties. *J Biol Chem* **268**, 15621-5.

Musacchio, A., Cantley, L.C. and Harrison, S.C. (1996), Crystal structure from the breakpoint cluster region-hoology region from phosphoinositide 3-kinase p85α subunit. *Proc Natl Acad Sci, USA* **93**, 14373-78.

Nakata, H. and Watanabe, Y. (1998). Comparative study of p120 GTPase-activating protein and its point mutant in the pleckstrin homology domain. *Cell Signal* **10**, 211-6.

Nantel, A., MohammadAli, K., Sherk, J., Posner, B. I. and Thomas, D. Y. (1998). Interaction of the Grb10 adapter protein with the Raf1 and MEK1 kinases. *Journal Of Biological Chemistry* **273**, 10475-10484.

Neer, E. J. (1994). G-Proteins - Critical Control Points For Transmembrane Signals. *Protein Science* **3**, 3-14.

Nikolic, M., Chou, M. M., Lu, W. G., Mayer, B. J. and Tsai, L. H. (1998). The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. *Nature* **395**, 194-198.

Nishibe, S., Wahl, M. I., Hernandez Sotomayor, S. M., Tonks, N. K., Rhee, S. G. and Carpenter, G. (1990). Increase of the catalytic activity of phospholipase C-gamma 1 by tyrosine phosphorylation. *Science* **250**, 1253-6.

Nobes, C. and Hall, A. (1994). Regulation and function of the Rho subfamily of small GTPases. *Curr Opin Genet Dev* **4**, 77-81.

Nobes, C. D. and Hall, A. (1995). Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem Soc Trans* **23**, 456-9.

Obermeier, A., Tinhofer, I., Grunicke, H. H. and Ullrich, A. (1996). Transforming potentials of epidermal growth factor and nerve growth factor receptors inversely correlate with their phospholipase C gamma affinity and signal activation. *Embo J* **15**, 73-82.

Obermeier, A., Ahmed, S., Manser, E., Yen, S. C., Hall, C. and Lim, L. (1998). PAK promotes morphological changes by acting upstream of Rac. *Embo J* **17**, 4328-39.

Olenik, C., Barth, H., Just, I., Aktories, K. and Meyer, D. K. (1997). Gene expression of the small GTP-binding proteins RhoA, RhoB, Rac1, and Cdc42 in adult rat brain. *Molecular Brain Research* **52**, 263-269.

Oligino, L., Lung, F. D. T., Sastry, L., Bigelow, J., Cao, T., Curran, M., Burke, T. R., Wang, S. M., Krag, D., Roller, P. P. and King, C. R. (1997). Nonphosphorylated peptide ligands for the Grb2 Src homology 2 domain. *Journal Of Biological Chemistry* **272**, 29046-29052.

Olson, M. F., Pasteris, N. G., Gorski, J. L. and Hall, A. (1996). Faciogenital dysplasia protein (FGD1) and Vav, two related proteins required for normal embryonic development, are upstream regulators of Rho GTPases. *Curr Biol* **6**, 1628-33.

Olson, M. F., Paterson, H. F. and Marshall, C. J. (1998). Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1 [see comments]. *Nature* **394**, 295-9.

Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. and Nishizuka, Y. (1989). Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc Natl Acad Sci U S A* **86**, 4868-71.

Orioli, D. and Klein, R. (1997). The Eph receptor family: axonal guidance by contact repulsion. *Trends Genet* **13**, 354-9.

Ottinger, E.A., Botfield, M.C. and Shoelson, S.E. (1998). Tandem SH2 domains confer high specificity in tyrosine kinase signalling. *J Biol Chem* **273**, 729-35.

Park, I., Chung, J., Walsh, C. T., Yun, Y., Strominger, J. L. and Shin, J. (1995). Phosphotyrosine-independent binding of a 62-kDa protein to the src homology 2 (SH2) domain of p56lck and its regulation by phosphorylation of Ser-59 in the lck unique N-terminal region. *Proc Natl Acad Sci U S A* **92**, 12338-42.

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D. and Ullrich, A. (1986). The complete primary structure of protein kinase C--the major phorbol ester receptor. *Science* **233**, 853-9.

Pawson, T. (1995). Protein modules and signalling networks. *Nature* **373**, 573-80.

Pawson, T. and Scott, J. D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075-2080.

Pei, Z., Yang, L. and Williamson, J. R. (1996). Phospholipase C-gamma 1 binds to actin-cytoskeleton via its C-terminal SH2 domain in vitro. *Biochem Biophys Res Commun* **228**, 802-6.

Pellicena, P., Stowell, K. R. and Miller, W. T. (1998). Enhanced phosphorylation of Src family kinase substrates containing SH2 domain binding sites. *J Biol Chem* **273**, 15325-8.

Pendergast, A. M., Muller, A. J., Havlik, M. H., Maru, Y. and Witte, O. N. (1991). BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. *Cell* **66**, 161-71.

Peppelenbosch, M. P., Qiu, R. G., de Vries Smits, A. M., Tertoolen, L. G., de Laat, S. W., McCormick, F., Hall, A., Symons, M. H. and Bos, J. L. (1995). Rac mediates growth factor-induced arachidonic acid release. *Cell* **81**, 849-56.

Perona, R., Montaner, S., Saniger, L., Sanchez Perez, I., Bravo, R. and Lacal, J. C. (1997). Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes Dev* **11**, 463-75.

Phizicky, E. M. and Fields, S. (1995). Protein-protein interactions: methods for detection and analysis. *Microbiol Rev* **59**, 94-123.

Polakis, P. and McCormick, F. (1993). Structural requirements for the interaction of p21ras with GAP, exchange factors, and its biological effector target. *J Biol Chem* **268**, 9157-60.

Puius, Y. A., Mahoney, N. M. and Almo, S. C. (1998). The modular structure of actin-regulatory proteins. *Curr Opin Cell Biol* **10**, 23-34.

Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. and Woodgett, J. R. (1991). Phosphorylation of c-jun mediated by MAP kinases. *Nature* **353**, 670-4.

Qiu, R. G., Abo, A., McCormick, F. and Symons, M. (1997). Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. *Mol Cell Biol* **17**, 3449-58.

Quilliam, L. A., Khosravi Far, R., Huff, S. Y. and Der, C. J. (1995). Guanine nucleotide exchange factors: activators of the Ras superfamily of proteins. *Bioessays* **17**, 395-404.

Quilliam, L. A., Lambert, Q. T., Mickelson Young, L. A., Westwick, J. K., Sparks, A. B., Kay, B. K., Jenkins, N. A., Gilbert, D. J., Copeland, N. G. and Der, C. J. (1996). Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein and potential effector of Rho protein signaling. *J Biol Chem* **271**, 28772-6.

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J. and Davis, R. J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem* **270**, 7420-6.

Rameh, L. E., Chen, C. S. and Cantley, L. C. (1995). Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell* **83**, 821-30.

Reid, T., Furayashiki, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P. and Narumiya, S. (1996). Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the Rho-binding domain. *Journal Of Biological Chemistry* **271**, 13556-13560.

Ridderstrom, M., Saccucci, F., Hellman, U., Bergman, T., Principato, G. and Mannervik, B. (1996). Molecular cloning, heterologous expression, and characterization of human glyoxalase II. *J Biol Chem* **271**, 319-23.

Ridley, A. J. and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-99.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-10.

Ridley, A. J. (1995). Rho-related proteins: actin cytoskeleton and cell cycle. *Curr Opin Genet Dev* **5**, 24-30.

Rivero Lezcano, O. M., Marcilla, A., Sameshima, J. H. and Robbins, K. C. (1995). Wiskott-Aldrich syndrome protein physically associates with Nck through Src homology 3 domains. *Mol Cell Biol* **15**, 5725-31.

Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D. and Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras [see comments]. *Nature* **370**, 527-32.

RodriguezViciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A. and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89**, 457-467.

Rommel, C. and Hafen, E. (1998). Ras - a versatile cellular switch. *Current Opinion In Genetics & Development* **8**, 412-418.

Roychowdhury, S. and Rasenick, M. M. (1997). G protein beta1gamma2 subunits promote microtubule assembly. *J Biol Chem* **272**, 31576-81.

Rozakis Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G. and et al. (1992). Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360**, 689-92.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993). The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1 [see comments]. *Nature* **363**, 83-5.

Rudel, T., Zenke, F. T., Chuang, T. H. and Bokoch, G. M. (1998). Cutting edge: p21-activated kinase (PAK) is required for Fas-induced JNK activation in Jurkat cells. *Journal Of Immunology* **160**, 7-11.

Rui, L. and Carter-Su, C. (1998). Platelet-derived growth factor (PDGF) stimulates the association of SH2-Bβ with PDGF receptor and phosphorylation of SH2-Bβ. *J Biol Chem* **273**, 21239-45.

Sanchez, P., De Carcer, G., Sandoval, I. V., Moscat, J. and Diaz Meco, M. T. (1998). Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62. *Mol Cell Biol* **18**, 3069-80.

Saras, J., Franzen, P., Aspenstrom, P., Hellman, U., Gonez, L. J. and Heldin, C. H. (1997). A novel GTPase-activating protein for Rho interacts with a PDZ domain of the protein-tyrosine phosphatase PTPL1. *J Biol Chem* **272**, 24333-8.

Sastry, L., Lin, W., Wong, W. T., Di Fiore, P. P., Scoppa, C. A. and King, C. R. (1995). Quantitative analysis of Grb2-Sos1 interaction: the N-terminal SH3 domain of Grb2 mediates affinity. *Oncogene* **11,** 1107-12.

Schaber, M. D., Garsky, V. M., Boylan, D., Hill, W. S., Scolnick, E. M., Marshall, M. S., Sigal, I. S. and Gibbs, J. B. (1989). Ras interaction with the GTPase-activating protein (GAP). *Proteins* **6,** 306-15.

Schafer, D. A. and Cooper, J. A. (1995). Control of actin assembly at filament ends. *Ann Rev Cell Dev Biol* **11,** 497-518.

Schaffhausen, B. (1995). SH2 domain structure and function. *Biochim Biophys Acta* **1242,** 61-75.

Scheffzek, K., Klebe, C., Fritz Wolf, K., Kabsch, W. and Wittinghofer, A. (1995). Crystal structure of the nuclear Ras-related protein Ran in its GDP-bound form. *Nature* **374,** 378-81.

Schlessinger, J. (1997). Direct binding and activation of receptor tyrosine kinases by collagen. *Cell* **91,** 869-72.

Schmitz, U., Ishida, T., Ishida, M., Surapisitchat, J., Hasham, M. I., Pelech, S. and Berk, B. C. (1998). Angiotensin II stimulates p21-activated kinase in vascular smooth muscle cells - Role in activation of JNK. *Circulation Research* **82,** 1272-1278.

Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D. and Emr, S. D. (1993). Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science* **260,** 88-91.

Segal, A. W. and Abo, A. (1993). The Biochemical Basis Of the Nadph Oxidase Of Phagocytes. *Trends In Biochemical Sciences* **18,** 43-47.

Seger, R. and Krebs, E. G. (1995). The MAPK signaling cascade. *Faseb J* **9,** 726-35.

Selbie, L. A. and Hill, S. J. (1998). G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. *Trends Pharmacol Sci* **19,** 87-93.

Sells, M. A. and Chernoff, J. (1997). Emerging from the Pak: the p21-activated protein kinase family. *Trends in Cell Biology* **7**, 162-167.

Settleman, J., Albright, C. F., Foster, L. C. and Weinberg, R. A. (1992). Association between GTPase activators for Rho and Ras families. *Nature* **359**, 153-4.

Seykora, J. T., Myat, M. M., Allen, L. A. H., Ravetch, J. V. and Aderem, A. (1996). Molecular determinants of the myristoyl-electrostatic switch of MARCKS. *J Biol Chem* **271**, 18797-802.

Shaw, G. (1996). The pleckstrin homology domain: An intriguing multifunctional protein module. *Bioessays* **18**, 35-46.

Shimohama, S., Sasaki, Y., Fujimoto, S., Kamiya, S., Taniguchi, T., Takenawa, T. and Kimura, J. (1998). Phospholipase C isozymes in the human brain and their changes in Alzheimer's disease. *Neuroscience* **82**, 999-1007.

Shrivastava, A., Radziejewski, C., Campbell, E., Kovac, L., McGlynn, M., Ryan, T. E., Davis, S., Goldfarb, M. P., Glass, D. J., Lemke, G. and Yancopoulos, G. D. (1997). An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. *Mol Cell* **1**, 25-34.

Sin, W.C. (1994). Isolation and characterisation of α2-chimaerin cDNA encoding a p21^{rac} GTPase-activating protein with an SH2 domain. *Ph.D. Thesis.*

Smith, M. R., Court, D. W., Kim, H. K., Park, J. B., Rhee, S. G., Rhim, J. S. and Kung, H. F. (1998). Overexpression of phosphoinositide-specific phospholipase Cgamma in NIH 3T3 cells promotes transformation and tumorigenicity. *Carcinogenesis* **19**, 177-85.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J. and et al. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767-78.

Songyang, Z., Shoelson, S. E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T. and et al. (1994). Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol Cell Biol* **14**, 2777-85.

Sorokin, A., Reed, E., Nnkemere, N., Dulin, N. O. and Schlessinger, J. (1998). Crk protein binds to PDGF receptor and insulin receptor substrate-1 with different modulating effects on PDGF- and insulin-dependent signaling pathways. *Oncogene* **16,** 2425-2434.

Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J. and Lax, I. (1994). Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* **79,** 1015-24.

Sulciner, D. J., Irani, K., Yu, Z. X., Ferrans, V. J., Goldschmidt Clermont, P. and Finkel, T. (1996). rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for Nf-kappaB activation. *Mol Cell Biol* **16,** 7115-21.

Sun, H. and Tonks, N. K. (1994). The coordinated action of protein tyrosine phosphatases and kinases in cell signaling. *Trends Biochem Sci* **19,** 480-5.

Sun, H. Q., Kwiatkowska, K. and Yin, H. L. (1995). Actin monomer binding proteins. *Curr Opin Cell Biol* **7,** 102-10.

Svitkina, T. M., Verkhovsky, A. B. and Borisy, G. G. (1996). Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. *J Cell Biol* **135,** 991-1007.

Symons, M., Derry, J. M. J., Karlak, B., Jiang, S., Lemahieu, V., McCormick, F., Francke, U. and Abo, A. (1996). Wiskott-Aldrich Syndrome Protein, a Novel Effector For the Gtpase Cdc42hs, Is Implicated In Actin Polymerization. *Cell* **84,** 723-734.

Takahashi, K., Sasaki, T., Mammoto, A., Takaishi, K., Kameyama, T., Tsukita, S. and Takai, Y. (1997). Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. *J Biol Chem* **272,** 23371-5.

Tan, E. C., Leung, T., Manser, E. and Lim, L. (1993). The Human Active Breakpoint Cluster Region-Related Gene Encodes a Brain Protein With Homology to Guanine-Nucleotide Exchange Proteins and GTPase-Activating Proteins. *Journal Of Biological Chemistry* **268,** 27291-27298.

Tanaka, K. and Takai, Y. (1998). Control of reorganization of the actin cytoskeleton by Rho family small GTP-binding proteins in yeast. *Curr Opin Cell Biol* **10**, 112-6.

Tapon, N., Nagata, K., Lamarche, N. and Hall, A. (1998). A new Rac target POSH is an SHS-containing scaffold protein involved in the JNK and NF-kappa B signalling pathways. *Embo Journal* **17**, 1395-1404.

Taylor, J. M., Hildebrand, J. D., Mack, C. P., Cox, M. E. and Parsons, J. T. (1998). Characterization of graf, the GTPase-activating protein for rho associated with focal adhesion kinase. Phosphorylation and possible regulation by mitogen-activated protein kinase. *J Biol Chem* **273**, 8063-70.

Teo, M. (1994). Purification and Functional Characterisation of Brain and Recombinant Chimaerin, a p21rac GTPase activating protein. *Ph.D. Thesis.*

Teo, M., Manser, E. and Lim, L. (1995). Identification and molecular cloning of a p21cdc42/rac1-activated serine/threonine kinase that is rapidly activated by thrombin in platelets. *J Biol Chem* **270**, 26690-7.

Thissen, J. A., Gross, J. M., Subramanian, K., Meyer, T. and Casey, P. J. (1997). Prenylation-dependent association of Ki-Ras with microtubules. Evidence for a role in subcellular trafficking. *J Biol Chem* **272**, 30362-70.

Thompson, A. D., Braun, B. S., Arvand, A., Stewart, S. D., May, W. A., Chen, E., Korenberg, J. and Denny, C. (1996). EAT-2 is a novel SH2 domain containing protein that is up regulated by Ewing's sarcoma EWS/FLI1 fusion gene. *Oncogene* **13**, 2649-2658.

Thornalley, P. J. (1993). The glyoxalase system in health and disease. *Mol Aspects Med* **14**, 287-371.

Threadgill, R., Bobb, K. and Ghosh, A. (1997). Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. *Neuron* **19**, 625-34.

Toker, A. and Cantley, L. C. (1997). Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387**, 673-6.

Tolias, K. F., Cantley, L. C. and Carpenter, C. L. (1995). Rho family GTPases bind to phosphoinositide kinases. *J Biol Chem* **270**, 17656-9.

Toure, A., Dorseuil, O., Morin, L., Timmons, P., Jegou, B., Reibel, L. and Gacon, G. (1998). MgcRacGAP, a new human GTPase-activating protein for Rac and Cdc42 similar to Drosophila rotundRacGAP gene product, is expressed in male germ cells. *J Biol Chem* **273**, 6019-23.

Trahey, M. and McCormick, F. (1987). A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* **238**, 542-5.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. *Curr Opin Genet Dev* **4**, 96-101.

Treisman, R. (1996). Regulation of transcription by MAP kinase cascades. *Curr Opin Cell Biol* **8**, 205-15.

Tsukita, S., Oishi, K., Sato, N., Sagara, J., Kawai, A. and Tsukita, S. (1994). ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J Cell Biol* **126**, 391-401.

Tsukita, S., Yonemura, S. and Tsukita, S. (1997). ERM proteins: head-to-tail regulation of actin-plasma membrane interaction. *Trends Biochem Sci* **22**, 53-8.

Turunen, O., Wahlstrom, T. and Vaheri, A. (1994). Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. *J Cell Biol* **126**, 1445-53.

Ullrich, A. and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-12.

Van Aelst, L., Barr, M., Marcus, S., Polverino, A. and Wigler, M. (1993). Complex formation between RAS and RAF and other protein kinases. *Proc Natl Acad Sci U S A* **90**, 6213-7.

Van der Geer, P., Henkemeyer, M., Jacks, T. and Pawson, T. (1997). Aberrant Ras regulation and reduced p190 tyrosine phosphorylation in cells lacking p120-Gap. *Mol Cell Biol* **17**, 1840-7.

Van Horn, D. J., Myers, M. G., Jr. and Backer, J. M. (1994). Direct activation of the phosphatidylinositol 3'-kinase by the insulin receptor. *J Biol Chem* **269,** 29-32.

van Weering, D. H., de Rooij, J., Marte, B., Downward, J., Bos, J. L. and Burgering, B. M. (1998). Protein kinase B activation and lamellipodium formation are independent phosphoinositide 3-kinase-mediated events differentially regulated by endogenous Ras. *Mol Cell Biol* **18,** 1802-11.

Von Willebrand, M., Williams, S., Saxena, M., Gilman, J., Tailor, P., Jascur, T., Amarante-Mendes, G.P., Green, D.R. and Mustelin, T. (1998). Modification of phosphatidylinositol 3-kinase SH2 domain binding properties by abl- or lck-mediated tyrosine phosphorylation at tyr-668. *J Biol Chem* **273,** 3994-4000.

Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D. and Miyamoto, S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* **9,** 2723-35.

Vogel, W., Gish, G. D., Alves, F. and Pawson, T. (1997). The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol Cell* **1,** 13-23.

Wade, R. H. and Hyman, A. A. (1997). Microtubule structure and dynamics. *Curr Opin Cell Biol* **9,** 12-7.

Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J., Overduin, M. and et al. (1992). Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides [see comments]. *Nature* **358,** 646-53.

Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D. and Kuriyan, J. (1993). Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. *Cell* **72,** 779-90.

Walker, J. E., Arizmendi, J. M., Dupuis, A., Fearnley, I. M., Finel, M., Medd, S. M., Pilkington, S. J., Runswick, M. J. and Skehel, J. M. (1992). Sequences of 20 subunits of NADH:ubiquinone oxidoreductase from bovine heart mitochondria. Application of a novel strategy for sequencing proteins using the polymerase chain reaction. *J Mol Biol* **226,** 1051-72.

Wang, L. H. and Strittmatter, S. M. (1996). A family of rat CRMP genes is differentially expressed in the nervous system. *J Neurosci* **16,** 6197-207.

Wang, L. H. and Strittmatter, S. M. (1997). Brain CRMP forms heterotetramers similar to liver dihydropyrimidinase. *J Neurochem* **69,** 2261-9.

Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A. and Narumiya, S. (1996). Protein kinase N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho. *Science* **271,** 645-648.

Way, M., Pope, B. and Weeds, A. G. (1992). Are the conserved sequences in segment 1 of gelsolin important for binding actin? *J Cell Biol* **116,** 1135-43.

Wegner, A. (1976). Head to tail polymerization of actin. *J Mol Biol* **108,** 139-50.

Weissbach, L., Settleman, J., Kalady, M. F., Snijders, A. J., Murthy, A. E., Yan, Y. X. and Bernards, A. (1994). Identification of a human rasGAP-related protein containing calmodulin-binding motifs. *J Biol Chem* **269,** 20517-21.

Weng, G., Li, J., Dingus, J., Hildebrandt, J. D., Weinstein, H. and Iyengar, R. (1996). Gbeta subunit interacts with a peptide encoding region 956-982 of adenylyl cyclase 2. Cross-linking of the peptide to free Gbetagamma but not the heterotrimer. *J Biol Chem* **271,** 26445-8.

Wirth, J. A., Jensen, K. A., Post, P. L., Bement, W. M. and Mooseker, M. S. (1996). Human myosin-IXb, an unconventional myosin with a chimerin-like rho/rac GTPase-activating protein domain in its tail. *J Cell Sci* **109,** 653-61.

Witke, W., Podtelejnikov, A. V., Di Nardo, A., Sutherland, J. D., Gurniak, C. B., Dotti, C. and Mann, M. (1998). In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly. *Embo J* **17,** 967-76.

Wittinghofer, A. and Nassar, N. (1996). How Ras-related proteins talk to their effectors. *Trends Biochem Sci* **21,** 488-91.

Woodford-Thomas, T. A., Rhodes, J. D. and Dixon, J. E. (1992). Expression of a protein tyrosine phosphatase in normal and v-src-transformed mouse 3T3 fibroblasts. *J Cell Biol* **117**, 401-14.

Wu, W. J., Leonard, D. A., R, A. C. and Manor, D. (1997). Interaction between Cdc42Hs and RhoGDI is mediated through the Rho insert region. *J Biol Chem* **272**, 26153-8.

Wu, Y., Spencer, S. D. and Lasky, L. A. (1998). Tyrosine phosphorylation regulates the SH3-mediated binding of the Wiskott-Aldrich syndrome protein to PSTPIP, a cytoskeletal-associated protein. *J Biol Chem* **273**, 5765-70.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270**, 1326-31.

Xu, W. Q., Harrison, S. C. and Eck, M. J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. *Nature* **385**, 595-602.

Yamada, K. M. and Miyamoto, S. (1995). Integrin transmembrane signaling and cytoskeletal control. *Curr Opin Cell Biol* **7**, 681-9.

Yamada, K. M. and Geiger, B. (1997). Molecular interactions in cell adhesion complexes. *Curr Opin Cell Biol* **9**, 76-85.

Yamamoto, T., Matsui, T., Nakafuku, M., Iwamatsu, A. and Kaibuchi, K. (1995). A novel GTPase-activating protein for R-Ras. *J Biol Chem* **270**, 30557-61.

Yang, W., Tabrizi, M., Berrada, K. and Yi, T. (1998). SHP-1 phosphatase C-terminus interacts with novel substrates p32/p30 during erythropoietin and interleukin-3 mitogenic responses. *Blood* **91**, 3746-55.

Yenush, L., Makati, K. J., Smith Hall, J., Ishibashi, O., Myers, M. G., Jr. and White, M. F. (1996). The pleckstrin homology domain is the principal link between the insulin receptor and IRS-1. *J Biol Chem* **271**, 24300-6.

Yoshida, H., Watanabe, A. and Ihara, Y. (1998). Collapsin response mediator protein-2 is associated with neurofibrillary tangles in Alzheimer's disease. *J Biol Chem* **273**, 9761-8.

Yu, J., Zhang, Y., McIlroy, J., Rordorf-Nikolic, T., Orr, G.A. and Backer, J.M. (1998a). Regulation of the p85/p110 phosphatidylinositol 3-kinase: stabilisation and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol* **18**, 1379-87.

Yu, J., Wjasow, C. and Backer J.M. (1998b). Regulation of the p85/p110α phosphatidylinositol 3-kinase. *J Biol Chem* **273**, 30199-203.

Yuan, B. Z., Miller, M. J., Keck, C. L., Zimonjic, D. B., Thorgeirsson, S. S. and Popescu, N.C. (1998). Cloning, characterization, and chromosomal localization of a gene frequently deleted in human liver cancer (DLC-1) homologous to rat RhoGAP. *Cancer Res* 58, 2196-9.

Yuan, S., Miller, D. W., Barnett, G. H., Hahn, J. F. and Williams, B. R. (1995). Identification and characterization of human beta 2-chimaerin: association with malignant transformation in astrocytoma. *Cancer Res* **55**, 3456-61.

Zalcman, G., Closson, V., Camonis, J., Honore, N., Rousseau Merck, M. F., Tavitian, A. and Olofsson, B. (1996). RhoGDI-3 is a new GDP dissociation inhibitor (GDI). Identification of a non-cytosolic GDI protein interacting with the small GTP-binding proteins RhoB and RhoG. *J Biol Chem* **271**, 30366-74.

Zervos, A. S., Gyuris, J. and Brent, R. (1993). Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites [published erratum appears in Cell 1994 Oct 21;79(2):following 388]. *Cell* **72**, 223-32.

Zheng, Y., Cerione, R. and Bender, A. (1994a). Control of the yeast bud-site assembly GTPase Cdc42. Catalysis of guanine nucleotide exchange by Cdc24 and stimulation of GTPase activity by Bem3. *J Biol Chem* **269**, 2369-72.

Zheng, Y., Bagrodia, S. and Cerione, R. A. (1994b). Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. *J Biol Chem* **269**, 18727-30.

Zheng, Y., Wong, M. L., Alberts, B. and Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. *Nature* **378,** 578-83.
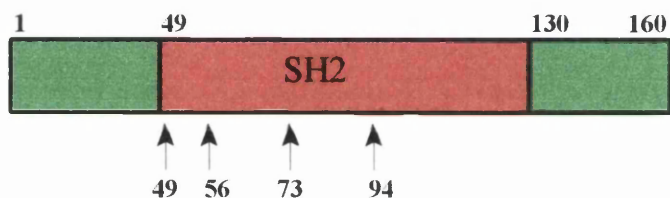
Zheng, Y., Zangrilli, D., Cerione, R. A. and Eva, A. (1996). The pleckstrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting. *J Biol Chem* **271,** 19017-20.

Zhou, M. M. and Fesik, S. W. (1995). Structure and Function Of the Phosphotyrosine Binding (Ptb) Domain. *Progress In Biophysics & Molecular Biology* **64,** 221-235.

APPENDICES
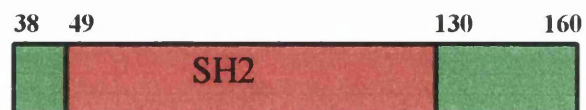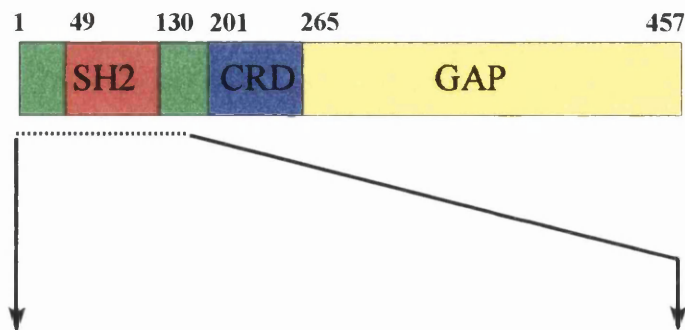
Appendix 1: The Different α2 Chimaerin SH2 Domain Constructs Used

A schematic representation of the domain structure of full length α2 chimaerin is shown at the top of the diagram, the two different SH2 domain constructs beneath it. The SH2 domain is indicated in red, sequences flanking the SH2 domain in green, the cysteine rich/phorbol ester binding domain in blus and the GTPase activating domain in brown. In each part of the diagram, the number of the first amino acid of each distinct part of the protein is shown with the exception of the final number given, which refers to the last amino acid of the protein sequence. The two different SH2 domain constructs used differed in the length of their amino-terminal sequences. In general, Construct A was used to produce proteins for affinity chromatography experiments and Construct B if the fusion proteins was to be radioactively labelled. Point mutations were produced in the chimaerin SH2 domain at the sites marked on Construct B. These proteins were expressed exclusively with the full amino-terminal sequences of α2 chimaerin (i.e. Construct B).

```
CGCCTTTACAATGGCCCTGACCCTGTTTGATACAGATGAATATAGACCTCCTGTTTGGAA    60
ATCTTACTTATATCAGCTACAACAGGAAGCCCCTCATCCTCGAAGAATTACCTGTACTTG    120
CGAGGTGGAAAACAGACCAAAGTATTATGGAAGAGAGTTTCATGGCATGATCTCCAGAGA    180
AGCAGCCGACCAGCTCTTGATTGTGGCTGAGGGGAGCTACCTCATCCGGGAGAGCCAGCG    240
GCAGCCAGGGACCTACACTTTGGCTTTAAGATTTGGAAGTCAAACCAGAAACTTCAGGCT    300
CTACTACGATGGCAAGCACTTTGTTGGGGAGAAACGCTTTGAGTCCATCCACGATCTGGT    360
GACTGATGGCTTGATTACTCTCTATATTGAAACCAAGGCAGCAGAATACATTGCCAAGAT    420
GACGATAAACCCAATTTATGAGCACGTAGGATACACAACCTTAAACAGAGAGCCAGCATA    480
CAAAAAACATATGCCAGTCCTGAAAGAGACACATGATGAGAGAGATTCTACAGGCCAGGA    540
TGGGGTGTCAGAGAAAAGGTGACATCACTTGTTAGAAGAGCAACTCTGAAAGAAAACGA    600
GCAAATTCCAAAATATGAAAAGATTCACAATTTCAAGGTGCATACATTCAGAGGGCCACA    660
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TACCACTAAGCGGCCAATGGTGGTAGACATGTGCATCAGGGAGATTGAGTCTAGAGGTCT    900
TAATTCTGAAGGACTATACCGAGTATCAGGATTTAGTGACCTAATTGAAGATGTCAAGAT    960
GGCTTTCGACAGAGATGGTGAGAAGGCAGATATTTCTGTGAACATGTATGAAGATATCAA    1020
CATTATCACTGGTGCACTTAAACTGTACTTCAGGGATTTGCCAATTCCACTCATTACATA    1080
TGATGCCTACCCTAAGTTTATAGAATCTGCCAAAATTATGGATCCGGATGAGCAATTGGA    1140
AACCCTTCATGAAGCACTGAAACTACTGCCACCTGCTCACTGCGAAACCCTCCGGTACCT    1200
CATGGCACATCTAAAGAGAGTGACCCTCCACGAAAAGGAGAATCTTATGAATGCAGAGAA    1260
CCTTGGAATCGTCTTTGGACCCACCCTTATGAGATCTCCAGAACTAGACGCCATGGCTGC    1320
ATTGAATGATATACGGTATCAGAGACTGGTGGTGGAGCTGCTTATCAAAAACGAAGACAT    1380
TTTATTTTAAATTTTTAATTTGAGGGGAAAAGAAATGTTTTACAGATGAAGGAATGTTTT    1440
ATAGTAATTTAATTTGCTCCTGTAGCTGCATTATTTCTTGATTAGAGGTTTGGGCATATA    1500
ACCAGATTAAAGTGAAGGAACTTTCTGTTGTTTTTGTAGCACCGCTCAGCTGTCTTGTAA    1560
AACAGTGAACACACGCTTTCTGGTTCTAGTAATCCTGGGTGTTTATCACGTTCAGAGAAA    1620
CTCAAGCTATTGCATGATTAGCCCCCTATCTGGCAAGGAAACCCCATACAGAAGAAACAA    1680
CAAACCTGCGCCTGCACCGCCTCTGCGTCCTGGGTAGTCTGTGCTTGTAATCCAGCATGT    1740
TTCACAGAGTAAGCCTGTTGTGACTTTGCTTTTGGGGTCTATGTCATTGGTTTCTGATGC    1800
TTGTACAAACACGCACACACAAATGGATAAAACAGCACCTCTGGCTGTTACATTACCATA    1860
AACCATATCACATGCCTACATTTTACAAATGATTTCTGGTTTCTCTTAGTTCTTCTCTAA    1920
CATAGTACTTTCTTTCCAGCAAAAGCAAAATGTGTTTTCAGATTTGTTACTTTAATAAAG    1980
GTTATCCATACCAATAAAAAGTGTACAACACAGCATTTTCTGTTAAATTATTATTGGTTT    2040
TCAGTTGTAATTTGGTATTTTTTCTGGCATGCGTTTATTAATTTATTAAATTGGCTTTTA    2100
GAAATAAAAAATATTGATAGCTTACTTTTTTCTCATAAGAAT    2142
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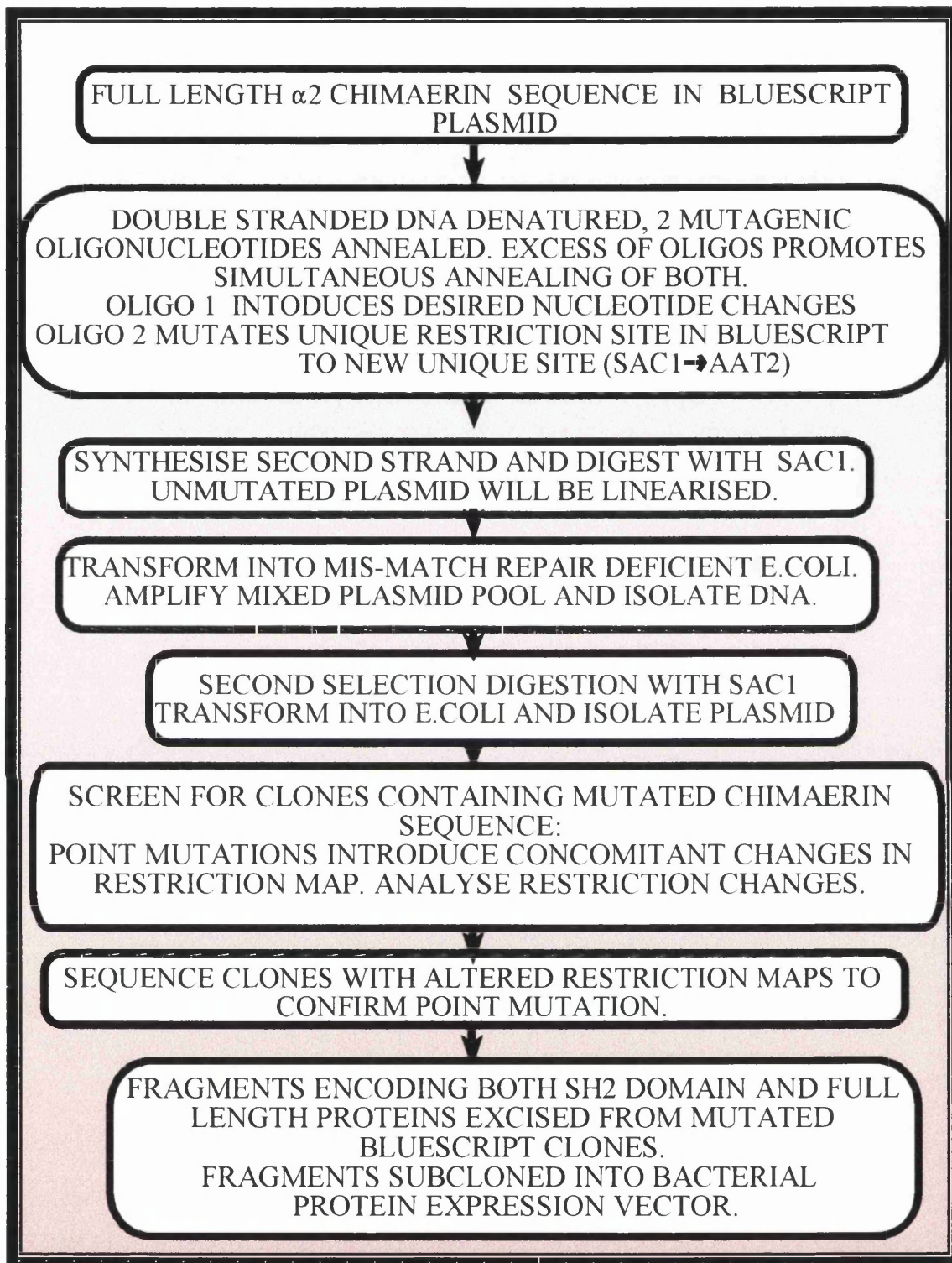
A summary of the mutations introduced in the chimaerin SH2 domain by primers GCF 3-6:

3. GAG→TGG: E^{49}→W $^{141-169}$CATCGGATGAAACCATATTCCATAATAC

4. AGA→CTA: R^{56}→L $^{164-191}$GTCGGCTGCTTCTAGGGAGATCATGCC

5. CGG→CTG: R^{73}→L $^{212-241}$CCGCTGGCTCTCCAGGATGAGGTAGCTCC

6. AAC→CAC: N^{94}→H $^{274-304}$GTAGAGCCTGAAGTGTCTGGTTTGACTTCC

Appendix 2A: Site Directed Mutagenesis of the Chimaerin SH2 Domain

The complete nucleotide sequence of the α2 chimaerin cDNA is shown (Genebank# HSA2CHIA_1). Coding sequences are indicated in bold type. The SH2 domain, the cysteine rich domain and the GAP domain are indicated in red, blue and green type respectively. The nucleotide marked in yellow indicates the point where the sequence common to α1 and α2 chimaerin begins. Codons in the SH2 region altered by site-directed mutagenesis to encode a different amino acid are underlined; the nucleotides altered within these codons are marked in brown. The nucleotide changes introduced and the consequent amino acid changes are summarised. The primers used are shown in a 5'→3' orientation; the nucleotides of the chimaerin sequence to which each primer was designed are indicated in superscipt.

FULL LENGTH α2 CHIMAERIN SEQUENCE IN BLUESCRIPT PLASMID

↓

DOUBLE STRANDED DNA DENATURED, 2 MUTAGENIC OLIGONUCLEOTIDES ANNEALED. EXCESS OF OLIGOS PROMOTES SIMULTANEOUS ANNEALING OF BOTH.
OLIGO 1 INTODUCES DESIRED NUCLEOTIDE CHANGES
OLIGO 2 MUTATES UNIQUE RESTRICTION SITE IN BLUESCRIPT TO NEW UNIQUE SITE (SAC1➡AAT2)

↓

SYNTHESISE SECOND STRAND AND DIGEST WITH SAC1. UNMUTATED PLASMID WILL BE LINEARISED.

↓

TRANSFORM INTO MIS-MATCH REPAIR DEFICIENT E.COLI. AMPLIFY MIXED PLASMID POOL AND ISOLATE DNA.

↓

SECOND SELECTION DIGESTION WITH SAC1 TRANSFORM INTO E.COLI AND ISOLATE PLASMID

↓

SCREEN FOR CLONES CONTAINING MUTATED CHIMAERIN SEQUENCE:
POINT MUTATIONS INTRODUCE CONCOMITANT CHANGES IN RESTRICTION MAP. ANALYSE RESTRICTION CHANGES.

↓

SEQUENCE CLONES WITH ALTERED RESTRICTION MAPS TO CONFIRM POINT MUTATION.

↓

FRAGMENTS ENCODING BOTH SH2 DOMAIN AND FULL LENGTH PROTEINS EXCISED FROM MUTATED BLUESCRIPT CLONES.
FRAGMENTS SUBCLONED INTO BACTERIAL PROTEIN EXPRESSION VECTOR.

Appendix 2B: Strategy for Generating Point Mutations in the α2 Chimaerin SH2 Domain

The procedure used to prepare site-directed mutants of the α2 chimaerin SH2 domain is

outlined in the flow chart above. Following this method, around 5% of the plasmids obtained were mutated. Analysis of clones by restriction digestion after the second selection digest allowed the rapid identification of clones that might contain the desired mutation. This reduced overall time of the procedure because it was then necessary to sequence a very limited number of clones. As mutations were produced in the full length $\alpha2$ chimaerin sequence it was possible to subclone either the SH2 region (see Construct B, Appendix 2A) or the full length sequence into pGEX-2TK for subsequent use.