UNDERSTANDING THE FUNCTION OF THE PH AND CaLB DOMAINS OF p120GAP THROUGH THE IDENTIFICATION OF INTERACTING PROTEINS

by

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A thesis submitted in the conformity with the requirements for the degree of Master of Science
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ABSTRACT

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The GTPase-activating protein, p120GAP, is a negative regulator of the proto-oncogene product p21ras. GAP contains multiple domains, all of which may be involved in the regulation of this protein and therefore Ras to various incoming signals. Of these domains, very little is known of the function of the pleckstrin homology (PH) and Ca²⁺-dependent phospholipid binding (CaLB) domains, but evidence suggests that they may be multifunctional domains involved in protein-protein or protein-lipid interactions. In hopes to gain a better understanding of the function of these domains in regulating GAP, the two-hybrid system was used to search for proteins that could interact with either of these two domains. This search was performed a total of three times, each time increasing the number of transformants under selection. However, no interacting proteins were found using this system. Other methods are now being employed to determine potential interacting partners of these two domains. These methods as well as some initial results are discussed.
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DEDICATIONS

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Chapter 1

GENERAL INTRODUCTION
INTRODUCTION

Cellular Signalling Through Ras GTPases

Ras proteins are critical regulators of intracellular pathways involved in numerous physiological functions including the proliferation of fibroblasts (Mulcahy et al., 1985), maturation of Xenopus oocytes (Korn et al., 1987), differentiation of neuronal cells (Hagag et al., 1986), and photoreceptor development in Drosophila (Simon et al., 1991) (Fig 1.1). p21GTP is a plasma membrane-associated guanosine triphosphatase (or small G-protein) whose biological activity is determined by the bound nucleotide. The cycling of Ras between an inactive GDP-bound and active GTP-bound conformations is directly regulated by guanine nucleotide exchange factors (GEFs), such as Sos, and GTPase-activating proteins (GAPs) (Trahey et al., 1988; Boguski and McCormick, 1993; Lowy and Willumsen, 1993) (Fig 1.2). It is the GTP-bound form of Ras that is biologically active and transmits signals to downstream effectors, such as Ral1, resulting in various cellular responses to particular extracellular signals (Pai et al., 1990; Satoh et al., 1992; Lowy and Willumsen, 1993). Mutationally activated forms of Ras are frequently associated with cancer, most commonly due to mutations in Ras which reduce its intrinsic GTPase activity and confer resistance to downregulation by GAPs (Bos, 1989; Lowy and Willumsen, 1993).

The first clues to the function of p21GTP acting downstream of tyrosine kinases in a mitogenic signalling pathway were based on the observations that microinjecting antibodies (Y13-259) that neutralize the biological activity of Ras also blocked the mitogenic activities of serum, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) and
blocked transformation by tyrosine kinase oncogenes, including v-src (Mulcahy et al., 1985; Smith et al., 1986). As well, studies using a dominant negative mutant allele of Ras (Asn-17) was found to inhibit ligand induced proliferation and differentiation in various cell types and revealed the requirement of Ras in the activation of the MAP kinases, ERK1 and ERK2, by a variety of extracellular stimuli (Feig and Cooper, 1988; deVries-Smits et al., 1992; Thomas et al., 1992; Wood et al., 1992).

There is growing evidence that Ras regulates several signalling pathways involving protein serine/threonine kinases, the best characterized of which is the Raf/MEK/ERK mitogen-activated protein (MAP) kinase cascade (Marshall, 1995). This MAP kinase pathway is only found in metazoans and is activated through Ras by extracellular mitogenic or differentiating factors. Stimulation of this pathway enhances transcription by promoting the activation of a number of transcription factors and also results in the activation of other enzymes including cytosolic phospholipase A2 (cPLA2). A second MAP kinase cascade, the MEKK/SEK/SAPK/JNK pathway, which involves Ras, has also been described. Unlike the Raf/MEK/ERK cascade, this second pathway is conserved from yeast to vertebrates and is activated in response to stress (Herskowitz, 1995; Marshall, 1995). Thus, the activation of Ras triggers at least two divergent MAP kinase cascades with different substrate and transcriptional specificities in response to various extracellular signals.

The activation of various growth factor receptor and cytoplasmic receptor tyrosine kinases induces plasma membrane-localization of Ras modulators through Src homology 2 (SH2) and Src homology 3 (SH3) domains, which are found in a variety of signalling proteins and recognize specific phosphotyrosine and proline-rich peptides, respectively (McCormick.
Figure 1.1: General Overview of Ras Signalling through Protein Tyrosine Kinases and G Protein-Coupled Receptors
Tyrosine kinases

(+) Exchange

Ras-GDP

Sos

Ras-GTP

GAP

Hydrolysis

Effectors (Raf, MEKK1, PI3-K?, Ral-GDS?, GAP?)

Figure 1.2: Ras GTPase Cycle Regulation and Signalling
1993; Pawson and Schlessinger, 1993; Schlessinger, 1994). Activation of certain heterotrimeric G protein-coupled receptors also leads to Ras activation although the mechanisms involved in this activation are just beginning to be elucidated (Ablas et al., 1993; van Corven et al., 1993; Winitz et al., 1993; Crepso et al., 1994; Koch et al., 1994; Mattingly et al., 1994). Since pleckstrin homology (PH) domains have been implicated in binding βγ subunits of G proteins and are present in many Ras modulators (Haslam et al., 1993; Masacchio et al., 1993; Mayer et al., 1993; Gibson et al., 1994; Touhara et al., 1994), there may be a variety of mechanisms to induce membrane association of these modulators and therefore couple various incoming signals to Ras.

**p120GAP, a Negative Regulator of Ras**

The Ras GTPase-activating protein, p120\(^{GAP}\), is a ubiquitously expressed protein of 120 kDa which stimulates the weak intrinsic GTPase activity of normal, but not oncogenic forms of Ras, returning Ras to its inactive GDP-bound form (Trahey and McCormick, 1987; Gibbs et al., 1988; Trahey et al., 1988; Vogel et al., 1988). The importance of p120\(^{GAP}\) as a negative regulator of Ras in vivo was revealed by the observation that overexpression of full length GAP or the C-terminus encoding its catalytic domain suppressed transformation in NIH 3T3 cells by c-src, v-src and c-ras, but not of oncogenic, activated forms of p21\(^{ca}\) (v-ras) (Zhang et al., 1990; DeClue et al., 1991; Nori et al., 1991). p120\(^{GAP}\) is a multidomain protein consisting of two SH2 domains, one SH3 domain as well as a pleckstrin homology (PH) and a Ca\(^{2+}\)-dependent phospholipid binding (CaLB) domain in addition to its C-terminal
Figure 1.3: Interactions of p120 Ras GAP Domains
catalytic domain (Clark et al., 1991; Koch et al., 1991; Masacchio et al., 1993; Mayer et al., 1993) (Fig 1.3). Proteins are often assembled from different combinations of modular domains, each with a particular function which may contribute to the regulation of the molecule in which they are found. These non-catalytic domains may allow GAP to respond, and therefore regulate Ras, in response to various incoming signals. Thus, identifying its partners is essential in order to gain an understanding of its regulation.

**GAP Complex Formation and Subcellular Localization**

A common feature of GEFs and GAPs is that they are normally cytosolic and therefore must translocate to the plasma membrane in order to access Ras. The regulation of subcellular localization of these modulators may therefore be a general mechanism to regulate Ras. The importance of this regulation is exemplified by the observations in which targeting GAP or its catalytic domain to the plasma membrane is inhibitory to growth (Clark et al., 1993; Huang et al., 1993). Activation of the EGF or PDGF receptor tyrosine kinases, transformation by the v-Src tyrosine kinase as well as ionophore-induced elevation of intracellular Ca^{2+} causes partial localization of \( p_{120^{\text{MAP}}} \) to the particulate fraction, containing cellular membranes (Molloy et al., 1989; Moran et al., 1991; Gawler et al., 1995a). GAP forms a complex with activated EGF and PDGF receptors (Kaplan et al., 1990; Kazlauskas et al., 1990; Soler et al., 1993) through its SH2 domains with the amino-terminal SH2 domain having a greater affinity than the carboxy-terminal SH2 domain for the activated tyrosine autophosphorylated receptors (Anderson et al., 1990; Moran et al., 1990). In addition, GAP is phosphorylated on tyrosine and serine residues in cells stimulated by growth factors or in
cells transformed by a variety of oncogenic tyrosine kinases or activated transforming mutants of c-src (Molloy et al., 1989; Ellis et al., 1990; Kaplan et al., 1990; Bouton et al., 1991; Moran et al., 1991), and forms distinct complexes with two cellular proteins, called p62 and p190 (Ellis et al., 1990; Moran et al., 1991; Molloy et al., 1992).

GAP is primarily a monomer in the cytoplasm of normal, unstimulated cells, however, upon EGF stimulation or cell transformation by v-src, the majority of cytosolic GAP associates with p190, both of which are phosphorylated on tyrosine, but are primarily phosphorylated on serine residues (Ellis et al., 1990; Moran et al., 1991). The assembly of this complex is dependent on phosphorylation (Moran et al., 1991; Bryant et al., 1995). From experiments using p190 expressed in baculovirus and/or GST fusion proteins expressed in bacteria, both SH2 domains of GAP as well as tyrosine phosphorylation of p190 appear to be required for maximal binding (Bryant et al., 1995; Ellis et al., 1995). A minor fraction of GAP becomes associated with the particulate fraction which is more highly tyrosine phosphorylated than cytosolic GAP. A subpopulation of this membrane localized GAP associates with the highly tyrosine phosphorylated protein p62. Unlike the cytosolic GAP-p190 complex, the complex between GAP and p62 is localized in the cytosol as well as at membranes (Moran et al., 1991). Again, using fusion proteins expressed in bacteria, p62 was found to associate with the amino-terminal SH2 domain of GAP (Moran et al., 1990). Furthermore, using a dominant negative mutant of Ras, p21ras (Asn-17), the formation of both of these complexes was found to be independent of GAP binding to Ras·GTP (Pronk et al., 1993). p190 has been found to be a GTPase-activating protein in vitro for the rho/rac family of proteins (Settleman et al., 1992a; Settleman et al., 1992b). Members of the rho subfamily
of small GTPases play a role in controlling the organization of the actin cytoskeleton, where rho is involved in the formation of actin stress fibres and rac stimulates actin polymerization at the plasma membrane to produce membrane ruffles and lamellipodia in response to growth factor stimulation (Ridley et al., 1992; Ridley and Hall, 1992; Hall, 1994). The function of p62 remains unknown.

Tyr-460 of human GAP (corresponding to Tyr-457 of bovine GAP), which lies just C-terminal to its second SH2 domain, is the major tyrosine residue phosphorylated by both the EGF receptor and activated Src kinase (Liu and Pawson, 1991; Park et al., 1992). Inhibition of GAP might be a mechanism to activate Ras, however, no direct link between tyrosine phosphorylation of p120<sup>GAP</sup> and inhibition of its catalytic activity has been demonstrated (Gibbs et al., 1990; Moran et al., 1991). Interestingly, in v-src transformed cells, tyrosine phosphorylation of GAP enhances its association with p62 at membranes compared with the cytoplasm, although the GAP<sup>F145</sup> mutant used in these experiments, which lacks the major tyrosine phosphorylation site, does not completely abolish its association with p62 (Park and Jove, 1993).

Overall, there is evidence that activation of protein tyrosine kinases induces GAP to become phosphorylated and to associate with two phosphoproteins, mediated by its SH2 domains. This association may regulate other cellular events, as in the case of p190 and actin cytoskeleton organization, or may be involved in regulating GAP's localization or association with other proteins. Since p62 and the activated EGF and PDGF receptors bind to the same SH2 domain of GAP, binding of p62 may dissociate GAP from the receptor (Anderson et al., 1990; Moran et al., 1990; Moran et al., 1991). In addition, since the GAP-p190 complex
is cytosolic, formation of this complex may prevent both GAP and p190 access to their membrane-localized substrates (Moran et al., 1991; Bryant et al., 1995).

\( p120^{\text{GAP}} \) may act as a Downstream Effector of p21^{\text{ras}}

The site of p21^{\text{ras}} that interacts with \( p120^{\text{GAP}} \) partly overlaps with the effector region of Ras. Mutations within this region not only prevented \( p120^{\text{GAP}} \) binding but also blocked the biological activity of Ras (Sigal et al., 1986; Willumsen et al., 1986; Adari et al., 1988; Calés et al., 1988). These observations lead to the proposal that in addition to its role as a negative regulator of Ras, GAP or a GAP-associated protein may also generate downstream signals (Adari et al., 1988; Calés et al., 1988). This proposed effector role of GAP is consistent with the observation that oncogenic Ras mutants that are resistant to downregulation by \( p120^{\text{GAP}} \) retain their ability to interact with GAP (Trahey and McCormick, 1987; Vogel et al., 1988). The N-terminal region of GAP has been shown to control gene expression (Medema et al., 1992), control cell morphology (McGlade et al., 1993), inhibit germinal vesicle breakdown (GVBD) in \textit{Xenopus} oocytes (Duchesne et al., 1993), uncouple G_k from atrial muscarinic receptors (m2) thereby inhibiting potassium (K^+ [ACh]) channel opening (Martin et al., 1992), inhibit muscarinic receptor-dependent (m1,m3,m5) transformation of NIH 3T3 cells (Mattingly et al., 1994; Xu et al., 1994) and complements a myristylation-defective, nontransforming mutant of v-src in a cell transformation assay in NIH 3T3 cells (DeClue et al., 1993). Clearly, GAP appears to be a multifunctional protein.

Of particular interest is the uncoupling of G_k from muscarinic receptors (m2) (Martin et al., 1992) and inhibition of GVBD in \textit{Xenopus} oocytes (Duchesne et al., 1993) in which
the N-terminal region of GAP acts downstream and independent of Ras, as would be expected of an effector function. p120\textsuperscript{GAP} was found to inhibit muscarinic K\textsuperscript{[ACh]} channel opening in isolated atrial cell membranes, but only in the presence of p21\textsuperscript{ras}\textsuperscript{GTP}. The effects of Ras and GAP did not appear to interfere with the interaction of G\textsubscript{k} with the K\textsuperscript{+} channel directly, since the inhibition could be overcome by adding G\textsubscript{k}α prebound to GTP\textgamma{}S. Rather, it was concluded that Ras and GAP prevented coupling of G\textsubscript{k} to the muscarinic receptor (m2) which would therefore limit the amount of G\textsubscript{k}·GTP available to open the K\textsuperscript{+} channels. (Yatani et al., 1990). Later, it was found that deletion of the first SH2 domain and the SH3 domain of p120\textsuperscript{GAP} significantly decreased this observed inhibition of K\textsuperscript{+} channel opening and that deletion of the catalytic domain of GAP relieved the requirement for Ras in this process (Martin et al., 1992). This lead to the proposal that the binding of Ras to the catalytic domain of GAP causes a conformational change in GAP exposing its N-terminal domains allowing effector function (Martin et al., 1992).

Microinjection of either a monoclonal antibody directed against the SH3 domain of p120\textsuperscript{GAP} or peptides which encompass the SH3 domain or correspond to amino acids which lie at the surface of the SH3 domain, blocked germinal vesicle breakdown (GVBD) in Xenopus oocytes, induced by insulin or by the oncogenic p21\textsuperscript{ras} (Lys-12) protein, but not of progesterone which involves a Ras independent pathway. This lead to the suggestion that the SH3 domain of p120\textsuperscript{GAP} is essential for Ras-mediated events (Duchesne et al., 1993). Recently, it was found that injection of this monoclonal antibody or these peptides blocked the activation of the maturation-promoting factor (MPF) complex (cyclin B-Cdc2) which is required for GVBD by oncogenic Ras. Furthermore, this observed inhibition was
accomplished by preventing synthesis of the c-mos proto-oncogene product, a 39 kDa serine/threonine protein kinase required for the activation of Cdc2 (Pomerance et al., 1996). A rasGAP-SH3-associated protein has also been identified recently in mammalian cells, called G3BP (Parker et al., 1996). This cytosolic 68kDa protein is homologous with members of the heterogeneous nuclear ribonucleoprotein (hnRNP) superfamily. It was therefore suggested that Ras, through GAP and G3BP, may regulate translational efficiency or stability of mRNAs. Consistent with the model proposed by Martin et al. (1992), coimmunoprecipitation of GAP and G3BP only occurred in proliferating cells or in cells transformed with p21Har(r) (Val-12), but not in cells expressing a dominant negative Ras mutant, p21Har(r)(Asn-17) (Parker et al., 1996). Generally, there is evidence to suggest that p120Har, at least in some systems, acts not only as a modulator of Ras but as a downstream effector as well.

**Pleckstrin Homology (PH) Domains**

As previously mentioned, GAP contains multiple domains which may enable it to interact with other molecules as well as Ras in response to various signals. Of these domains, the least well understood are the PH and CaLB domains, both of which appear to be multifunctional domains capable of interacting with both proteins and lipids. The pleckstrin homology (PH) domain was originally identified as an internal repeat of approximately 100 amino acids at the N and C termini of pleckstrin, which is the major substrate of PKC in platelets (Tyers et al., 1988). Since its discovery, sequence alignment programs have led to the identification of over 80 proteins containing putative PH domains (Haslam et al., 1993;
Figure 1.4: Diagrammatic Representation of the Domain Structures of Selected PH-Domain-Containing Proteins.

PH: Pleckstrin homology; SH2: Src homology 2; SH3: Src homology 3; CaLB: Ca$^{2+}$-dependent phospholipid binding; C2: conserved region 2; PTB: phosphotyrosine binding; DH: Dbl homology; IQ: IQ motif

β-ARK: β-adrenergic receptor kinase; Btk: Bruton's tyrosine kinase; Akt/Rac/PKB: AKT8 retrovirus proto-oncogene/related to A and C kinase/Protein kinase B; IRS-1: Insulin receptor substrate; PLC: Phospholipase C; Ras-GRF: Guanine nucleotide releasing factor for Ras; Sos: Son of Sevenless; GAP: GTPase-activating protein.
Mayer et al., 1993; Gibson et al., 1994; Parker et al., 1994). PH-containing proteins are ubiquitous in eukaryotes ranging from single-cell organisms such as yeast, to invertebrates and vertebrates. The PH domain is found in many signalling and cytoskeletal proteins, many of which are associated with plasma or organellar membranes. Proteins that contain PH domains include regulators of small GTP-binding proteins, ser/thr kinases, cytoskeletal proteins, Tec tyrosine kinase family members, putative adaptor molecules, phospholipase C isoforms (Masacchio et al., 1993; Gibson et al., 1994), and at least one GTPase (Dynamin) (Ferguson et al., 1994) (Fig 1.4). This frequency with which PH domains are found as well as the types of molecules that contain them suggest that PH domains may be important signalling modules analogous to SH2 and SH3 domains.

**PH Domain Structure**

The secondary structure was first predicted by computer analysis (Masacchio et al., 1993) and later confirmed by nuclear magnetic resonance (NMR) and crystallographic structural studies. The 3-dimensional structure of the N-terminal PH domain of pleckstrin (Yoon et al., 1994) and the PH domain of β-spectrin (Macias et al., 1994) were solved by NMR. The crystal structure of the PH domain from human dynamin (Ferguson et al., 1994) (Fig 1.5), PLC-γ1 (Ferguson et al., 1995) and β-spectrin (Hyvönen et al., 1995) have also been determined.

Despite the very weak sequence identity between PH domains, the structural similarity is very high. The PH domain core is composed of 7 β strands folded into 2 antiparallel β sheets which are capped at one corner by a C-terminal α helix. One β sheet is composed of
the first 4 β strands and the second sheet is composed of the last 3 β strands and has been described as an antiparallel β sandwich (Ferguson et al., 1994) (Fig 1.5). Just as with SH2 and SH3 domains (Cohen et al., 1995) the amino- and carboxy-termini of the PH domain are close to each other which has been proposed to enable insertion of these domains into a variety of proteins during evolution without disrupting the overall structure of the proteins. Opposite of the C-terminal α helix are 3 variable loops which are the regions where insertions and deletions occur in the different PH domains, such as with β-spectrin and PLCγ. Between strands 3 and 4 (variable loop 2) there is an insertion of an α helix in β-spectrin and an insertion which contains two SH2 and one SH3 domain in PLCγ.

Each of the above solved PH domains are electrostatically polarized. The more conserved side of the domain, including the C-terminal α helix, is a region of negative potential, while the 3 variable loops form a positively charged surface. Interestingly, an X-linked immunodeficiency (xid) mutation in Bruton's tyrosine kinase (Btk), which leads to the disruption of B cell maturation and immune deficiency, maps on the positively charged surface. This mutation, in which an arginine is substituted with a cysteine in mice, is located at position 28 in the middle of β strand 2 (Rawlings et al., 1993; Thomas et al., 1993). This residue is exposed to the surface and could be involved in a ligand binding site (Gibson et al., 1994; Macias et al., 1994). A tryptophan located in the middle of the C-terminal α helix is the only amino acid that is invariant in all PH domains (Masacchio et al., 1993). The hydrophobic side chains from the α helix project into the well packed hydrophobic core that help maintain the core structure (Gibson et al., 1994).

Despite the absence of significant sequence homology, it has recently been found that
Figure 1.5: The Three-Dimensional Structure of the PH Domain from Human Dynamin

Coordinates were obtained from the publicly accessible Brookhaven Protein Data Bank and the figure was constructed using Ras Mac Molecular Graphics, Roger Sayle, Oct. 1994, version 2.5.
the phosphotyrosine binding (PTB) domain of the adaptor proteins Shc and IRS-1 adopt the same fold as PH domains (Zhou et al., 1995; Eck et al., 1996). However, there is no conserved tryptophan in these domains and they do not display the same polarization observed with PH domains. In contrast to SH2 domains, PTB domains recognize residues amino-terminal to the phosphotyrosine in the context of an "NPXpY" motif. The C-terminal α helix, β strand 5 and the 3₁₀ turn connecting β strands 4 and 5 of the IRS-1 PTB domain are all involved in recognizing the insulin receptor phosphopeptide. The amino-terminal residues of the phosphopeptide (pY-3 to pY-8) lie in a groove parallel to the C-terminal α helix and β strand 5 and form hydrogen bonds with β strand 5, basically forming another strand to the domain, and this is further stabilized by contacts with the C-terminal α helix.

The phosphotyrosine itself interacts with arginines that extend from β strand 5 and the loop between β strands 6 and 7 (variable loop 3) which donate hydrogen bonds to the phosphate oxygens (Eck et al., 1996).

The general orientation of the particular phosphopeptide, derived from TrkA or the insulin receptor, is similar when complexed with the PTB domains of Shc or IRS-1, respectively. However, residues involved in contacting the phosphotyrosine are not conserved. For example, Shc has a large insertion between β strands 1 and 2 (variable loop 1), that is not present in IRS-1, which contains an arginine that interacts with the phosphotyrosine (Zhou et al., 1995). Additionally, the Shc PTB domain prefers a bulky hydrophobic residue at position pY-5 (Trüb et al., 1995) which cannot be accommodated by the PTB domain of IRS-1 (Eck et al., 1996). In any event, due to the structural similarity, PTB domains may therefore represent a second class of PH domains.
which possess their own specific ligand.

**Function of PH Domains**

**Binding of PH Domains to Proteins**

Many groups have tried to determine whether PH domains bind a specific class of ligand. The C-terminal region of the β-adrenergic receptor kinase (βARK), which includes a PH domain, was found to bind to βγ subunits of heterotrimeric G proteins (Pitcher et al., 1992; Koch et al., 1993). By using GST fusion proteins and a direct binding assay or by measuring the ability to block Gβγ-mediated membrane translocation of βARK, PH domains from 9 proteins have been shown to associate with Gβγ, to varying extents.

By using a series of truncated PH domains derived from Ras-GRF, the Gβγ binding domain was crudely defined to β strand 4 to the α helix and sequences just distal to this (Touhara et al., 1994). This suggests that such interactions could localize effector molecules in Gβγ-mediated signalling. However, these interactions were produced *in vitro* using high concentrations of purified components and the affinity of the PH domains for Gβγ is very low compared to that of intact βARK. Furthermore, in competition experiments, a 28-residue peptide that contains only the last 9 residues of the βARK PH domain and residues C-terminal to it, inhibited association of βARK with Gβγ (Koch et al., 1993), indicating that most of the PH domain appears dispensable for this interaction.

Others have found that certain PH domains bind PKC (Konishi et al., 1994; Yao et al., 1994). The PH domain of Btk, a member of the Tec tyrosine kinase family, when fused to GST bound both Ca²⁺-dependent (α, βI, βII) and Ca²⁺-independent (ε, ζ) PKC isoforms
in mast cell lysates (Yao et al., 1994). Btk was an in vitro substrate for PKC. serine phosphorylation of Btk by PKC downregulated Btk tyrosine kinase activity. As well, GST PH fusion proteins containing the xid mutation (mentioned above), displayed a lower affinity for PKC (Yao et al., 1994). Similarly, by using GST fusion proteins, PKCζ bound to the PH domain of the ser/thr kinase Racβ/Akt in vitro. By transiently expressing full length PKCζ and mutant PKCζ, that lacks the C-terminal catalytic domain in COS-7 cells, the regulatory domain in the N-terminus of PKCζ was sufficient for binding to the PH domain of Racβ (Konishi et al., 1994).

**Binding of PH Domains to Membrane Lipids**

By centrifugation studies, Fesik and co-workers reported binding of several PH domains, including pleckstrin, to lipid vesicles containing negatively charged phospholipids such as phosphatidylinositol 4.5- bisphosphate (PtdIns(4,5)P₂) and suggested that this binding serves to anchor PH domain-containing proteins to the plasma membrane (Harlan et al., 1994). Analysis of NMR chemical shift differences upon binding to PtdIns(4,5)P₂ in detergent micelles showed that the N-terminal half involving the positively charged surface of the PH domain was involved in the interaction. The PH domains of spectrin and pleckstrin have also been found to bind to PtdIns(4,5)P₂ and to Ins(1,4,5)P₃ (Hyvonen et al., 1995; Lemmon et al., 1995). However, these interactions are quite weak (Kₐ > 30μM) and others have not been able to detect binding of the PH domain of dynamin to phospholipid vesicles by using gel filtration studies (Ferguson et al., 1994; Lemmon et al., 1995). Others have also argued against this hypothesis due to the low conservation of surface residues and the lack of a
sufficient hydrophobic pocket revealed by solved structures (Gibson et al., 1994) suggesting that these interactions may not be physiologically relevant for all PH domains.

The exception to this appears to be the PH domain of PLC-γ1. The amino-terminal region of PLC-γ1, which contains a PH domain (Parker et al., 1994), is required for its relatively high affinity (Kd=1 μM) binding to vesicles that contain PtdIns(4,5)P₂. Removal of the amino-terminal 60 amino acids from PLC-γ1 abolishes this binding (Cifuentes et al., 1993). Ins(1,4,5)P₃, the product of PtdIns(4,5)P₂ hydrolysis by PLC-γ1, also binds PLC-γ1 and was found to inhibit this binding of PLC-γ1 to vesicles that contain PtdIns(4,5)P₂ as well as inhibit its activity in vitro (Cifuentes et al., 1994; Yagisawa et al., 1994). Again, this interaction with Ins(1,4,5)P₃ involves the amino-terminal region of PLC-γ1 (Cifuentes et al., 1994; Yagisawa et al., 1994). Furthermore, the isolated PH domain of PLC-γ1 has been found to bind with relatively high affinity to both PtdIns(4,5)P₂ (Kd=1.7 μM) and Ins(1,4,5)P₃ (Kd=0.21 μM) in a stereo-specific manner, where Ins(1,4,5)P₃ competes directly with PtdIns(4,5)P₂ binding (Lemmon et al., 1995). This product inhibition is similar to that observed with the SH2 domain of PI 3-kinase where its product, PtdIns(3,4,5)P₃, competes with tyrosine-phosphorylated proteins for binding to the SH2 domain of its p85 subunit (Rameh et al., 1995).

The structure of the PH domain of PLC-γ1 complexed with Ins(1,4,5)P₃ has been solved (Ferguson et al., 1995). Ins(1,4,5)P₃ binds in the centre of the positively charged region of the PH domain, opposite from the C-terminal α helix, where all three phosphates, in particular the 4' and 5' phosphates, interact with residues found primarily between β strands 1 and 2 (variable loop 1) and β strands 3 and 4 (variable loop 2). Thus, Ins(1,4,5)P₃ and
PtdIns(4,5)P₂ are specific ligands for PH domains, at least for the PH domain of PLCᵦ₁.

**Other Proposed Ligands of PH Domains**

Finally, it has also been postulated that the role of PH domains may be to recognize short peptide sequences containing phosphorylated ser/thr residues analogous to SH2 domains (Gibson et al., 1994). Solved structures reveal putative ligand binding clefts (Macias et al., 1994) surrounded by many positively charged residues, suggesting binding to a negatively charged ligand. Furthermore, the weak surface conservation in the putative ligand-binding site is consistent with recognizing small ser/thr residues in the context of short variable peptide sequences (Gibson et al., 1994). Thus the evidence, including those revealed by solved structures, suggests a role for PH domains in signal transduction involving protein-protein and protein-lipid interactions comparable to SH2 and SH3 domains and the low sequence homology observed between them may dictate a specificity for a particular ligand.

**Ca²⁺-Dependent Phospholipid Binding (CaLB) Domains**

The other ill-defined domain of p₁20⁹⁰⁶₉, the CaLB domain, is yet another motif common to a variety of signalling proteins. The Ca²⁺-dependent phospholipid-binding (CaLB) domain was originally observed as a motif in various Ca²⁺-responsive signalling proteins including cPLA₂, activation of which results in the production of arachidonic acid and lysophosphatidylcholine (Clark et al., 1991). This 43 amino acid motif in GAP corresponds to roughly the central third of the conserved region 2 (C2) motifs of Ca²⁺-dependent PKC isoforms and the synaptic vesicle membrane protein, synaptotagmin.
Figure 1.6: Diagrammatic Representation of the Domain Structures of Selected CaLB/C2-Domain-Containing Proteins.

CaLB: Ca$^{2+}$-dependent phospholipid binding; C2: conserved region 2; PH: Pleckstrin homology; SH2: Src homology 2; SH3: Src homology 3

GAP: GTPase-activating protein; cPLA$_2$: Cytosolic phospholipase A$_2$; PLC: Phospholipase C; PKC: Protein kinase C; p170: a novel phosphatidylinositol 3-kinase
(Perin et al., 1990; Nishizuka, 1992; Gawler et al., 1995a). This motif is approximately 25% identical to sequences found in cytosolic phospholipase A2 (cPLA2), phospholipase C-γ1 (PLC-γ1), protein kinase C-γ (PKC-γ), and synaptotagmin/p65, all of which display Ca2+-dependent activity or membrane localization (Clark et al., 1991). Other C2/CaLB containing proteins include rabphilin-3A (Shirataki et al., 1993), the recently cloned mammalian Ras GTPase-activating proteins Gap1m (Maekawa et al., 1994) and GAP1filp (Cullen et al., 1995) as well as a novel class of phosphatidylinositol 3-kinases (MacDougall et al., 1995; Moltz et al., 1996; Virbasius et al., 1996) (Fig 1.6).

Function of C2 CaLB Domains

C2 CaLB Domain Binding to Membrane Lipids

C2/CaLB domains were initially described as Ca2+-dependent phospholipid binding motifs that can mediate the translocation of proteins to the membrane (Nishizuka, 1992). Indeed, a fragment containing the CaLB motif of cPLA2 showed Ca2+-dependent association with membrane vesicles in vitro (Clark et al., 1991). The C2A domain of synaptotagmin bound negatively charged phospholipids (phosphatidylinositol (PI) and phosphatidylserine (PS)), in a Ca2+-dependent manner in vitro (Davletov and Südhof, 1993; Chapman and Jahn, 1994; Fukuda et al., 1996), as does a carboxy-terminal fragment of rabphilin-3A containing both its C2 domains (Yamaguchi et al., 1993). The importance of C2 domains in vivo was revealed by experiments in which injection of cells with antibodies that recognize or peptides that correspond to the C2 domain of synaptotagmin blocked Ca2+-induced exocytosis (Bommert et al., 1993; Elferink et al., 1993). As well, the C2B domain of
synaptotagmin II mediates Ca$$^{2+}$$-independent binding to inositol-1,3,4,5-tetrakiphosphate (IP$_1$) (Fukuda et al., 1994), as does GAP$^{IP}$ which also contains two of these C2/CaLB domains (Cullen et al., 1995). However, this association appears to be a specific property of these proteins since the C2 domains of PKC$\alpha$ and rabphilin-3A do not exhibit this association with IP$_1$ (Fukuda et al., 1994).

Recently, our lab has found that the CaLB motif of p120$^{130K}$ is a functional domain which confers Ca$$^{2+}$$-dependent membrane localization both \textit{in vitro} and \textit{in vivo}, and that GAP, but not a mutant lacking the CaLB domain, translocates to the particulate fraction in response to ionophore-induced elevation of intracellular Ca$$^{2+}$$ (Gawler et al., 1995a). Deletion mutation analysis defined the minimal boundaries for \textit{in vitro} phospholipid-binding activity to amino acids 612-643 of p120$^{130K}$ (Gawler et al., 1995b).

\textit{C2 CaLB Domain Binding to Proteins}

In addition to binding phospholipids, C2/CaLB domains can also mediate interactions between proteins. A candidate membrane associated C2/CaLB receptor protein was first described by Mochly-Rosen \textit{et al.} (1991). These receptors for activated C-kinase (RACKs) were first identified in the Triton-insoluble material of the particulate fraction of neonatal rat hearts (Mochly-Rosen \textit{et al.}, 1991). This Triton-insoluble fraction is also where cytoskeletal elements are found. Binding of PKC to the Triton-insoluble fraction occurred in the presence of the PKC activators, Ca$$^{2+}$$ (1mM) and PS (20$\mu$g/ml) and association was increased by addition of PMA/DAG (0.1$\mu$M) \textit{in vitro} (Mochly-Rosen \textit{et al.}, 1991). This binding was abolished when the particulate fraction was treated with trypsin prior to the Triton extraction.
suggesting that PKC bound to this fraction through proteins. This binding was also found to be concentration-dependent, specific, and saturable. Furthermore, PKC did not bind through its substrate binding site (Mochly-Rosen et al., 1991). Other experiments revealed that the C2 domains of synaptotagmin (p65) bound these particulate proteins in vitro (Mochly-Rosen et al., 1992) as well as another C2 containing protein, PLC-γ1 (Disatnik et al., 1994) but, unlike PKC, these proteins bound in a Ca²⁺- and phospholipid-independent manner. From these results, it was suggested that the C2 region contains at least part of the RACK binding site (Mochly-Rosen et al., 1992). Recently, a cDNA encoding a 36 kDa protein (RACK1) was cloned from a rat brain expression library by screening for proteins that bound to PKC in the presence of PS, DAG, and Ca²⁺ in an overlay assay (Ron et al., 1994). RACK1 contains seven WD40 motifs and therefore belongs to a superfamily of proteins, including the β subunit of G proteins, which have 5 to 8 repeats of these WD40 motifs which are thought to be involved in protein-protein interactions (Neer et al., 1994). There is additional evidence for protein-protein interactions by C2/CaLB domains. The C2B domain mediates Ca²⁺-dependent dimerization of synaptotagmin (Chapman et al., 1996), and Ca²⁺-independent binding to AP-2, adapter protein 2, which is believed to be necessary for the assembly of clathrin-coated pits (Zhang et al., 1994). Other observations made in our lab lead to the conclusion that the GAP CaLB domain has two membrane binding sites: a low affinity, nonsaturable site which corresponds to the phospholipid interaction, and a higher affinity, saturable site which likely corresponds to a membrane-associated protein (Gawler et al., 1995a). Therefore, there is evidence for Ca²⁺-dependent interactions with both phospholipids and proteins by the CaLB domain of p120GAP. The CaLB domain may play a role in
membrane localization of p120GAP, and thereby facilitate Ras regulation in response to elevated intracellular Ca\(^{2+}\).

\textit{C2 Domain Structure}

The three dimensional structure of the first C2 domain (C2A) of synaptotagmin I was recently solved (Sutton \textit{et al.}, 1995). The C2A domain is composed of 8 \(\beta\)-strands folded into 2 four-stranded antiparallel \(\beta\) sheets forming a compact \(\beta\) sandwich. At the core of the crystal structure conserved residues corresponding to the CaLB domain were localized and described as forming a "C2 key" into which calcium bound. Calcium was found to bind between two polypeptide loops located within this "C2 key" motif coordinated mainly by aspartate residues which are clustered within this region. It remains to be determined whether the flanking sequences which are not generally conserved among CaLB-containing proteins are required to support the C2 structure of the synaptotagmin C2 domain.

\textit{Conclusion}

The well established functions of SH2 and SH3 domains has demonstrated the importance of protein-protein interactions in mediating signal transduction. In spite of the low sequence similarity within the PH and CaLB domain families, these domains may each have their own specific ligand(s) that dictates their specific function. Since there is evidence implicating both the PH and CaLB domains of GAP in protein-protein interactions, I have focused my research on identifying proteins that interact with these domains in order to determine their function in Ras signalling.
Chapter 2

UTILIZING THE TWO-HYBRID SYSTEM TO SEARCH FOR INTERACTING PROTEINS WITH THE PH OR CaLB DOMAINS OF \( p120^{\text{GAP}} \)
2.1 Introduction

In order to establish an understanding of the function of the PH and CaLB domains of p120^GAP, I searched for proteins that interact with these domains using the yeast two-hybrid system (Durfee et al., 1993). Such interacting proteins could regulate p120^GAP either indirectly by regulating subcellular localization or directly by affecting its activity. Since GAP may function as a downstream effector in Ras signalling (Martin et al., 1992), proteins that interact with CaLB and PH domains may help effect downstream signalling events. The two-hybrid system is a highly sensitive method that can be used to identify proteins that interact with a protein of interest or to delineate residues that are critical for an interaction (Fields and Sterngland, 1994). An attractive feature of this system is that it results in the immediate isolation of the cDNA encoding the interacting protein. This system has been very successful in the detection of many protein-protein interactions. Some of these include the identification of an interaction between c-Raf-1 and either Ras or MEK-1 (Van Aelst et al., 1993; Vojtek et al., 1993), or members of the 14-3-3 family of proteins (Fantl et al., 1994; Freed et al., 1994); PKCα and a novel substrate called PICK1 (Staudinger et al., 1995); and Ras with a guanine nucleotide dissociation stimulator for Ral (ralGDS), which is another member of the Ras protein family (Hofer et al., 1994; Kikuchi et al., 1994). As well, this system has also been used to define the residues required for the interaction between the p85 and p110 subunits of PI 3-kinase (Holt et al., 1994). This success prompted me to use yeast two-hybrid analysis to search for proteins that could interact with the PH and CaLB domains of p120^GAP.
2.2 General Strategy: Two-Hybrid System

This genetic approach to identify and study protein-protein interactions was first described by Fields and Song (1989) and later revised by Durfee et al. (1993). The first step requires constructing plasmids that encode two sets of fusion (hybrid) proteins. One hybrid consists of the protein (or a portion of the protein) of interest fused to the DNA-binding domain of the yeast transcriptional activator Gal4. The other hybrid consists of the Gal4 activation domain fused to protein sequences encoded by a cDNA library. If the two proteins expressed in yeast interact, the resulting complex is able to activate transcription of reporter genes which contain binding sites for Gal4, the upstream activating sequence from GAL1 (UAS1), in their promoters (Fig 2.1).

The reporter genes most commonly used are the E. coli lacZ gene encoding β-galactosidase, and a yeast gene involved in amino acid biosynthesis, such as HIS3. To screen for interacting proteins, a yeast reporter strain (Y153) expressing the Gal4 DNA-binding domain hybrid is transformed by the Gal4 activation domain library. Cells are then selected for His’ prototrophy, by plating on media lacking histidine, and later screened for β-galactosidase activity, which produces blue colonies on filters containing X-gal. Library-derived plasmids that pass the initial selection and screening process (ie. His’ and blue colonies) are considered potential positives. These plasmids are then isolated and retested to eliminate any false positives, that is, to see if this effect is reproducible and dependent only on the protein of interest. Those that pass this second screen are only then considered true positives.
Figure 2.1: The Two-Hybrid System:
A) Neither hybrid protein can activate transcription of reporter genes on their own.
B) If the two hybrid proteins interact, Gal4 regains the ability to activate transcription of reporter genes.
2.3 Methods and Materials

Bacterial and Yeast Strains

The *Saccharomyces cerevisiae* strain, Y153 (MATα, *leu2-3,112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δ, gal80Δ, *URA3::GAL-lacZ, LYS2::GAL-HIS3*), carries two chromosomally located reporter genes, the *E. coli lacZ* gene and the yeast *HIS3* gene, whose expression is regulated by Gal4. The *lacZ* gene is under the control of the *GAL1* promoter and the regulatory sequences of the *HIS3* gene have been replaced by the *GAL1 UAS*1. It also carries mutations in genes involved in amino acid biosynthesis (*trp1-901, leu2-3,112*) allowing selection of cells carrying the DNA-binding (pAS1) and activation (pSE1107) domain plasmids. The strain is also deleted for *GAL4* to ensure that endogenous Gal4 is absent, and deleted for *GAL80*, whose product normally inhibits Gal4 function, to avoid a requirement for galactose in the medium which would normally dissociate Gal80 from Gal4 inducing transcription from the *GAL1* promoter (Schneider and Guarente, 1991). *E. coli JA226 (hsdR, hsdM, leuB6, lop11, thi, recBC*, *strR*) was used to recover library-derived plasmids from yeast. The strain carries a *leuB6* mutation which can be complemented by the yeast *LEU2* gene carried on the library-derived plasmid (pSE1107).

Plasmids

For details of the construction of expression plasmids see Durfee *et al.* (1993). The cDNA fragment of interest is subcloned into the plasmid pAS1. In this plasmid, the *ADH1* (alcohol dehydrogenase 1) promoter drives expression of a fusion transcript encoding the
nuclear localization sequence from SV40 large T antigen, the Gal4 DNA-binding domain (a.a. 1-147; Keegan et al. 1986), and the protein of interest. The plasmid also encodes an in frame hemagglutinin (HA) epitope so that the fusion protein is tagged between the Gal4 DNA-binding domain and the protein of interest. It also contains the ColE1 origin and the 2μ origin for replication in E. coli and for high copy autonomous replication in yeast, respectively, and the TRP1 gene for selection in yeast. The other plasmid, pSE1107 carries a cDNA library expressed from the ADH1 promoter, which drives the expression of a fusion transcript encoding the SV40 large T antigen nuclear localization signal, the Gal4 activation domain II (a.a. 768-881; Ma and Ptashne 1987), and protein sequences encoded by a B cell cDNA library. The plasmid also contains the ColE1 origin and the 2μ origin for replication in E. coli and yeast, respectively, as well as the LEU2 gene for selection in yeast and E. coli. Both plasmids also contain the gene encoding β-lactamase conferring ampicillin resistance.

**Media and Materials**

Yeast drop out media and XY medium were prepared as described (Rose et al. 1990). Bacterial M9 + ampicillin medium was prepared as described (Sambrook et al. 1989) with the addition of 0.001% vitamin B1 (thiamine). Dextrose and ammonium sulfate (ultrapure) were purchased from Baker. Bacto-agar, yeast nitrogen base without amino acids or ammonium sulfate (YNB-AA/AS), Bacto-peptone, and Bacto-yeast extract were purchased from Difco. Amino acids and 3-aminotriazole (3-AT) were purchased from Sigma. Calf-intestinal alkaline phosphatase (CIP) and 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were from Boehringer Mannheim. Restriction endonucleases, E. coli DNA polymerase I large fragment
(Klenow). T4 polynucleotide kinase, Vent DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. 12CA5 ascites fluid was kindly provided by Mike Tyers. 3-AT was prepared as a 1M stock in sterile water, filter sterilized and stored at 4°C for up to two weeks. X-gal was prepared as a 2% (20mg/ml) stock by dissolving in sterile dimethylformamide and stored in the dark at -20°C. Glass beads (0.45-0.55mm; from Sigma) were washed by soaking for 1 hour in concentrated nitric acid, rinsed thoroughly with water and dried. Herring carrier DNA was prepared as described by Schiestl and Gietz (1989).

Construction of Gal4 DNA-binding domain plasmids

pAS1 was digested with BamH1, treated with DNA polymerase I large (Klenow) fragment to create blunt ends and with calf-intestinal alkaline phosphatase (CIP) to remove 5' phosphate groups, and purified using the Geneclean kit (BIO 101). Polymerase chain reaction (PCR) was used to amplify domain-encoding regions (PHCaLB, PH, and CALB) using 5'- and 3'-oligonucleotides as primers and Vent DNA polymerase. These blunt PCR products were 5' phosphorylated using T4 polynucleotide kinase, purified from agarose gels using Qiaex and subsequently ligated into pAS1. The nucleotide sequence of the pAS1-PHCaLB plasmid was verified by DNA sequencing and used in the two-hybrid screen.

Western Blot Analysis

Two 10ml cultures of each sample were made from a saturated (2 × 10^8 cells/ml) yeast culture and grown to mid-log phase (1 × 10^7 cells/ml; OD_600=0.3-0.5). Cells were pelleted by centrifugation at 2500 rpm for 5 min at 4°C, washed in 5ml SQ water, and centrifuged again.
Pellets were resuspended and pooled, and transferred to a fresh microfuge tube. Pelleted cells were either stored overnight at -70°C or lysed the same day. Cells were lysed in 100μl buffer 3 + protease inhibitors (0.1% NP40, 250mM NaCl, 50mM NaF, 5mM EDTA, 50mM Tris-Cl 7.5; 10μg/ml aprotinin, 10μg/ml leupeptin, 5μg/ml pepstatin A, 500μM PMSF, 100μg/ml TPCK), 100μl acid-washed glass beads were added and vortexed 8·30 sec at 4°C. Cells were checked periodically for lysis by phase contrast microscopy. At >75% lysis, the mixture was centrifuged at 14000 rpm for 15 min at 4°C and the supernatant was transferred to a new tube. The protein concentration was determined by Bradford protein assay and ~40μg total protein was boiled in SDS sample buffer (62.5 mM Tris-Cl (pH 6.8), 10% (w/v) glycerol, 2% (w/v) SDS, 0.1% (v/v) Bromphenol Blue, 5% (v/v) 2-ME) and resolved by SDS-polyacrylamide electrophoresis (SDS-PAGE) on a 10% gel. The proteins were then transferred onto nitrocellulose and incubated with blocking buffer (20mM Tris-Cl 7.4, 150mM NaCl, 0.05% Tween-20, 5% milk powder) for 1 h. The nitrocellulose filter was then washed 3·5 min in TBST (20mM Tris-Cl 7.4, 150mM NaCl, 0.05% Tween-20) and incubated with a HA-specific monoclonal antibody (12CA5) used at a 1:10,000 dilution for 1 h. The filters were washed again and incubated with horseradish peroxidase-conjugated secondary antibodies used at a 1:10,000 dilution for 1 h followed by washing and chemiluminescence detection (Amersham) on Kodak RP film.

**Lithium Acetate (LiAC) Transformation of Yeast**

10ml of cells were prepared for each transformation (Schiestl and Geitz, 1989). The appropriate saturated yeast culture (2·10⁸ cells/ml) was diluted 1/100 in the appropriate
media and grown at 30°C with good aeration until the cells reached a concentration of \( \sim 1 \cdot 10^7 \) (mid-log phase; \( \text{OD}_{600}=0.3-0.5 \)). To achieve a 2- to 3-fold higher transformation efficiency, the cells were diluted at this stage to \( 2 \cdot 10^6 \) cells/ml and grown until the cells again reached mid-log phase. The cells were then centrifuged at 2500 rpm for 5 min at 4°C. The cells were washed in 5 ml sterile SQ water and centrifuged again. The cells were resuspended in 100μl consisting of 50μg carrier DNA, 1·LiAC/TE (0.1M LiAC, 10mM Tris-Cl 7.5, 1mM EDTA), and 1μg transforming DNA and transferred to a 1.5 ml eppendorf tube. Six hundred microlitres of 40% PEG/LiAC/TE (40% polyethylene-glycol (PEG 4000 or 3350), 1·LiAC/TE) was added to the solution and mixed by pipetting. The mixture was incubated at 30°C for 1 h and at 42°C for exactly 15 min. The cells were then washed with 1 ml TE, resuspended in 200–400μl TE and 100–200μl was plated onto an appropriate drop-out plate. This method usually provides a transformation efficiency of \( 1 \cdot 10^4 \) transformants/μg DNA.

**Selective Recovery of Plasmids from Yeast**

**A) Yeast miniprep**

Cells from 1.5 ml of a saturated (\( 2 \cdot 10^8 \) cells/ml) culture, grown in medium lacking leucine (-Leu), were pelleted by centrifugation and resuspended in 200μl lysis buffer (10mM Tris-Cl 7.5, 2mM EDTA, 0.1M NaCl, 2% Triton X-100, 1% SDS). Four hundred microlitres of acid-washed glass beads and 200μl phenol/chloroform were added and the mixture was vortexed for 2 min and centrifuged at 14000 rpm for 7 min. The supernatant was then removed, 200μl phenol/chloroform added, vortexed for 10 sec and centrifuged for 10 min. The upper layer was removed and transferred to a new tube. Four hundred microlitres 100%
ethanol was added, left to stand for 5 min at room temperature and centrifuged at 14000 rpm for 10 min. The pellet was washed with 70% ethanol, dried and resuspended in 100\(\mu\)l TE - RNase. Total yeast DNA was then treated with glassmilk (Geneclean) and eluted twice with TE for a total volume of 50\(\mu\)l.

B) Preparation of competent E. coli cells (JA226)

A fresh overnight culture of E. coli JA226 cells, grown in Luria broth (LB), was diluted 40-fold into 1L of fresh media and incubated at 37°C with good aeration until cells reached an absorbance of 0.4-0.5 at 550nm. The cells were chilled on ice for 5-10 min and centrifuged at 5000 rpm for 10 min at 4°C. The cell pellets were placed on ice, resuspended in 500ml ice-cold 100mM CaCl\(_2\), incubated on ice for 30 min with occasional swirling, and centrifuged at 5000 rpm for 10 min at 4°C. The cells were resuspended in 40 ml ice-cold 100mM CaCl\(_2\), 15% glycerol, aliquoted in 200\(\mu\)l volumes, left on ice at 0-4°C for 12-24 h, froze in ethanol-dry ice and stored at -70°C.

C) Transformation into JA226

Ten microlitres of total yeast DNA was used for transformation with 200\(\mu\)l quickly thawed competent cells. The mixture was incubated on ice for 30 min and heat shocked for 2-5 min at 42°C. Each tube was incubated for 1 h at 37°C in 400\(\mu\)l LB media, aliquots were plated on LB + amp plates, incubated overnight at 37°C and subsequently patched onto M9 + amp plates.
The lift assay was performed essentially as described by Breeden and Nasmyth (1985). Colonies from selection plates lacking tryptophan, leucine, and histidine + 25mM 3-AT (-Trp -Leu -His + 3-AT) were picked and simultaneously patched onto two plates lacking tryptophan and leucine (-Trp -Leu) (one master plate and one for the β-gal lift assay) and grown at 30°C for 2 days. Patches were transferred onto Whatman 3MM paper by replica plating and grown overnight at 30°C overlaid on a -Trp -Leu plate. Patches of cells were permeabilized by freezing in liquid nitrogen for 15 sec and thawed at room temperature. Filters were then overlaid on Whatman 3MM paper saturated with X-gal/Z buffer (60mM Na₂HPO₄, 40mM NaH₂PO₄, 10mM KCl, 1mM MgSO₄, 38mM β-mercaptoethanol, pH 7.0; 2ml + 0.2 mg/ml X-gal per 10cm plate) and incubated at 30°C for 30 min to overnight for colour development.

Library Screening

The library search was performed as described by Durfee et al. (1993). Y153 was transformed with pAS1-PHCaLB using LiAc method, and grown on plates lacking tryptophan (-Trp) at 30°C for 48 h. A single colony was grown in -Trp medium at 30°C and transformed with library DNA. Aliquots were taken from the transformation mix to determine the transformation efficiency and grown on -Trp -Leu plates. The remainder of the transformation mix was then grown on -Trp -Leu -His + 25mM 3-AT at 30°C for 6 days. 3-aminotriazole (3-AT), a chemical inhibitor of imidazole glycerol phosphate dehydrogenase encoded by the HIS3 gene, was added to prevent histidine
prototrophy due to residual HIS3 expression by the GAL1-HIS3 fusion. His colonies were then screened for β-galactosidase activity using the filter lift assay. Library-derived plasmids (pSE1107) that were considered potential positives (i.e., His and blue colonies) were selectively recovered in *E. coli* (JA226) and transformed back into yeast containing pAS1 alone, pAS1-PHCaLB (the protein of interest), or pAS1-SNF1 (a yeast serine/threonine kinase required for the regulatory response to glucose starvation) or pAS1-lamin (an intermediate filament protein associated with the nuclear envelope) and grown on -Trp -Leu plates. Those transformants expressing β-gal activity only in the presence of pAS1-PHCaLB but not in the presence of pAS1-SNF1 or pAS1-lamin were considered true positives.

### 2.4 Results

Before searching the library for interacting proteins, I tested whether the Gal4 DNA-binding domain fusion proteins were expressed in yeast by western blotting. As shown in Figure 2.2, the PHCaLB and PH fusion proteins were expressed and migrated at their predicted molecular weights of ~44 kDa and ~34 kDa, respectively. However, the CaLB fusion protein was not expressed. Since I wished to search simultaneously for proteins that could bind to both of these domains, I decided to use the pAS1-PHCaLB plasmid, which encoded both the PH and CaLB domains of p120^SHAP^, as bait for my two-hybrid screen. Additionally, the region between the PH and CaLB domains may be important in stabilizing either one or both domains thereby improving the likelihood of proper folding. In control experiments, pAS1 exhibited residual β-galactosidase activity which was detected after
Figure 2.2: Western Blot Analysis of the HA Epitope-Tagged Gal4 DNA-Binding Domain Fusion Proteins.

Both the PHCaLB and PH fusion proteins expressed and migrated at their predicted molecular weights. A non-specific 12CA5 cross reactive species is found in all lanes at ~ 50 kDa.
incubating the cells ~8 h to overnight at 30°C with X-gal/Z buffer solution. This activity was eliminated once the PHCaLB sequences were subcloned into this plasmid. Neither pAS1 nor pAS1-PHCaLB conferred growth on selective plates (-Trp -Leu -His + 25mM 3-AT). pAS1-Cln3 and pAS1-SNF1 (or lamin) were kindly provided by Mike Tyers and were used as positive and negative controls, respectively in the β-gal assay. pAS1-SNF1 (or lamin) did not exhibit any β-gal activity. By contrast, pAS1-Cln3 exhibited strong β-gal activity after ~1 h. Since the hybrid protein containing the DNA-binding domain of Gal4 and PHCaLB sequences (pAS1-PHCaLB) was unable to activate transcription by itself, this fusion protein was suitable for use as bait in a two-hybrid search.

I performed this two-hybrid search a total of three times, each time increasing the number of transformants under selection in order to maximize representation of the cDNA library expressing potential interacting proteins. In the first search, a total of 3.4×10⁴ transformants were placed under selection. Of these, only two clones exhibited β-gal activity. The cDNAs from these positive clones were recovered in *E. coli* and retransformed back into yeast expressing the Gal4 DNA-binding domain alone (pAS1 plasmid), or containing two different Gal4 DNA-binding domain hybrids (pAS1-PHCaLB or pAS1-SNF1 plasmids) and then tested for β-gal activity. Clones were considered false positives if the interaction with pAS1-PHCaLB was not reproduced, or if they displayed β-gal activity in the presence of an unrelated fusion protein (SNF1). Both of the initial two clones were eliminated as false positives (data not shown).

In a second screen, 1 million transformants were placed under selection. Of these transformants, ~3000 colonies were screened for β-gal activity. Of these, 17 potential
positives were found. These potential positives all grew on selective plates and expressed β-
gal activity to varying degrees (as determined by the extent of blue colour and the time at
which colour was detected). The cDNA containing plasmids were recovered and transformed
into the Y153 strain either alone, or with pAS1 alone, pAS1-PHCaLB, or pAS1-lamin.
Those transformants showing β-gal activity only in the presence of pAS1-PHCaLB were
considered true positives. As shown in Figure 2.3, all of these potential positives were
eliminated as false, except for 8 and 12 which I could not confirm due to an inability to
recover the plasmids containing these cDNAs (*). Three of these clones (9,11,13) were able
to activate transcription of the reporter gene on their own. This is most likely due to the
cDNAs encoding proteins involved in transcription (Bartel et al., 1993; Fields and
Sternglanz,1994). These clones might represent the same protein since all three had the same
sized insert.

I performed a final screen in which 5 million transformants were placed under
selection. Of the ~4500 clones which were obtained through selection of HIS3 expression,
20 also displayed β-gal activity. All 20 potential positives were eliminated as false, as shown
in Figure 2.4, mainly because of their inability to interact with pAS1-PHCaLB upon re-
transformation.
TABLE 2.1: Results From Second Two-Hybrid Screen

One million transformants were placed under selection. Of the ~3000 clones tested, 17 exhibited \( \beta \)-gal activity. Library-derived plasmids that were considered potential positives (1-17) were isolated and retransformed into the Y153 strain alone, or into yeast carrying the pAS1 plasmid alone (encoding the Gal4 DNA-binding domain (DBD)), or carrying two different DBD hybrid plasmids (pAS1-PH CalB or pAS1-lamin). Transformants were assayed for the presence of \( \beta \)-gal activity.

\( \beta \)-gal activity is shown relative to residual \( \beta \)-gal activity exhibited by cells carrying only the DBD (pAS1) plasmid (+), and relative to the positive control, pAS1-Cln3 (++++), which exhibited strong \( \beta \)-gal activity after ~1h. (-) represents white transformants which do not exhibit \( \beta \)-gal activity. (*) unable to recover the plasmids containing these cDNAs in \textit{E. coli}.

Those transformants expressing \( \beta \)-gal activity only in the presence of pAS1-PH CalB, but not in the presence of pAS1-lamin were considered true positives. All potential positives were eliminated as false.
### β-Galactosidase Activity

<table>
<thead>
<tr>
<th>Positive Clone</th>
<th>~ size of insert (kb)</th>
<th>pAS1</th>
<th>pAS1 PHCalB</th>
<th>pAS1 SNF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.75</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1.75</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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**TABLE 2.2**: Results From Third Two-Hybrid Screen

Five million transformants were placed under selection. Of the ~4500 clones tested, 20 exhibited β-gal activity. Library-derived plasmids that were considered potential positives (1-20) were isolated and retransformed into the Y153 strain carrying the pAS1 plasmid alone (encoding the Gal4 DNA-binding domain (DBD)), or carrying two different DBD hybrid plasmids (pAS1-PHCalB or pAS1-SNF1). Transformants were assayed for β-gal activity.

β-gal activity is shown relative to residual activity exhibited by cells carrying only the DBD (pAS1) plasmid (+), and relative to the positive control, pAS1-Cln3 (+++). (-) represents white transformants which do not exhibit β-gal activity.

Those transformants expressing β-gal activity only in the presence of pAS1-PHCalB, but not in the presence of pAS1-SNF1 were considered true positives. All potential positives were eliminated as false.
2.5 Discussion

Two main classes of false positives typically arise in two-hybrid library searches (Bartel et al., 1993; Fields and Stern, 1994). One class contains library-derived plasmids that activate reporter gene expression entirely on their own. As previously mentioned, this is often caused by the cDNA encoding a protein involved in transcription. Another class of false positives has also been described that activate transcription only in the presence of a DNA-binding domain hybrid but not in the presence of the DNA-binding domain alone. These proteins again are thought to be transcription factors that access the promoter of the reporter gene when overexpressed. However, these should be largely eliminated in the initial screen because the promoter of the HIS3 reporter gene and the GAL1 promoter of the GAL1-lucZ fusion share little in common except that they both contain the GAL1 UAS$_g$ sequences (the Gal4 binding sites). However, these can also be eliminated by testing for β-gal activity in the presence of another unrelated protein, such as SNF1.

There are a number of reasons why this system may have failed to identify interacting partners of the PH and CaLB domains of p120$^{GAL}$. One explanation is that the PHCaLB fusion protein did not fold properly or folded in such a way that each of these domains could not access interacting proteins. In that sense, it may have been preferable to use these domains individually so that they could efficiently associate with other proteins. Another possibility is that a posttranslational modification, such as phosphorylation, is required for an interaction. This explanation is consistent with the hypothesis that PH domains may recognize serine/threonine phosphorylated peptides (Gibson et al., 1994). Recently, another group attempted this approach to identify proteins that could interact with certain PH
domains and were also unsuccessful (Ferguson et al., 1994). Alternatively, an interaction with the CaLB domain may require cofactors such as Ca\textsuperscript{2+} and/or lipids which may not be available or present in sufficient amounts in the yeast nucleus. It has also been suggested that if the amino-terminal sequences of either protein are involved in the interaction, the DNA-binding domain or the activation domain of Gal4, which are fused to the N-terminus of the proteins, could interfere with these sequences to access interacting proteins (Fields and Sternglanz, 1994). In agreement with this, I have found proteins that can interact with the N-terminal half of the PH domain which may not be accessible in the context of the Gal4 PH fusion protein (see next chapter). Finally, it is also possible that clones encoding interacting proteins were not isolated from yeast. It is possible that the same yeast cell contains more than one library-derived plasmid. Since the cDNA plasmid used for eliminating false positives was recovered from only one *E. coli* transformant per potential positive, some of the plasmids which may have encoded an interacting protein would not have been isolated (Durfee et al., 1993).
Chapter 3

FUTURE DIRECTIONS
Initial Results and Future Directions

The PH Domain of p120^c^B^P^ Binds to at Least Three Phosphoproteins

Since the two-hybrid system failed to identify interacting proteins of the PH and CaLB domains of p120^c^B^P^, I used an in vitro binding assay with purified GST fusion proteins from E. coli immobilized on glutathione agarose beads to isolate proteins from Rat2 cell lysates. Since it was recently found that the PH domain of Btk bound PKC (Yao et al., 1994) and PH domains have been speculated to bind phosphorylated serine/threonine peptide sequences (Gibson et al., 1994), I used an in vitro kinase assay to detect precipitated proteins. At least 3 proteins bound GST fusion proteins in a PH dependent manner. These three phosphoproteins migrated at approximately 90, 65 and 45 kDa. I performed phosphoamino acid analysis which showed exclusively phosphorylated serine in the PH-associated proteins. Thus I speculate the GAP PH domain may interact with a novel serine kinase in vivo. From deletion analysis, it appears that the C-terminal half of the domain is dispensable for binding to these proteins (data not shown).

The PH Domain Recognizes a Short Peptide Sequence within p90

I wanted to determine if the PH domain could recognize a short peptide sequence of any of these proteins. I found that GST PH fusions specifically bound denatured p90, but not p65 or p45. This suggests that the PH domain probably recognizes a short peptide sequence within the 90 kDa protein. Alternatively, the 90 kDa protein refolds very quickly so that tertiary structural determinants can be recognized by GST PH. In any event, this result
suggests that the 90 kDa protein may be the true binding partner of the PH domain and that the 65 and 45 kDa proteins are brought down as a complex/substrates. To investigate this, I will perform renaturation kinase assays within the gel itself, in which the kinase will be phosphorylated, but its substrates will not.

**GAP Associates with a 90 kDa Protein in vivo**

Recently, I performed an *in vitro* kinase assay of GAP immunoprecipitated from Rat 2 cell lysates and found a species which comigrated with the 90 kDa protein. I will perform phosphopeptide mapping of these two proteins to determine if they are the same protein, but at this point I cannot conclude that they are. I am also testing if this comigrating band binds to the PH domain of GAP *in vivo*. I have engineered a KT3 epitope-tagged, PH-deleted GAP construct and will perform an *in vitro* kinase assay on this mutant GAP immunoprecipitated with KT3 antibodies. As well, I plan to determine which stimuli induces this association and whether this interaction between GAP and p90 are dependent on Ras activation.

Interestingly, Martin *et al.* (1992) (see section on p120^GAP^) "detected a phosphoprotein of ~90 kDa in atrial cell membrane fractions that binds to the noncatalytic regions of GAP *in vitro*." They did not state if this "noncatalytic region" of GAP refers to the entire amino-terminus or just the SH2-SH3 domains of GAP. Also in this study, it was found that deletion of the SH2-SH3 region of GAP does not completely abolish inhibition of channel opening whereas deletion of the entire noncatalytic region of GAP does. Furthermore, a construct containing just the SH2-SH3-SH2 regions had a similar effect on
inhibition as a construct containing the entire noncatalytic region, but required twice the concentration to achieve this (Martin et al., 1992). These results are consistent with the notion that the PH domain of GAP may also be required for inhibition of K⁺ channel opening. It is intriguing that I have found a 90 kDa protein in fibroblasts that may link G protein coupled receptors and Ras signalling.

**Other Objectives**

One of my main priorities is to purify enough p90, p65, and p45 by affinity chromatography for microsequencing. The identity of these proteins will aid in the choice of experiments to do next. If any are novel, then naturally they will be cloned.

It is of interest to note that GAP is phosphorylated on tyrosine and serine after stimulation of cells with growth factors or when transformed by oncogenic tyrosine kinases (Molloy et al., 1989; Ellis et al., 1990; Kaplan et al., 1990; Kazlauskas et al., 1990; Moran et al., 1991). This phosphorylation may affect GAP activity or may influence the formation of complexes involved in effector function or in localization. If one of p90, p65, or p45 proteins is a kinase, then I plan to determine if GAP is a substrate of the kinase. If so, then I will initially test if this phosphorylation affects GAP activity in vitro as well as determining which stimuli cause this potential kinase to become phosphorylated and/or activated.

As mentioned previously, PH domains have been proposed to recognize phosphorylated serine/threonine peptides (Gibson et al., 1994). Since p90, p65, and p45 are phosphorylated on serine residues and the PH domain appears to recognize a short peptide sequence within p90, I plan to determine if phosphorylation of these proteins is required for
binding to the PH domain of GAP. It has also been found that PtdIns(4,5)P$_2$ and Ins(1,4,5)P$_1$ bind to the N-terminal half of the PH domain (Harlan et al., 1994; Ferguson et al., 1995). To determine if negatively charged phospholipids such as PtdIns(4,5)P$_2$ compete for the same site on the PH domain of GAP as these proteins, I plan to perform competition experiments using various phospholipids.

**GAP Interacts with Recombinant RACK1 in Vitro**

We have recently received the vector encoding the maltose binding protein RACK1 fusion protein (MBP-RACK1) from Dr. Mochly-Rosen. To determine if the CaLB domain of p120$_{GI}$AP can interact with RACK1, I performed in vitro binding experiments using full-length GAP immunoprecipitated from Rat2 cell lysates and recombinant MBP-RACK1. p120$_{GI}$AP bound to MBP-RACK1 in the presence of PS and Ca$^{2+}$ but not without. In other experiments, this interaction was found to be dependent only on Ca$^{2+}$. Although I have not determined the minimal concentration of Ca$^{2+}$ required for this interaction, full binding was still present when the Ca$^{2+}$ was lowered to 0.1mM, but was inhibited when the Ca$^{2+}$ exceeded 10mM.

I have not yet been able to compete out this interaction with GST CaLB, nor have I been able to consistently detect an interaction between GST CaLB and MBP-RACK1 in vitro. This could be due to a number of reasons: the affinity for the isolated CaLB domain could be much lower than that of intact GAP, sequences which lie outside of this domain are also required for the interaction with RACK1, or the CaLB domain is not involved in the interaction.
The PH Domain of p120<sup>34β</sup> Binds to Endogenous RACK1

By incubating various GST fusion proteins encoding different domains of GAP with Rat2 cell lysates, I have found that GST PH domain fusion, but not the GST CaLB domain fusion protein, binds to endogenous RACK1 in a Ca<sup>2+</sup>-independent manner. Again by using various truncated GST PH fusion proteins, the N-terminal half of the domain is involved in this interaction. This was quite surprising at first, since I believed that the CaLB domain would have been involved. however, it has been speculated that PH domains may bind to proteins with WD40 motifs.

As mentioned earlier, some PH domains associate with G<sub>11</sub> subunits (Touhara et al., 1994). Additionally, fusion proteins containing the PH domains of βARK and β-spectrin were found to bind to a C-terminal fragment of G<sub>11</sub> in vitro which contained 5 of its 7 WD40 motifs (Wang et al., 1994). For this reason, it was suggested that PH domains may bind proteins containing these motifs and that the varying affinities of PH domains for G<sub>11</sub> observed (Touhara et al., 1994) may reflect the preference for different PH domains for different proteins containing these motifs such as RACK1 (Shaw, 1996).

GAP Associates with RACK1 in vivo

In coimmunoprecipitation experiments using different mutants of GAP in which the CaLB or PH domains were deleted, I determined that only wild-type GAP associates with RACK1 in vivo. This suggests that the CaLB domain is also important for this interaction in vivo or that deletion of this domain causes GAP to fold in such a way that the PH domain is unable to access RACK1. Surprisingly, the association between GAP and RACK1 appears
to only occur in quiescent cells. So far I have found that cells stimulated with ionomycin and PMA, lose the association between GAP and RACK1. I plan to use other agonists as well to determine which other pathways may cause disassociation. I also plan to further delineate the minimal sequences of the PH domain, and RACK1, required for their interaction. In any event, RACK1 may be the link bringing GAP and activated PKC together.

*Other Objectives*

I plan to determine the intracellular localization of RACK1 by immunoflorescence. as well, it will be interesting to determine the localization of GAP and the domain deletion mutants of GAP in response to extracellular stimuli and to see how this may affect their colocalization with RACK1. I also plan to microinject these domains into intact cells and determine the effects this will have on such things as GAP localization, Ras activation, cell growth or other effects in response to various stimuli. Our lab has expertise in microinjection and I plan to learn these methods to support my own research project. I would also like to make stable lines expressing wild-type GAP or the above mentioned domain deletion mutants in GAP- fibroblasts, that I have at my disposal, to directly determine the importance of these domains in the regulation of GAP without possible interference of endogenous GAP.

Finally, I also plan to make stable lines expressing these GAP constructs and v-src in these GAP- fibroblasts. It has already been established that GAP partially localizes at the membrane and associates with p190 and p62 in Src transformed cells (Ellis *et al.*, 1990; Moran *et al.*, 1991). However, unlike activated receptors and tyrosine phosphorylated p62, p190 binding cannot readily be mimicked *in vitro* using bacterially expressed GAP SH2
domains (Anderson et al., 1990; Moran et al., 1990). Point mutations in the FLVR sequence of both SH2 domains of GAP, where the arginine is required for pTyr binding, does not completely abolish the association between GAP and p190 (Bryant et al., 1995). Additionally, both GAP and p190, in the GAP-p190 complex, contain relatively little phosphorylated tyrosine residues (Ellis et al., 1990). It has therefore been suggested that additional factors, other than the SH2-pTyr interaction may contribute to their association (Moran et al., 1991; McGlade et al., 1993; Bryant et al., 1995). It may be that another domain in GAP, such as the CaLB or the PH domain is involved in an initial interaction and the SH2-pTyr interaction stabilizes the association. The same may be true for the interaction between GAP and RACK1, involving both the PH and CaLB domains, mentioned earlier.

Conclusion

Through these studies, I hope to identify proteins that interact with the PH and CaLB domains of p120^{GAP}. RACK1 is a good candidate for one such protein, as are the p90, p65 and p45 proteins I have identified. These interactions will be studied using a variety of approaches both in vitro and in vivo. Establishing the protein targets of the PH and CaLB domains of p120^{GAP} will contribute to a better understanding of the function of p120^{GAP} and the Ras signalling pathway.
REFERENCES


