Characterization of regulatory mechanisms of CdGAP, a negative regulator of the small GTPases Rac1 and Cdc42

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Abstract:

The Rho GTPases form a diverse family of proteins which regulates numerous cell processes from cytoskeletal reorganization to control of gene expression. They are negatively regulated by GTPase-activating proteins (GAPs). Cdc42-GTPase-Activating Protein (CdGAP) is a negative regulator of the Rho GTPases Rac1 and Cdc42, whose aberrant signaling is involved in pathological processes such as tumorigenesis and metastasis. In this study, we set out to identify regulatory mechanisms of CdGAP. We show that CdGAP exists as a long isoform of 1425 amino acids in addition to the shorter 820 amino acid isoform that was previously described. We also identify RSK-1, ERK1 and GSK-3 as interacting partners of CdGAP, with ERK1 and GSK-3 interacting with the proline-rich domain (PRD) of CdGAP, a region known to be necessary for CdGAP regulation. We characterize the phosphorylation of CdGAP and show that *in vivo* CdGAP is mostly phosphorylated on serine residues. RSK-1, ERK1 and GSK-3 are all able to phosphorylate CdGAP in the PRD, and, in the case of ERK1 and GSK-3, we have identified Thr-776 as a site phosphorylated by both kinases. The site can be phosphorylated *in vivo*, and its phosphorylation leads to decreased CdGAP activity. We have also identified a novel potential mechanism of RhoGAP protein regulation, namely the serum-induced upregulation of CdGAP mRNA levels. We characterize the mechanism by which this occurs and demonstrate that Rho GTPase activity is necessary for this upregulation to occur. In summary, we have identified and characterized novel regulatory mechanisms for CdGAP, a negative regulator of Rac1 and Cdc42.

Résumé

La famille des protéines GTPases Rho contrôle une grande variété de processus cellulaire comme la réorganisation du cytosquelette ou le contrôle de l'expression des gènes. Ils sont inhibés par les protéines "GTPase-activating proteins" (GAP). Cdc42-GTPase-Activating Protein (CdGAP) est un inhibiteur des GTPases Rac1 et Cdc42. Une dérégulation de ces deux protéines provoque des processus pathologiques comme le développement de tumeurs et de métastases. Dans ce projet, nous cherchons à identifier les mécanismes de régulation de CdGAP. Nous démontrons que, en plus de la forme courte de CdGAP déjà connue, il existe une forme longue de 1425 acides aminés. Nous avons aussi identifiés des partenaires qui interagissent avec CdGAP tels que RSK-1, ERK1 et GSK-3. Ces deux dernières protéines se lient au domaine riche en proline (PRD) de CdGAP, connu pour être important pour sa régulation. Nous avons caractérisé la phosphorylation de CdGAP et démontré que les résidus phosphorylés sont pour la plupart des sérines. RSK-1, ERK1 et GSK-3 sont toutes les trois capables de phosphoryler le domaine PRD de CdGAP et pour ERK-1 et GSK-3 nous avons trouvé que le site de phosphorylation était la thréonine 776. Ce résidu peut être phosphorylé *in vivo* et sa phosphorylation induit une diminution de l'activité de CdGAP. De plus, nous avons identifié que l'expression du ARN messager de CdGAP est augmentée en réponse au sérum. Ceci pourrait aussi représenter un nouveau mécanisme de regulation des protéines RhoGAP. Nous avons caractérisé ce mécanisme par lequel il a lieu et prouvé que l'activité des Rho GTPases est nécessaire pour cette augmentation de l'expression. En

résumé, nous avons identifié et caractérisé des nouveaux mécanismes de régulation de CdGAP.

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Abbreviations:

3-AT	3-amino-1,2,4-triazole
ABR	Active BCR-related
Abl	Abelson tyrosine kinase
ActD	Actinomycin D
ANXL	Annexin-like
AP-1	Activator protein 1
APC	Adenomatous polyposis coli
ARAP	ArfGAP, RhoGAP, Ankyrin repeat, Ras-associating (RA), and five PH domains
Arf	ADP-ribosylation factor
Arp2/3	Actin-related protein 2 and 3 complexes
ATP	Adenosine 5'-triphosphate
BCR	Breakpoint cluster region
BSA	Bovine serum albumin
CaMKII	Ca 2+ /Calmodulin-Dependent Protein Kinase II
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division cycle 42
CdGAP	Cdc42 GTPase-activating protein
CdGAP-l	CdGAP long isoform
CdGAP-s	CdGAP short isoform
cDNA	Complementary DNA

Chp	Cdc42 homologue protein
Cool	Cloned out of library
CREB	Cyclic AMP Response Element-binding Protein
CSF-1	Colony Stimulating Factor 1
DBC2	Deleted in breast cancer 2
DEF	Docking for ERK FXFP
DH	Dbl Homology
DHR1	Dock180 homology region 1
DHR2	Dock180 homology region 2
DLC-1	Deleted in Liver Cancer 1
DRB	5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside
ECM	Extracellular matrix
Ect2	Epithelial Cell Transforming Sequence 2
EGF	Epidermal growth factor
ELMO	Engulfment and cell motility protein
EMT	Epithelial–mesenchymal transition
ERK	Extracellular-signal regulated kinases
ERM	Ezrin/radixin/moesin
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
FilGAP	Filamin A-associated RhoGAP

FRAT1	Frequently rearranged in advanced T-cell lymphomas GAP GTPase-activating protein
GEF	Guanine Nucleotide Exchange Factor
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanosine diphosphate
GLUT4	Glucose transporter-4
GMIP	Gem interacting protein
GRAF	GTPase regulator associated with FAK
Grit	GTPase regulator interacting with TrkA
GSK-3	Glycogen Synthase Kinase 3
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HGF	Hepatocyte growth factor
HIF-1	Hypoxia-Inducible Factor 1
ICAP-1	Integrin cytoplasmic domain-associated protein-1
IGF-1	Insulin-like growth factor 1
IκB	Inhibitor of kappa B
JNK	c-jun N-terminal kinase
kDa	Kilodalton
LARG	Leukemia Associated RhoGEF
LiCl	Lithium Chloride
LPA	Lysophosphatidic acid
MAPK	Microtubule associated protein-2 kinases

MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MBP	Myelin Basic Protein
MEF	Mouse embryonic fibroblast
MEK	MAP/ERK kinase
MEKK1	MEK kinase 1
MgcRacGAP	Male Germ Cell RacGAP
Miro	Mitochondrial Rho
MMP-1	Matrix metalloproteinase 1
MMP-2	Matrix metalloproteinase 2
mRNA	Messenger ribonucleic acid
MTOC	Microtubule-organizing center
mTOR	Mammalian target of rapamycin
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartic acid
PAK	P21-activated kinase
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDZ	Domain present in PSD-95, Dlg, and ZO-1/2
PH	Pleckstrin Homology

PI3K	Phosphatidylinositol 3-kinase
PKC	Protein Kinase C
PKN	Protein kinase N
PMA	Phorbol 12-myristate 13-acetate
PRC1	Protein-regulating cytokinesis 1
PRD	Proline-rich domain
PX	Phox homology
RA-RhoGAP	Rap-activated RhoGAP
Rab	Ras in brain
Rac	Ras-related-C3 botulinum toxin substrate
Ral	Ras-like
Ran	Ras-related nuclear protein
Rap	Ras-proximal
Ras	Rat sarcoma
RGS	Regulator of G protein Signaling
Rho	Ras homologous
RICS	RhoGAP involved in the β-catenin-N-cadherin and NMDA receptor signaling
Rif	Rho in filopodia
ROCK	Rho-associated coiled-coil-containing protein kinase
RSK	p90 Ribosomal S6 Kinase
SAM	Sterile alpha motif
SH2	Src homology 2

SH3	Src homology 3
siRNA	Small interfering RNA
Sos	Son of Sevenless
SRE	Serum Response Element
START	StAR-related lipid-transfer domain
STAT5A	Signal transducer and activator of transcription 5A
TCGAP	TC10/Cdc42 GTPase activating protein
TCL	TC10-like
TTF	Translocation three-four
VCA	Verprolin homology motif, a cofilin homology motif, and an acidic motif
VEGF	Vascular endothelial growth factor
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP family Verpolin-homologous protein
WRCH	Wnt-responsive Cdc42 homolog

Contributions of Authors to Manuscripts:

This thesis is structured in a manuscript-based form. In accordance with faculty regulations, manuscripts that are co-authored by others must be accompanied by an explicit statement as to who contributed to the work, and to what extent the contribution was made. Copyright waivers from the publishers, as well as from co-authors can be found in the appendix.

Chapter 2:

My contribution to the manuscript titled "Extracellular Signal-regulated Kinase-1 (ERK-1) interacts with and phosphorylates CdGAP at an important regulatory site" involved the creation of the His-tagged PRD T776A construct, as well as intellectual contributions to the design of the experiments. Ibtissem Triki performed the Western blot of mouse tissues in Figure 2.1B. All other experiments were performed by Joseph Tcherkezian. Joseph Tcherkezian, Nathalie Lamarche-Vane and I contributed to the writing of the manuscript.

Chapter 3:

My contributions to the manuscript titled "Glycogen synthase kinase-3 phosphorylates CdGAP at a consensus ERK 1 regulatory site" involved designing and performing all experiments shown. Joseph Tcherkezian made intellectual contributions to the design of

the experiments. The anti-phospho-Thr-776 antibody was created by Ibtissem Triki. Nathalie Lamarche-Vane and I both contributed to the writing of the paper.

Chapter 4:

My contributions to the manuscript titled "Rho GTPases regulate the serum-induced upregulation of CdGAP mRNA" involved designing and carrying out all experiments shown. Nathalie Lamarche-Vane and I both contributed to the writing of the manuscript.

This thesis includes the following manuscripts already published:

Chapter 2:

Extracellular Signal-regulated Kinase-1 (ERK-1) interacts with and phosphorylates CdGAP at an important regulatory site. Mol Cell Biol. 2005 25:6314-6329

Chapter 3:

Glycogen synthase kinase-3 phosphorylates CdGAP at a consensus ERK 1 regulatory site. J Biol Chem. 2007 282:3624-3631

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 General Introduction

Cells are the structural foundation of all living organisms (1). Organisms can consist of one cell acting alone, or multiple cells acting in concert. One defining feature common to all cells is the ability to respond to the environment surrounding the cell. Whether it is a single-celled organism which needs to find food, or needs to know whether it is appropriate to divide because conditions are favorable, or a cell within a tissue of a multi-cellular organism which is monitoring what its neighboring cells are doing so that it can act accordingly and stop proliferating when it no longer needs to, or take up sugar when it is present to drive metabolism, cells are constantly reading their environment and responding to it.

In the context of multicellular organisms this ability to read an environment correctly and respond appropriately can often mean the difference between a healthy functioning organism and one that is dysfunctional. For example, a cell that ignores environmental cues signaling that it is inappropriate to grow and divide can become a tumor. Similarly, a cell that has lost its ability to recognize insulin will not take up glucose when necessary. Cells also can be subverted by false signals sent by mutated proteins that remain active inappropriately, or by viral proteins which can mimic signals and force a cell to perform in ways favorable for the virus at the expense of the host. These are but a few examples of how the inability of a cell to correctly respond to its environment can be detrimental to the entire organism.

It is thus imperative to understand how a cell responds to its environment, both under normal and pathological states, as this will lead to the knowledge necessary to

design strategies and therapies to manage the problems that arise from inappropriate responses to environment.

In this thesis, the regulation of CdGAP is examined. CdGAP is a negative regulator of Cdc42 and Rac1, which are involved in a multitude of signaling pathways that a cell uses to sense and respond to its environment. Aberrant signaling of Cdc42 and Rac1 pathways can lead to cell proliferation abnormalities such as tumorigenesis.

1.1 The Ras Superfamily

In 1964, a virus which caused solid tumors in mice was identified (2). This was followed a few years later by the identification of another virus with similar effects (3). Subsequent sequencing of these viruses led to the discovery that they were highly homologous (4-7), and they were subsequently named Kirsten-Ras and Harvey-Ras, after their discoverers (8). Further studies went on to show that these viral proteins were highly related to a 21 kDa protein found in most mammalian cells (called cellular Harvey-Ras), and it was hypothesized that a normal cell functions well with its basal level of Ras, but, when transformed with a viral Ras, its growth becomes deregulated due to the over-expression of Ras, or through activating mutations in the viral protein, leading to increased Ras signaling (9). It was also discovered that these 21 kDa proteins had the ability to bind GTP (10, 11), and furthermore exhibited GTPase activity (12). Thus, Ras became the founding member of the small GTPase superfamily.

This superfamily has grown from its initial membership of Ras to include approximately 159 small GTPases encoded in the human genome (13). The small GTPases are characterized by their small size, approximately 20-40 kDa in size, (14), their ability to bind guanine nucleotides, and their generally weak GTPase activity (15). Their roles in cells are numerous, and vary from signal transduction leading to control of gene expression to control of membrane trafficking (14). They are broadly regulated by three classes of proteins: the Guanine Nucleotide Exchange Factors (GEFs), which stimulate the exchange of GDP for GTP, thus activating the GTPase; the Guanine Nucleotide Dissociation Inhibitors (GDIs), which generally bind the GDP bound form of the GTPase locking it in an inactive conformation; and the GTPase-activating proteins

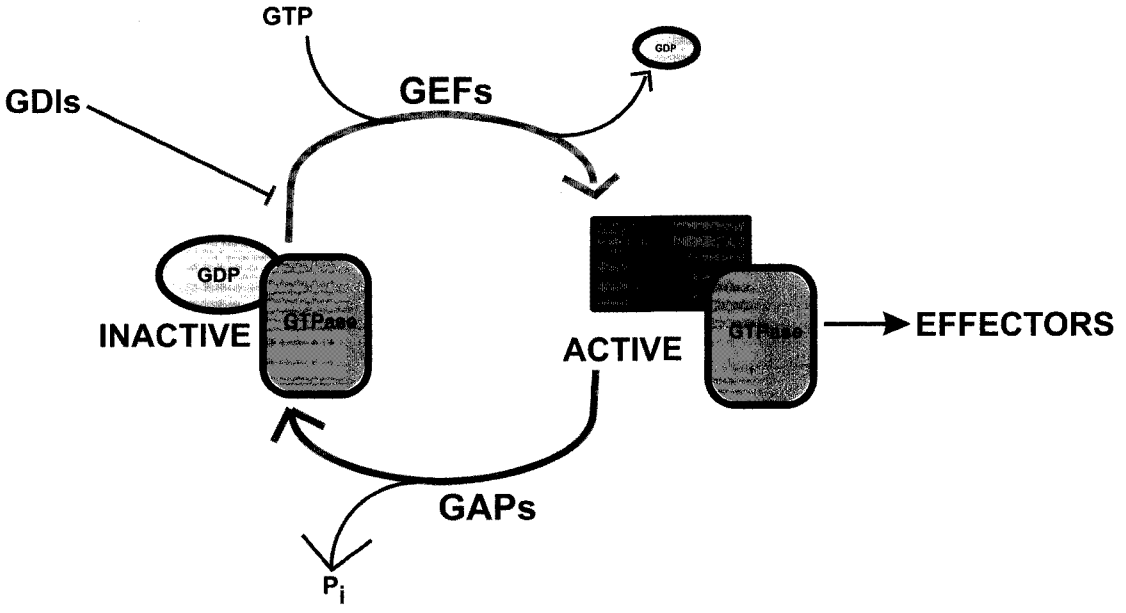
(GAPs), which enhance the catalytic activity of the GTPases leading to the hydrolyis of GTP to GDP, thereby inactivating the GTPase (Fig. 1.1) (14). The small GTPases are broadly classified into five families: the Ras subfamily, the Rho subfamily, the Rab subfamily, the Arf subfamily and the Ran subfamily (16).

1.1.1 The Ras Subfamily

The Ras subfamily of small GTPases is comprised of approximately 35 members, which share the highest homology to Ras (13). These proteins are most strongly implicated in regulating cell proliferation (17), apoptosis (18), morphology (17), and differentiation (19). The Ras subfamily in general is characterized by having C-terminal prenylation sites and, generally due to these modifications, plasma membrane localization (13).

The first three members of the Ras subfamily are the cellular versions of H-Ras, and K-Ras, as well as N-Ras (20), which was also identified as a viral oncogene. Ras proteins mainly signal by activating kinases within signal transduction pathways. One notable example is the activation of Raf by Ras downstream of growth factor signaling, which leads to the activation of the MEK-ERK MAP kinase cascade (21, 22). Raf has been implicated quite strongly in tumorigenesis, and is frequently mutated in human cancers (23). Another well studied example is the activation of PI3 kinases by Ras (23) which can lead to activation of downstream kinases such as AKT which is involved in cell survival. Interestingly, Ras promotes cell survival via AKT activation, but can also promote apoptosis through the Raf pathway (24), indicating a need for balance in Ras signaling.

Figure 1.1: The GTPase Cycle. The Ras superfamily of small GTPases cycle between an inactive GDP-bound state and an active GTP-bound state which allows the GTPase to interact with effectors. This cycle is broadly regulated by three classes of proteins, the Guanine Nucleotide Exchange Factors (GEFs), the Guanine Nucleotide Dissociation Inhibitors (GDIs), and the GTPase-Activating Proteins (GAPs). The GEFs stimulate the exchange of GDP for GTP, thus activating the GTPase. The GDIs generally bind the GDP-bound form of the GTPase and lock it in an inactive conformation. The GAPs enhance the catalytic activity of the GTPase, leading to GTP hydrolysis and a return to the inactive GDP-bound state.


GTP
GDP
GDIs
GEFs
GDP
INACTIVE
GTPase
ACTIVE
GTPase
EFFECTORS
GAPs
P_i

The Rap proteins (Ras-proximal) are a four member branch of the Ras subfamily tree. Interestingly, the effector domains of the Rap proteins are nearly identical to those of the Ras subfamily (25). As with the Ras proteins, Raf and PI3 kinase are the major effectors of the Rap proteins.

The Ral (Ras-like) proteins differ from Rap and Ras proteins, as they do not use Raf and PI3 kinase as effectors, possibly due to some differences in the effector binding domains (13). The Ral proteins can synergize with the Ras proteins to promote tumorigenesis, however, activated Ral proteins on their own are insufficient (26).

The above mentioned GTPases constitute roughly one-third of the Ras subfamily and represent the best characterized members. Some other notable members are the R-Ras proteins, which like the Ras branch generally activate Raf and PI3 kinase, and also cause tumorigenesis (13). The Rit and Rin proteins, which, while lacking the C-terminal prenylation box present in most Ras subfamily members, are still localized to the plasma membrane (27). Interestingly, some members of the Ras subfamily, like Rig, act as tumor suppressors (28). As a whole, the Ras subfamily functions as a master regulator of cell growth and proliferation, both in positive and negative fashions.

1.1.2 The Rab Subfamily

The Rab (Ras in brain) subfamily of small GTPases is easily the largest, comprising approximately 70 members in humans (13). Although originally identified in brain tissue, most Rabs are ubiquitously expressed (29). Like the Ras proteins Rab proteins have C-terminal prenylation motifs allowing for membrane insertion (29). They are involved in membrane trafficking, and, in general, are active when attached to

membranes, and inactive when in the cytosol (30). The GDP/GTP cycle operates with a cytoplasmic shuttling mechanism, where, when bound to GDP, the Rab protein is interacting with a GDI, which holds it in the cytosol. At an appropriate time, a GDI Displacement Factor (GDF) displaces the GDI, and the Rab protein is free to attach to membranes. When attached to the membrane, the Rab can interact with GEFs which are also membrane-bound, which then activate the Rab protein. Finally, either through its own slow catalysis alone, or with the help of a GAP protein, the GTP is hydrolyzed to GDP, whereby a GDI will bind the Rab protein and bring it back to the cytosol (31). The roles of the various Rab family members generally fall into one of three categories, membrane fusion, vesicle budding and vesicle movement (32).

1.1.3 The Ran Subfamily

The Ran (Ras-related nuclear protein) (33) is a strange member of the small GTPase superfamily. It is sometimes placed as a member of the Rab subfamily (13), whereas at other times it is placed as its own subfamily (14). When considered as its own subfamily, it becomes quite unique among the Ras superfamily, as it has only one member in humans (14). Ran is also notable for being the most abundant small GTPase in cells (16). Ran does share considerable homology to the Rab proteins (14), but functionally its role is quite different. The Ran proteins are involved in nucleo-cytoplasmic shuttling of proteins. Like all GTPases, the Ran proteins function in a GDP/GTP cycle. In the case of Ran proteins, the GDP-bound form is exclusively found in the nucleus. A GEF protein is then able to activate the Ran protein by stimulating exchange of GDP for GTP, and the GTP bound Ran shuttles out of the nucleus and into

the cytoplasm. In the cytoplasm, a GAP stimulates hydrolysis of the GTP, and the GDP bound form of Ran is then shuttled back to the nucleus. There are two main types of proteins that exploit this cycle: the importins and the exportins. The importins bind their substrate in the cytoplasm, without Ran, and when they enter the nucleus, and come into contact with GTP-Ran, they release their cargo. The exportins on the other hand first bind to Ran-GTP, and then to their substrate, and this complex is then exported from the nucleus. Once Ran-GTP is hydrolyzed to Ran-GDP, the complex disassembles and the exportin releases its cargo in the cytoplasm (34).

1.1.4 The Arf Subfamily

Arf (ADP-ribosylation factor) was first identified as a protein necessary for the regulation of adenlyl cyclase (35) and was subsequently shown to have homology to other small G proteins (36). There are now 30 members of the Arf family in humans (13). Unlike other members of the Ras superfamily, ARF proteins do not posses a C-terminal prenylation site. They do in general have an N-terminal myristolation site, and an N-terminal amphipathic helix (13). The mechanism of membrane insertion of the Arf proteins is GTP binding-dependant. When GTP binding occurs, the conformation of the GTPase changes allowing it, as with other small GTPases, to interact with effectors. In addition, it positions the N-terminal amphipathic helix to be inserted into membranes, thus anchoring the GTPase (37). The N-terminal helix seems to be the primary mechanism of insertion into the membrane (38). In general, ARF proteins are involved in the budding of vesicles. ARF 1 and ARF 3 are involved in recruiting COPI or clathrin coat proteins to a newly forming vesicle at the Golgi apparatus, and may also be

implicated in the sorting of cargo to these vesicles (39). Sar1 is localized to ER membranes and is involved in the formation of COPII vesicles (39). Aside from their roles in membrane trafficking, some members of the ARF family may be involved in microtubule and cilia regulation (39).

1.1.5 The Rho Subfamily (in Brief)

The Rho (Ras homologous) subfamily contains 23 members in humans (13). RhoA, Rac1 and Cdc42 are its best known members, and this subfamily is most often connected with the functions of these proteins in the control of cytoskeletal dynamics (14). This subfamily also has many other notable roles in the functioning of a cell, such as control of gene expression (40, 41), membrane trafficking (42, 43), and cell polarity (44). The Rho subfamily will be expounded upon in the subsequent sections of this introduction.

In conclusion, the Ras superfamily of small GTPases comprises a diverse family of proteins, linked by their usage of a GDP/GTP cycle to regulate effector binding. Their functions are broad, and even within subfamilies, there can be a wide range of roles.

1.2 The Rho Subfamily GTPases

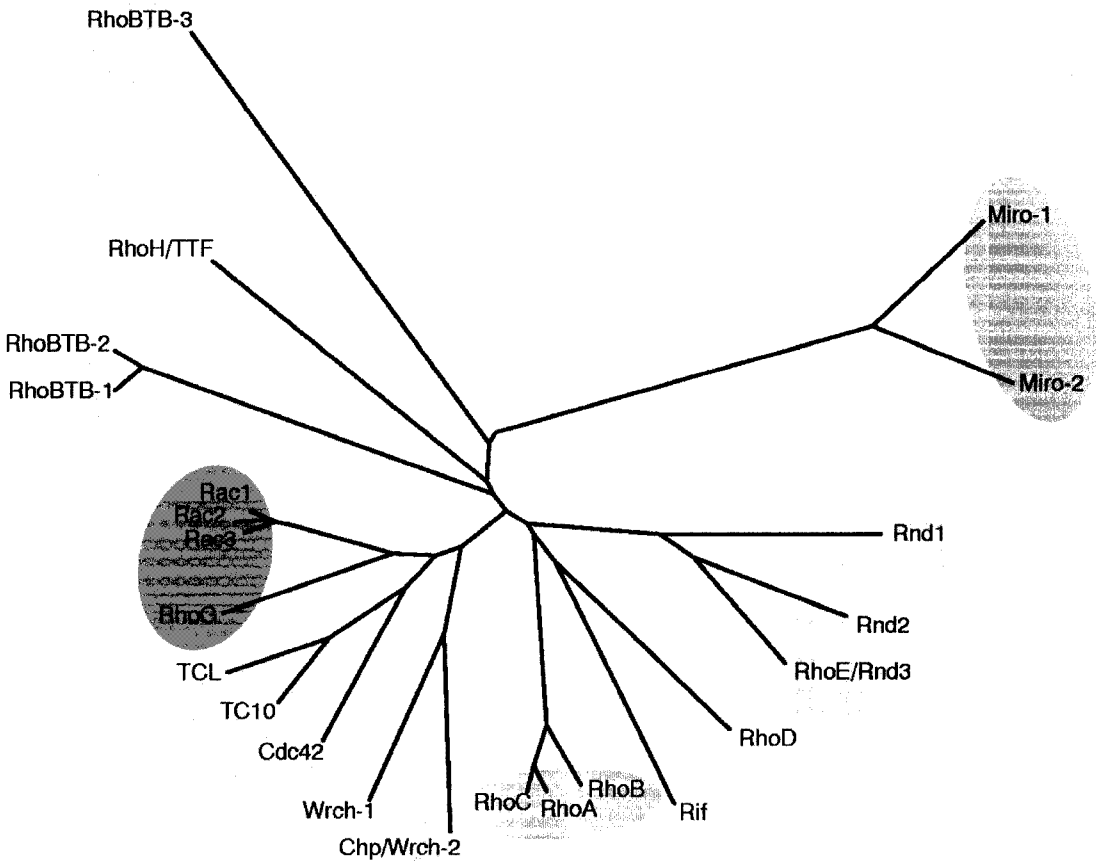
Rho was the first Ras homologue identified. It was found while screening for *Aplysia* genes homologous to human peptide hormones. A potential cDNA for human chorionic gonadotropin was isolated, but it turned out that it only had a 5 amino acid homology with human chorionic gonadotropin, but, its complementary strand coded a protein which had a 35% homology with the Ras protein (45). From this first homolog of Ras, the family grew to the 159 homologues of the Ras superfamily, including 23 members of the Rho subfamily.

Rho proteins set themselves apart from Ras proteins by the presence of a sequence known as the "Rho insert" within the functional domain (46). The Rho proteins share on average thirty percent homology with Ras, and anywhere from 40 to 95 percent homology with other Rho subfamily members (47). As with other small GTPases, the Rho subfamily cycles between a GDP-bound off state and a GTP-bound on state, that is regulated by GEFs, GAPs and GDIs. The Rho proteins can be broadly classified into 8 branches: RhoA and its related GTPases, Rac1 and its related GTPases, Cdc42 and its related GTPases, the RhoBTB GTPases, the Miro GTPases, the Rnd GTPases, RhoD and Rif, and, RhoH (Fig 1.2) (47).

1.2.1 The RhoA Related GTPases

Although Rho was first identified in *Aplysia*, its first major characterizations came a couple of years later in yeast. Yeast Rho1 shows 70 percent homology to *Aplysia*

Figure 1.2: The Rho Subfamily. There are 23 members of the Rho subfamily. They are broadly classified into 8 branches: The RhoA related GTPases, comprising RhoA, B and C, the Rac1 related GTPases, comprising Rac1, 2 and 3 as well as RhoG, the Cdc42 related GTPases, comprising Cdc42, TC10, TCL, Wrch1 and Wrch2, the Miro GTPases, comprising Miro 1 and 2, the RhoBTB GTPases comprising RhoBTB 1, 2 and 3, the Rnd GTPases comprising Rnd 1, 2 and 3, the RhoD and Rif branch comprising these two GTPases, and the RhoH branch comprising this GTPase. This figure is modified from Wennerberg and Der, 2004 (47).


RhoBTB-3
RhoH/TTF
RhoBTB-2
RhoBTB-1
Rac1
Rac2
Rac3
RhoG
TCL
TC10
Cdc42
Wrch-1
Chp/Wrch-2
RhoC
RhoA
RhoB
Rif
RhoD
Rnd1
Rnd2
RhoE/Rnd3
Miro-1
Miro-2

Rho (48). Rho1 was found to be an essential gene, whose deleted function could not be compensated for by Ras, nor by cAMP kinase expression (which could rescue yeast with deletions of functional Ras) (48), showing that the Rho and Ras small GTPases, although related, had different functions.

In humans there are three members of the RhoA branch of the Rho subfamily: RhoA, RhoB and RhoC (49-51). Intense studies of human Rho led to the discovery of the role of RhoA in cell morphology, and specifically the finding that activated RhoA leads to the formation of stress fibres (52). Studies with RhoB and RhoC have shown that these two homologues are also capable of inducing stress fibre formation when activated (53-55).

RhoA and RhoC are predominantly localized to the plasma membrane of cells, whereas RhoB is normally localized to early endosomes and pre-lysosomal compartments (56). This could be in part explained by the differences in the prenylation of the three proteins; RhoA, RhoB and Rho C all possess a C-terminal CAAX box, which is a consensus region for prenylation. However, RhoA and RhoC are only able to have a geranyl-geranyl group attached to them, while RhoB is able to have either a geranyl-geranyl or a farnesyl group attached to it (57). When RhoB is forced to have a geranyl-geranyl group, it is found at the plasma membrane (58). This is quite interesting, since although this implies that RhoB can be localized to the plasma membrane, it is generally found at the endosomal/lysosomal compartments, indicating that it is normally farnesylated. This presents an interesting mechanism for the action of RhoB, which can be active at two different locations within the cell depending on its prenylation.

The roles of Rho proteins in cells are numerous. Many of the roles, such as involvement in cell motility, can be linked to the role of Rho in the control of the organization of actin structures, while other roles are independant of these abilities. Integrins, receptors involved in cell adhesion to ECM, depend on Rho to make strong attachments and this is dependant on the ability of Rho to activate two of its effectors, ROCK (Rho-associated coiled-coil-containing protein kinase) and mDia (59). mDia nucleates actin and is responsible primarily for creating unbranched, linear actin filaments (60). It consists of a C-terminal actin-nucleating region and a C-terminal profilin binding domain (involved in filament elongation) along with a C-terminal regulatory domain that binds the N-terminal half of the protein, essentially folding the protein shut (60). The N-terminal domain contains the Rho binding domain which interacts with GTP bound Rho. When Rho binds mDia, it releases the auto-inhibition of mDia, allowing actin nucleation to proceed (60). mDia is capable of both nucleating actin, as well as enhancing the elongation of actin filaments, the latter with the aid of profilin (60). If mDia alone is activated in the absence of Rho activation, stress fibres do not form; this is due to the absence of the activation of ROCK, which is necessary along with mDia for stress fibre formation to occur (59).

ROCK was the first known effector of Rho, and exists as two isoforms in mammals, ROCK1 and ROCK2, which are ninety-two percent similar in the kinase domain (61). ROCKs have an N-terminal kinase domain, followed by a coiled-coil region, which contains the Rho-binding domain and a C-terminal pleckstrin homology (PH) domain (61). Like mDia, ROCK is in an inactive conformation with the N-terminus binding to the C-terminus, and binding of Rho changes the conformation freeing the

kinase domain. Proteins such as Gem and Rad bind the coiled-coil region of ROCK 1 and 2, respectively, and inhibit the kinase activity of ROCK (61). During focal adhesion assembly, Rho activates ROCK which leads to ROCK phosphorylating and inactivating myosin light chain phosphatase. In addition to this, ROCK is able to phosphorylate the myosin light chain directly. This all leads to an increase in myosin light chain phosphorylation, which leads to increased myosin contractility which is necessary for proper focal adhesion formation (59). Other targets of ROCK include PI3 kinase which is inhibited by ROCK (62), the Lim kinases, which are activated by ROCK allowing them to phosphorylate and inactivate cofilin, an actin depolymerizing/severing protein, and various other proteins involved in actin binding such as α-adducin, and the ERM proteins (61). ROCK also affects other cytoskeletal proteins, such as intermediate filaments and microtubules. ROCK can phosphorylate vimentin, leading to impaired disassembly of vimentin filaments while on the other hand, neurofilament protein is impaired in its ability to form filaments after phosphorylation (61). Both Tau and Map-2 microtubule-associated proteins are phosphorylated by ROCK and this leads to decreased microtubule polymerization (61).

Rho is very important in cell motility. A motile cell attaches at its leading edge, which provides a strong anchoring point that can be used by actomyosin to create a strong contractile force to bring in the trailing edge of the cell. Rho is involved in generating the actomyosin contractility of motile cells (63).

In addition to the above roles, Rho proteins are also implicated in signal transduction leading to altered gene transcription. One interesting example is the regulation of Mal by Rho. Mal, a Serum Response Factor co-activator, normally binds

G-actin, and when Rho signaling leads to polymerization of G-actin into filaments, Mal is released from the G-actin and it goes to the nucleus to induce transcription (64). Rho is also involved in Jak/Stat transcription, mainly through activation of Stat3 and Stat5 (65). RhoB has been shown to inhibit AP1 leading to decreased transcription of TGF-β receptor type 2 (66). Interestingly, RhoB itself is transcriptionally regulated and is transiently upregulated upon cell stimulation of EGF, and unlike RhoA and RhoC the mRNA of RhoB is quite short-lived, with a thirty minute half life (67).

The Rho proteins are implicated in various stages of tumorigenesis and cancer. RhoA and RhoB are both able to promote tumorigenesis in fibroblasts, whereas RhoC is not (57). In the case of RhoB, this is particularly interesting as RhoB has been shown to antagonize tumor growth in animal models (68). In fact, RhoB expression is downregulated in many tumors (69). Although it is unable to transform fibroblasts, RhoC has roles nonetheless in cancer progression. RhoC is able to promote the upregulation of genes involved in invasion and metastasis, and it is upregulated in many metastatic cancers (70). Unlike the Ras proteins, activating mutations of RhoA, B and C do not seem occur during tumorigenesis, and instead, these proteins have their activity regulated by the activation or inactivation of their regulatory proteins or by up or down-regulation of the GTPases themselves (65).

1.2.2 The Rac1 Related GTPases

Rac (Ras-related-C3 botulinum toxin substrate) was first identified as two isoforms, Rac1 and Rac2, both of which were substrates of the C3 toxin (71). They were found to be ninety-two percent homologous to each other, and shared twenty-six to thirty

percent homology with Ras, and fifty-eight percent homology with Rho (71). Rac1 is ubiquitously expressed, whereas Rac2 is found only in cells of hematopoietic lineage (71). The next Rac branch member to be identified was RhoG. RhoG was found in a screen for late-induced genes following serum stimulation of hamster fibroblasts (72). RhoG is closely related to the Rac1 and Rac2 proteins and is ubiquitously expressed in adult human tissues (72). The fourth member of the Rac branch, Rac3, was identified in a search for homologues of Rac1, and it was found to have 77% identity to Rac1, 83% identity to Rac2 and 69% homology to RhoG (73). Rac3 seems to be expressed mainly in the brain, as well as the heart, placenta and pancreas (73). Rac1, 2, 3 and RhoG can all be prenylated with a geranyl-geranyl group which leads to plasma membrane localization (74).

The first major role of Rac1 was its involvement in the regulation of the actin cytoskeleton. Rac1 was shown to induce lamellipodia and membrane ruffling, and it was shown to be responsible for mediating the membrane ruffling downstream of growth factor receptor stimulation (75). Rac2 has also been implicated in control of actin structures (76, 77), as have Rac3 (78), and RhoG (79). It was initially thought that the ability of Rac1 to induce cytoskeletal changes was due to the activities of PAK (P21-activated kinase), a major effector of Rac1; however, numerous studies subsequently demonstrated that PAK activation by Rac1 is not necessary for Rac1 to induce cytoskeletal changes (80-82). The major effector of Rac1 in its regulation of actin structures is the WAVE protein. Active Rac interacts with SRA-1, and together these proteins bind WAVE, releasing inhibition caused by other WAVE interacting partners. This leads to changes in the conformation of WAVE allowing it to interact with Arp2/3,

resulting in actin polymerization (83). During cell motility, Rac1 will activate Scar1 and WAVE at the leading edge of the cell leading to ARP2/3 activation. This leads to actin-driven membrane protrusion (84). Rac1 activation is also central to the initial stages of focal complex assembly, which may help in anchoring the cell to the matrix, thus providing stable anchoring points for the actin cytoskeleton, which help in generating the forces necessary to protrude the membrane (84). In addition to its effects on WAVE, Rac1 stimulates the dissociation of gelsolin from actin. Gelsolin severs actin filaments, and stays bound to the barbed (fast-growing) end, preventing actin monomers from binding to this end. By promoting the dissociation of gelsolin from the actin filaments, Rac1 helps induce rapid addition of actin monomers to the barbed ends of actin filaments (85). Rac1 can also activate LIM kinase which phosphorylates and inhibits cofilin, preventing it from destabilizing actin filaments (86). Interestingly, there is evidence that the actin cytoskeleton can influence the amount of Rac1 in a cell, as disruption of the actin cytoskeleton with depolymerizing agents, such as cytochalasin B, leads to a decrease in Rac1 mRNA (87).

Although they are not necessary for Rac1 to control actin cytoskeleton dynamics, the PAK proteins are a major set of effectors of Rac1. The 6 PAK proteins are serine/threonine kinases, and are broadly classified into two sets: type I (PAKs1, 2 and 3) and type II (PAKs 4,5 and 6) (88). Active Rac1 binds and activates type I PAKs. Type I PAK proteins have a C-terminal kinase domain and an N-terminal RhoGTPase binding domain, as well as an auto-inhibitory domain. The auto-inhibitory domain binds the C-terminal of PAK, leaving the kinase domain inaccessible. Active Rac binds PAK, and prevents the auto-inhibitory domain from interacting with the C-terminal domain,

thus releasing the inhibition (89). Active Rac seems to only bind weakly to PAK 4, but does bind PAKs 5 and 6; however, binding of activated Rac to the type II PAKs does not seem to change their activity (88). This may be due to the fact that type II PAKs do not have an N-terminal autoinhibitory domain (88). Some substrates of PAK include Lim kinase, which is phosphorylated and activated by PAK (90). Some other roles of PAK in the control of the cytoskeleton are mediated by its interactions with filamin, an actin cross-linking protein that allows cross-linking of orthogonal filaments (91). The interaction of PAK filamin is crucial to its role in promoting lamellipodia formation (92). PAK can also phosphorylate and inhibit myosin light chain kinase, which leads to a decrease in acto-myosin contractility (93). p41-Arc, part of the Arp2/3 complex is another cytoskeletal-related substrate of PAK, and its phosphorylation by PAK is important for its correct localization to the Arp2/3 complex (94). PAK is also involved in various signal transduction cascades, being able to activate JNK, P38Map kinase and NF-κB pathways, as well as synergizing with Raf to activate ERK signaling pathways (95, 96).

Another role of Rac is in the regulation of NADPH oxidases. These oxidases serve to produce oxygen free radicals that are used for host defense, as well as, in small quantities, to signal for growth and transformation (97). Active Rac is involved in assembling a functional NADPH complex at the plasma membrane (97). Rac is also involved in cell survival and seems to play dual roles, depending on the situation. Rac can activate Bcl-2, a pro-survival protein, and Rac activity can lead to phosphorylation and inactivation of Bad, a pro-apoptotic protein. At the same time, there is some evidence that overexpression of Rac can also lead to apoptosis in some cell types (98).

Although activating mutations are a common mechanism in tumorigenesis mediated by Ras proteins, these mutations are rare in Rho subfamily GTPases. Rac may be the exception, as mutations within the Rac genes are found in some cancers; however, the role of these mutations is still unknown (99-101). The levels of Rac proteins are upregulated in many cancers, as are positive regulators (GEFs) of Rac (98). Aside from its roles in promoting cell proliferation and survival, and its roles in cell motility, Rac also governs further aspects of cancer, including invasion and metastasis. Rac has been shown to upregulate metalloproteinases such as MMP-2 (102). Under the control of VEGF, Rac can control vascular permeability which allows for metastasis to occur (103), and Rac also can stimulate angiogenesis through the production of reactive oxygen species by NADPH (104). Thus, Rac is involved in various stages of cancer, from tumorigenesis to angiogenesis and metastasis.

1.2.3 The Cdc42 Related GTPases

Cdc42 (Cell division cycle 42) was originally identified in the yeast *Saccharomyces cerevisiae*, in a screen for temperature-sensitive mutants that affect the cell cycle. At the non-permissive temperature, Cdc42 mutants were unable to form buds, yet they were still able to synthesize DNA, and nuclear division could occur (105, 106). Subsequent studies performed by generating a deletion of Cdc42 in the yeast confirmed that Cdc42 is an essential gene (44). It was found that the amino acid sequence of Cdc42 was 30% identical to human H-Ras and 53% identical to Rho (44). At the same time, a 25kDa GTP-binding protein, G25K, was purified from human placental and platelet membranes (107). Sequencing of G25K showed the predicted protein to be 80%

identical to yeast Cdc42, and 50% and 70% identical to Rho and Rac, respectively (108). G25K was able to rescue temperature-sensitive Cdc42 mutants in yeast, and was thus judged to be the human homolog of Cdc42 (108, 109). Cdc42 is found in all tissues in the mouse and in humans (110-112).

The role of Cdc42 in mammalian cells began to be elucidated when it was found that the EGF receptor was capable of stimulating phosphorylation of a small molecular weight GTPase that was subsequently identified as Cdc42 (113). Although the role of the phosphorylation was at the time unclear, it was noted that the phosphorylation was most efficient when the GTPase was devoid of guanine nucleotides (113) . Later studies have shown that EGF stimulates the activation of Src which then phosphorylates Cdc42. Phosphorylated Cdc42 is then able to interact with RhoGDI; this interaction is important for the transforming activity of Cdc42 (114, 115). Perhaps the first known functional role for Cdc42 in mammalian cells came from studies with the Dbl protein, a known oncogene, which was able to specifically stimulate the exchange of GDP for GTP of Cdc42. Dbl thus became the first GEF of Cdc42, and at the same time, this became the first example of an oncogene regulating a small GTPase (116). As the closest relatives of Cdc42 at the time, Rac and Rho were both involved in regulation of actin cytoskeletal structures, Cdc42 was tested for its ability to induce changes in the actin cytoskeleton, and it turned out that Cdc42 was able to stimulate filopodia and microspike formation (117, 118). From Cdc42, this branch of the Rho subfamily has expanded to a total of five members, including Cdc42, as well as TC10, TCL, Wrch1 and Wrch2 (47).

TC10 was identified in a screen for Ras-related genes in human teratocarcinoma (TC) cells (119), and TC10 mRNA is found in human heart and brain (111). In general,

TC10 has similar functions and binds similar regulators and effectors as Cdc42. Some notable exceptions include its inability to interact with WASP, and the fact that the membrane protrusions that it stimulates are generally longer and thinner than those produced by Cdc42 (111). TCL (TC10-like) was identified in a database screen of human and mouse ESTs looking for proteins with homology to the Rho subfamily GTPases (120). Murine TCL is expressed in the heart, lung and liver (120). TCL differs from TC10 and Cdc42 in regulation of actin structures, as it does not seem to induce filopodia, but rather forms ruffle-like protrusions on the dorsal end of cells (120).

Wrch-1 (Wnt-responsive Cdc42 homolog 1) was identified in a screen looking for proteins upregulated by sustained Wnt-1 activation (121). It shares 57% amino acid identity and 70% amino acid identity or similarity to Cdc42, and 30-55% similarity with other Rho subfamily GTPases. Unique for a Rho subfamily GTPase, its N-terminal region contains a polyproline region with several putative SH3 binding motifs (121). Binding of SH3 domain containing proteins, such as Grb2 to the polyproline region of Wrch-1 leads to increased interaction of active Wrch-1 with effectors (122). Wrch-1 is upregulated in mouse mammary tumors driven by Wnt-1, and is not present in normal mouse mammary tissue (121). In human tissues, Wrch-1 mRNA is ubiquitously expressed with the highest expression in brain, muscle, and placenta, and moderate expression in the liver, heart and lungs (121). Wrch-1 binds common effectors of Cdc42, and like Cdc42, can induce the formation of filopodia (121). Expression of Wrch-1 in cells can lead to increased cell proliferation, and, in fact, expression of Wrch-1 mimics the proliferative, tumorigenic effects of increased, sustained, Wnt-1 signaling (121).

Wrch-2, also known as Chp (Cdc42 homologue protein), was initially identified in a screen for interacting partners of PAK using a rat cDNA library (123) as Chp, and was also characterized from a bio-informatics screen for human proteins as a GTPase with homology to Cdc42 and Wrch-1 (124). Wrch-2 has 55.4% identity to Wrch-1 and 43.5% identity to Cdc42. Wrch-2, like Wrch-1, has an N-terminal polyproline region with putative SH3 binding sites, and the C-terminus of Wrch-2 does not contain the prenylation sites found in most Rho subfamily GTPases (123). Wrch-2 mRNA expression is high in the pancreas, placenta and brain, and is frequently increased in various human cancers, such as gastric, pancreatic, cervical and lung cancers (124). Wrch-2 is able to interact with effectors common to other Cdc42 branch members; however, expression of Wrch-2 elicits lamellipodia as opposed to filopodia (123).

Cdc42 and its related branch members are all involved in the control of actin structures. The ability of Cdc42 to control actin is most often linked to the activation of WASP. WASP proteins have an N-terminal GTPase binding domain, and a C-terminal VCA domain which binds both monomeric actin and Arp2/3 (125). The VCA domain of inactive WASP is bound to the GTPase-binding domain, keeping the protein in a closed conformation where it is not able to interact with Arp2/3. When active Cdc42 binds WASP, it displaces the VCA domain from the GTPase binding domain, allowing it to interact with and activate Arp2/3 (125). Recent evidence indicates that filopodia formation may also be mediated by formins (126, 127). Formins are capable of both nucleating actin, as well as extending actin filaments by binding to the barbed end and aiding in the addition of actin monomers (128). They consist of an N-terminal GTPase binding domain and a C-terminal diaphanous autoregulatory domain which flank the two

central formin homology domains (129). In the inactive protein, the autoregulatory domain is bound to the GTPase binding domain, preventing the formin homology domains from catalyzing actin filament extension. Much like the case of N-WASP, Cdc42 can bind the formin, thus displacing the autoregulatory domain, and leaving the formin domains in an active conformation (129).

In mammalian cells, the predominant localization of Cdc42 is at the Golgi apparatus (130), and this is due to its interaction with a subunit of the coatamer complex which localizes to Golgi membranes. (131). Cdc42 binding to the subunit of COPI blocks the ability of the microtubule motor dynein to bind COPI vesicles, and thus blocks microtubule-mediated transport of COPI vesicles in processes such as ER to Golgi transport (132). Cdc42 has been implicated in both endocytosis (133) and exocytosis (134), with both processes being dependant on the ability of Cdc42 to induce changes in the actin cytoskeleton. Other members of the Cdc42 family are also involved in membrane trafficking, such as TC10, which is involved in the transport of the GLUT4 receptor from intracellular membranes to the plasma membrane (135), and TCL which is involved in the trafficking of endocytosed vesicles to the early endosomal compartments (136).

Cdc42 has been demonstrated to be involved in the establishment of cell polarity. Cdc42 plays a role in the formation of adherens junctions during the formation of polarized epithelial sheets (137). Cdc42 is also involved in actin cytoskeleton changes and Golgi apparatus repositioning for more efficient secretion to the site of a T-cell synapse with an antigen presenting cell (138). Similar events are seen in healing of a scratch wound in a sheet of astrocytes, where, through the activation of the Par6/PKC

complex, Cdc42 activation leads to repositioning of the microtubule organizing center and Golgi apparatus to face perpendicular to the scratch site, and to direct membrane protrusions and cell migration, also perpendicular to the scratch site (139).

Like Rho and Rac, Cdc42 is able to transform cells. Although evidence had already existed for a mechanism whereby Dbl transforms cells through Cdc42 (116), the first direct evidence of the transformation capabilities of Cdc42 came from transfection of cells with constitutively active Cdc42, which was able to transform cells (140). Cdc42, through its activation of PAK participates in various signaling pathways which can contribute to cell proliferation, such as the JNK and P38 Map kinase pathways (141). Cdc42 has been shown to be upregulated in various breast cancers (142), and this may be due to the ability of Cdc42 to regulate the activity of c-cbl, leading to increased EGF receptor levels (143). Blocking Cdc42 activity has also been demonstrated to block the ability of v-Ras to induce phenotypes such as focus formation and anchorage-independant growth in NIH 3T3 cells, indicating that Cdc42 plays an essential role in Ras mediated transformation (144).

1.2.4 The RhoBTB GTPases

The founding members of the RhoBTB branch were originally identified in a cDNA screen of *Dictyostelium discoideum* looking for Ras related genes. RacA was isolated and found to be 74% identical to human Rac1, 62% identical to yeast Cdc42, 53% identical to rat RhoB, 58% identical to human TC10, and 64% identical to human RhoG (145). RacA does not contain C-terminal prenylation motifs, which are characteristic of Rho subfamily GTPases, but does contain two C-terminal BTB domains,

which are involved in hetero or homotypic interactions with other BTB domain-containing proteins (146). Three homologues of RacA were identified in humans, and named as RhoBTB1, 2 and 3 (146). All three RhoBTB proteins, as well as RacA possess a proline-rich region between the GTPase domain and the BTB domains, which may bind SH3 domain containing proteins (146). The GTPase domain of RhoBTB3 diverges from the other RhoBTB proteins, and from Rho subfamily proteins in general, and may not be a functional GTPase (146). Human RhoBTB1, 2 and 3 are ubiquitously expressed (147). RhoBTB2 was also identified as a tumor suppressor gene that was deleted in 3.5% of breast cancer cases, and is also called DBC2 (deleted in breast cancer 2) (148). Its role as a tumor suppressor may be mediated by its ability to downregulate cyclin D1 (149). RhoBTB2 has been shown to have its mRNA levels downregulated in bladder cancers (150). RhoBTB proteins also have roles in ER to Golgi transport, as has been demonstrated with RhoBTB2 (151). Contrary to the other Rho subfamily members, the RhoBTB proteins do not seem to have a role in actin re-organization (147).

1.2.5 The Miro GTPases

The Miro (Mitochondrial Rho) proteins were identified in searches of the human genome for additional Rho proteins. There are two Miro proteins, Miro-1 and Miro-2, which are 60% identical to each other (152). The Miro proteins have an N-terminal GTPase domain, which is related to the GTPase domains of the Rho subfamily, but, interestingly, they also possess a C-terminal GTPase domain which is not related to Rho GTPases. The Miro proteins lack C-terminal prenylation motifs, but do possess a C-terminal transmembrane domain (152, 153). Both Miro proteins are ubiquitously

expressed in humans (152). The Miro proteins were both found to co-localize with mitochondrial markers, and constitutively active Miro causes collapse of the mitochondrial network. Truncation of the C-terminal transmembrane domain abolishes the targeting of Miro to the mitochondrial membrane (152, 153). The effects of Miro on mitochondria may be through the proteins GRIF-1 and OIP106, both of which transport mitochondria along microtubules by binding to kinesin. Miro has been shown to interact with both of these proteins (153). Constitutively active Miro also induces caspase-mediated apoptosis, possibly through its perturbation of the mitochondrial network (152). Miro proteins do not seem to have any effects on the actin cytoskeleton (152).

1.2.6 The Rnd GTPases

The Rnd branch of the Rho subfamily derives its name from the discovery that Rnd1, when overexpressed, causes cells to round up (154). The first identified member of the Rnd family was RhoE, which was identified in a yeast two-hybrid screen looking for interacting partners of p190RhoGAP, a negative regulator of multiple Rho subfamily GTPases (155). RhoE shared 54% identity with RhoA, B and C, 46% identity with Rac1 and Rac2, 43% identity to Cdc42, and 27% identity with Ras (155). RhoE is GTPase deficient and incubation with a GTPase-activating protein known to interact with RhoE, p190RhoGAP, does not stimulate GTP hydrolysis, nor does incubation with concentrated whole cell extracts from a variety of tissues (155). When certain residues are mutated in RhoE to more closely match known GTPases, GTPase activity is conferred upon RhoE (155). Also, peculiar to other RhoGTPases, RhoE is farnesylated (most Rho proteins are geranylgeranylated) at its C-terminal region (155). RhoE, Rnd1, and Rnd2 share from 54

to 63% identity with each other, and RhoE was re-named to be Rnd3 due to its homology with Rnd1 and Rnd2 (154). When overexpressed in fibroblast cells, Rnd1 causes a dramatic loss of actin filaments, and causes cell rounding. Rnd3 acts in a similar fashion, whereas Rnd2 does not have any apparent effects on the actin cytoskeleton in fibroblasts (154). In neuronal cells, Rnd2 can affect neurite branching through its effector Rapostlin, which is able to bind both microtubules and N-WASP (156, 157). The effects of Rnd1 on actin are dependant upon it being in an active, GTP-bound state, and being prenylated (154). Rnd3 has been shown to inactivate the RhoA effector ROCK, and this inactivation blocks ROCK-induced stress fibre formation (158). Interestingly, it has also been shown that ROCK can phosphorylate Rnd3, leading to stabilization, and activation of the protein; Rnd3 devoid of this phosphorylation does not inhibit stress fibre formation (159). Unlike Rnd3, expression of Rnd2 in fact seems to stimulate ROCK pathways. Rnd2 activates its effector Pragmin, which is able to activate RhoA, leading to ROCK-mediated cell contraction (160). As with many other Rho subfamily members, the Rnd proteins have roles in cell proliferation. Rnd3 expression has been demonstrated to be induced by DNA damage, and leads to a cell cycle block that is upstream of the retinoblastoma checkpoint. Rnd3 expression is sufficient to block Ras-induced transformation of cells (161).

1.2.7 RhoD and Rif

RhoD was first identified through a PCR screen of mouse kidney cDNAs to find homologues of Rho and Rab proteins (162), and was also isolated as RhoHp1 in a screen of human placental cDNAs (163). RhoD shares 50 to 53% homology with other members

of the Rho GTPases, though more closely with RhoA than with Rac1 or Cdc42 (163). Expression of RhoD in a variety of cell lines leads to long, thin, actin-rich processes, both attached and motile, protruding from the plasma membrane, that are distinct from those elicited by Cdc42 and Rac expression (164). Inside the cell, RhoD is localized to the plasma membrane as well as to early endosomes (164). Overexpression of constitutively active RhoD leads to a change in shape of the endosomes, from having tubular extensions to becoming spherical. This is caused by a reduction in endosome motility, leading to decreased homotypic fusion of endosomes (164). The decrease in mobility of the endosomes is dependant on Hdia2C, a formin which is activated at the early endosome by RhoD (165). Constitutively active RhoD can also stimulate the dismantling of actin stress fibres and focal adhesions, and reduces cell motility (166). In addition RhoD is able to cause cell division defects in dividing *Xenopus* embryos; however, it does not affect nuclear division, implying that it may be blocking cytokinesis (166).

Rif (Rho in filopodia) was originally identified in a screen of human ESTs for homologues of Rho. Rif is 49% similar to Rac2, 48% similar to RhoD, 47% similar to RhoA and 43% similar to Cdc42 (167). When wild-type or constitutively active Rif is overexpressed in HeLa cells, the cells gain a hairy appearance due to the numerous filopodia extending from the cell (167). Furthermore, Rif is localized inside the filopodia, unlike Cdc42 (167, 168). The formation of filopodia by Rif is dependant on the ability of Rif to activate its effector mDia2 (169). Rif overexpression also causes a slight increase in the amount of actin stress fibres (167). The ability of Rif to control actin cytoskeleton reorganization is not dependant on Cdc42, nor on the activity of WASP, and likewise, Cdc42 induced filopodia are not blocked by expression of dominant

negative Rif, indicating that the two GTPases may use distinct mechanisms to induce filopodia formation (167).

1.2.8 RhoH

RhoH was initially identified as TTF (translocation three-four), in attempts to identify genes affected by chromosomal abnormalities in non-Hodgkin's lymphoma (170). RhoH has 43% identity with Rac1, 44% identity with RhoB and 45% identity with Cdc42, and is only expressed in hematopoietic cells (170). RhoH is also found in regions of chromosomal abnormalities in multiple myelomas (171), and RhoH is frequently mutated in lymphomas (172). RhoH expression does not alter the actin cytoskeleton (173). RhoH inhibits NF-κB signaling by preventing the degradation of the NF-κB inhibitor IκB, and is also able to block Rac and Cdc42 induced P38 MAP kinase signaling (173). RhoH does not seem to possess GTPase activity (173). RhoH can be regulated at the transcriptional level, as has been shown by stimulation with the phorbol ester PMA , which leads to a decrease in RhoH mRNA levels (173). PMA is a known mitogen and tumor promoting agent (174, 175). Since the activity of RhoH seems to oppose that of other Rho subfamily members, particularly by blocking certain signaling pathways leading to cell proliferation, it is likely that the roles of RhoH in various cancers may be as a tumor suppressor which is inactivated by mutations or chromosomal abnormalities.

1.3 The Guanine Nucleotide Exchange Factors (GEFs)

The Rho GEF proteins are broadly classified into two families: the Dbl Homology (DH) domain GEFs (DH-GEFs) and the DHR domain GEFs. There are 69 Dbl GEFs encoded in the human genome, and they are characterized by the presence of a DH domain in tandem with a PH (Pleckstrin Homology) domain (176). The DH domain is responsible for the GEF activity (i.e. for catalyzing the exchange of GDP for GTP), and the PH domain is important in the proper localization of the GEF to the plasma membrane (176). The DHR related GEFs comprise eleven GEFs encoded in the human genome, and are characterized by the presence of a DHR1 and a DHR2 domain (177). The DHR2 domain, which is also known as the Docker domain, is the domain responsible for the catalytic activity of these GEFs (177). Less is known about the function of the DHR1 domain, but it appears to be involved in localization of the GEF, and/or in regulation of the DHR2 domain (177, 178).

1.3.1 The Dbl Homology Domain Containing GEFs

The first GEF identified was the protein Dbl, which was identified as a proto-oncogene in diffuse B-cell lymphoma cells (179), which had no apparent similarity to other known (proto)-oncogenes (179). Dbl catalyzes the exchange of GDP for GTP, of Cdc42 and RhoA thereby activating these GTPases (116, 180). The region of Dbl involved in this catalysis is the same region of Dbl that is involved in transformation of cells, and is known as the DH domain. Dbl also contains a PH domain which is important for the oncogenic activity of Dbl (181). The PH domain is involved in localizing Dbl to cytoskeletal components, and the function of a defective PH domain

cannot be restored by the addition of a Ras prenylation sequence present to target Dbl to the plasma membrane (181). Dbl is found both in the cytoplasm, where it is inactive, and at the plasma membrane, where it is active. Upstream activators of Dbl can change its subcellular location from the cytosol to the plasma membrane (182). Dbl activation leads to the activation of ERK1 and ERK2, independant of an activation of Ras, possibly via Cdc42/PAK (183).

Shortly after the discovery of Dbl, other GEFs containing the Dbl homology domain began to be identified. These proteins are quite diverse and are regulated by a variety of mechanisms. Some GEFs are specific to one particular Rho GTPase, such as LARG which is a GEF for RhoA (184), or Tuba which is a GEF specific for Cdc42 (185). Other GEFs can activate multiple Rho GTPases, such as Vav and Ect2, which are both GEFs for RhoA, Rac1 and Cdc42 (186, 187), or Gef-H1 which is a GEF for RhoA and Rac1 (188). Trio possesses two GEF domains, one for Rac1 and RhoG, and another for RhoA activation (189, 190). The expression profiles of the different GEFs are variable; some GEFs like Gef-H1 (a GEF for RhoA and Rac1) are ubiquitously expressed (188), while others are more restricted in their expression, such as Vav, which is only expressed in hematopoietic cells (191).

Dbl homology GEFs often contain other domains which are involved in the function of the GEF. LARG (Leukemia Associated RhoGEF), a GEF for RhoA, possesses an RGS and a PDZ domain in addition to its DH and PH domains (184, 192). By means of the RGS domain, LARG is able to interact with heterotrimeric G proteins, leading to activation of LARG (184). LARG is also activated by activated IGF-1 receptor, which binds to and activates LARG through the PDZ domain of LARG (192).

Many Dbl homology RhoGEF proteins are regulated by intramolecular inhibition. This is seen for example with Vav, whose N-terminal region binds to the DH domain and blocks its access to GTPases (193). This inhibition can be released by tyrosine phosphorylation of the N-terminal region (193). Similar mechanisms are seen in other GEFs such as Ect2 and Sos (194, 195). GEFs can also be regulated by intermolecular inhibition, as is seen with Cool-2. Cool-2 can exist both as monomers and as dimers. When dimerized, Cool-2 acts as a GEF for Rac1. Monomeric Cool-2 is a GEF for both Rac1 and Cdc42; however, the monomeric form must be bound to PAK or to Cbl in order to have GEF activity (196). Another example of regulation of GEFs by proteins is seen with Gef-H1, which is negatively regulated by microtubule binding (188). Gef-H1 can thus act as a node between microtubules and actin cytoskeleton dynamics as regulated by RhoA and Rac1, which are both activated by Gef-H1 (188).

Post-translational modification of Dbl-homology GEF proteins can regulate their activity. Many GEFs are phosphorylated, and this can either enhance or inhibit their activity. Gef-H1 is negatively regulated by phosphorylation (197), while LARG, Vav, and Trio are all activated by phosphorylation (193, 198, 199). Ect2 is regulated by ubiquitination, which can either change the localization of Ect2 from perinuclear regions to axonal or dendritic processes in hippocampal neurons (200), or which can regulate levels of Ect2 by tagging it for degradation (200).

1.3.2 The DHR Domain Containing GEFs

In addition to the Dbl related GEFs, there are the DHR domain GEFs. The DHR2 domain is able to catalyze the exchange of GDP for GTP just like the Dbl domain (177).

The first DHR protein identified was DOCK180/ELMO. DOCK180 was known to be an upstream regulator of Rac1, but its mechanism was not understood, and it lacked any DH domains. It was found that the protein did in fact have GEF activity towards Rac1, and that the domain employed (DHR2) was common to other proteins (201). DOCK2, DOCK3, DOCK6 and DOCK9 (zizimin1) all contain this domain, and all these proteins, including DOCK180 also contain the DHR1 domain as well, which is important for membrane binding (201). DOCK2 is a GEF for Rac1, DOCK9 is a Cdc42 GEF, and DOCK7 may be a GEF for Cdc42 and RhoA (201). DOCK180 is activated downstream of integrin receptors to mediate Rac1 activation (202).

Although the aforementioned GEFs represent only a fraction of the GEFs it can easily be appreciated that they are a diverse family, that are regulated in a myriad of fashions, such as location with a cell type, location within the cell itself, regulation by intermolecular interactions or phosphorylation events that alleviate intramolecular inhibition. All of these mechanisms lead to a diverse array of options not only for the regulation of the GEFs themselves, but consequently for the regulation of the RhoGTPases in general.

1.4 The Guanine Nucleotide Dissociation Inhibitors (GDIs)

Guanine nucleotide dissociation inhibitors (GDIs) bind GTPases at the membrane and solubilize them, shuttling them to the cytosol. They are able to interact only with prenylated forms of the GTPase and lock the GTPase with the nucleotide present at the time of extraction, thus preventing exchange of GDP for GTP, or preventing GTPase

activity (203). RhoGDI was first discovered as a cytosolic binding partner of RhoB that prevented the exchange of GDP for GTP (204, 205). This GDI was found to bind only the GDP bound form of RhoB, and could bind it in the cytosol and prevent its transit to the membrane, as well as binding the membrane bound RhoB and removing it from the membrane (205, 206). In addition to being a GDI for RhoA and RhoB proteins, RhoGDI is also a GDI for Rac1 (207, 208). RhoGDI can bind to Cdc42-GDP, preventing its nucleotide exchange, but is also capable of binding to GTP bound Cdc42, preventing both its intrinsic and GAP-stimulated GTPase activity (209, 210). RhoGDI is also capable of binding to GTP bound Rac1 and RhoA, and preventing the GTPase activity of Rac1 as well as blocking it from GAP proteins (208, 211). As the family of RhoGDIs grew to three members, RhoGDI was renamed to RhoGDIα (212).

RhoGDIβ was originally identified as the gene D4 which is highly expressed in cells of lymphoid and myeloid origin and has high homology to RhoGDI (213), and it was also identified as Ly-GDI expressed in hematopoietic tissues (214). It was subsequently renamed as RhoGDIβ (212). RhoGDIγ can bind to both RhoA and Cdc42, and has been shown to be a GDI for Cdc42. It is expressed in the brain, pancreas, lung, kidney and testis, and inside the cell localizes to the cytosol, and to perinuclear regions and cytosolic vesicles (212, 215). RhoGDIα, which is ubiquitously expressed (205, 210), when deleted in mouse leads to defects in kidney function, and also defective male reproductive system development (216).

RhoGDIα, when in complex with RhoA is found in a phosphorylated state, and disruption of this phosphorylation dissociates RhoA from the RhoGDI (217). Phosphorylation mediated interaction of RhoA with RhoGDI can also have a protective

role for RhoA, rendering it resistant to ubiquitin-mediated proteasomal degradation (218). Phosphorylation of active, GTP-bound RhoA at the plasma membrane by PKA leads to the binding of RhoA-GTP to RhoGDI, and its subsequent sequestration to the cytosol, providing an alternate route to prevent effector binding to Rho-GTP (219). Interestingly though, phosphorylation of RhoGDI can act as a double-edged sword, as it also can promote dissociation of RhoGDI from RhoA, as has been shown downstream of BCR/Abl signaling, where RhoGDI is phosphorylated, leading to increased RhoA activation (220). This has also been observed in the case of Rac1, where phosphorylation of RhoGDIα by PKC likewise leads to a dissociation of Rac1 leading to Rac1 activation (221). PAK1 is also able to phosphorylate RhoGDIα and stimulate its dissociation from Rac1, leading to activated Rac1 (222). Phosphorylation of the GTPases can also affect the stability of the complex with RhoGDI. RhoA is phosphorylated by PKA (223), and this leads to an increased binding ability for RhoGDI, and Cdc42 is phosphorylated by both PKA and Src, both leading to increased ability to bind to RhoGDI (115, 223).

RhoGDI can be stimulated to dissociate from Rho proteins by protein-protein interactions, such as in the case of ERM proteins which can bind RhoGDI and cause its dissociation from RhoA, Rac1 and Cdc42 *in vitro*. *In vivo*, it seems that this may only lead to the activation of RhoA (224).

RhoGDI is involved in the transformation of cells by Cdc42. When a mutation is introduced into constitutively active Cdc42 that renders it incapable of binding to RhoGDIα, it is no longer able to transform cells (114). This may be due to altered localization of Cdc42, as with the RhoGDI binding defective mutation, Cdc42 only localizes to the Golgi membrane, and not to the cytosol or plasma membrane, where it is

also normally found (114). Thus, the RhoGDIs may play a role in shuttling GTPases to other locations within the cell.

The effect of RhoGDIs extends beyond modulators of Rho GTPase signaling. RhoGDIα is present in the nucleus as well as in the cytoplasm, and in the nucleus it is able to interact with and modulate the activity of estrogen receptor α (225). RhoGDIβ contains two caspase cleavage sites at its N-terminus, and the further of the two, when cleaved, leads to a loss of GDI ability (226). This form suppresses the metastatic ability of v-src transformed fibroblasts, and, in addition, enhances the adhesion of cells to fibronectin, laminin and collagens (226). The expression of this form of RhoGDIβ also promotes anoikis, and this could be part of its anti-metastatic mechanism (226).

There may also be as yet unclassified RhoGDI proteins that function as RhoGDIs, but with little to no homology with them. ICAP-1 inhibits intrinsic and GEF-mediated GDP for GTP exchange of both Rac1 and Cdc42 (227), and is able to remove Cdc42 from cell membranes, translocating Cdc42 to the cytosol (227). ICAP-1 does have some sequence homology to RhoGDI, and in its C-terminal region there is 34% similarity to the region of RhoGDI that binds the prenyl groups of RhoGTPases, with conservation of approximately 60% of critical residues for a RhoGDI-prenyl group interaction (227). It is unclear whether structurally ICAP-1 adopts a similar conformation to the prenylation binding site of RhoGDI (227, 228). Thus, ICAP-1 may be a distant relative of the RhoGDIs. P120 catenin is also able to bind to RhoA-GDP and prevent the dissociation of GDP, as well as blocking the ability of RhoGEFs to catalyze the exchange of GDP to GTP, and has no homology with the three RhoGDIs (229). Thus, like with the

RhoGEFs, there are other proteins with similar properties, but with different functional domains.

The RhoGDIs represent a family of Rho GTPase regulators with diverse functions, as they can act as inhibitors of the GTPases, shuttles to aid in their transport to different localizations in the cell, and they even can act to prevent degradation of the GTPases.

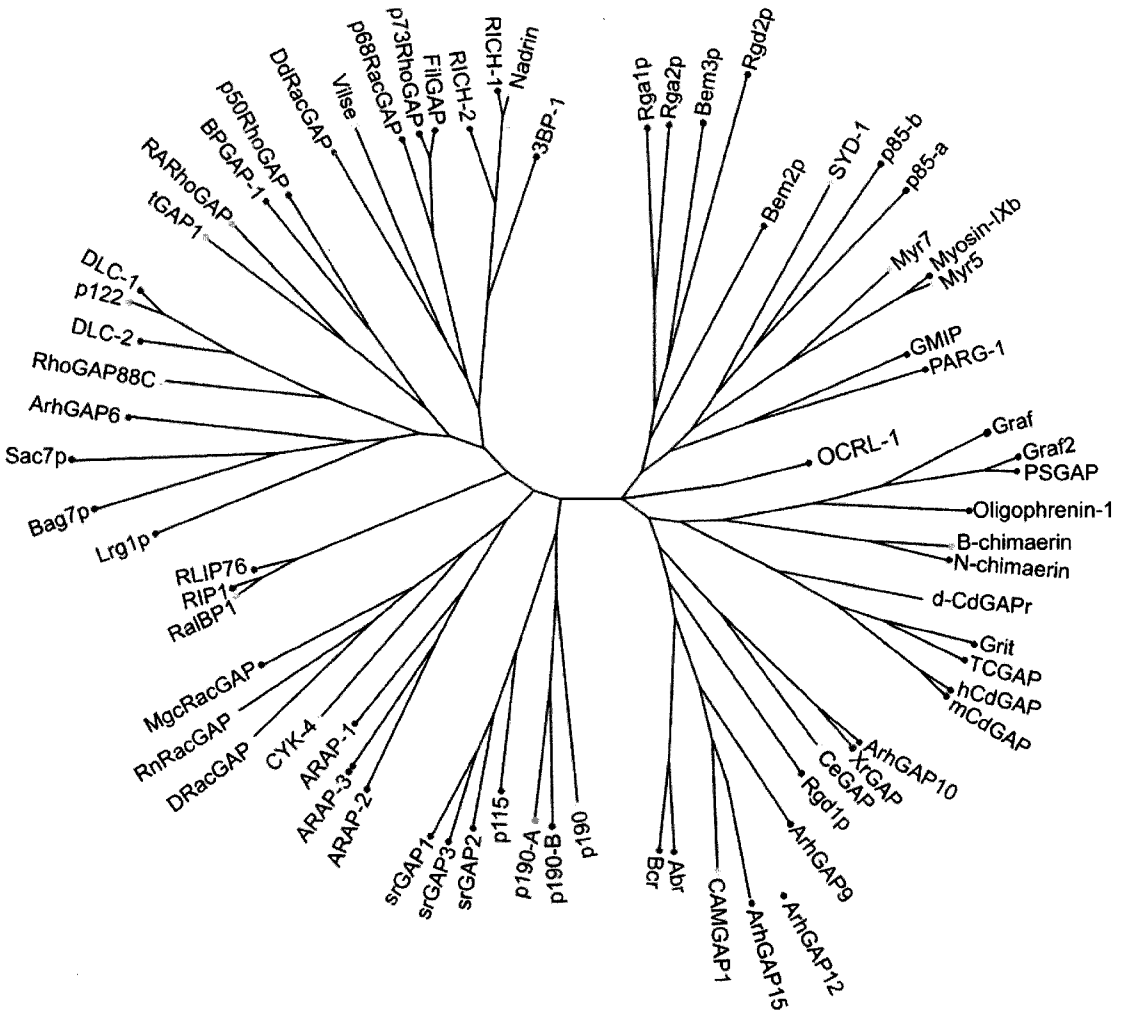
1.5 The GTPase-Activating Proteins (GAPs)

The Rho subfamily GTPase-Activating proteins (RhoGAPs) are a diverse family of proteins whose commonality lies in the presence of a RhoGAP domain, which stimulates Rho subfamily GTPases to hydrolyze GTP to GDP (Fig. 1.3). Thus, they are inactivators of the GTPases. The family consists of approximately 70 RhoGAPs encoded in the human genome (230). The RhoGAP proteins are regulated in a myriad of fashions, as is seen with the following RhoGAP proteins, which, although they only represent a small fraction of all the RhoGAPs, highlight the various roles and regulatory mechanisms of RhoGAP proteins.

p50RhoGAP

The first RhoGAP identified was p50RhoGAP, also known as Cdc42GAP, which was identified as a component in human spleen cytosolic extract capable of acting as a GAP for RhoA, Rac1 Cdc42 and TC10, but not for Ras (111, 231-233). p50RhoGAP is a

Figure 1.3: The RhoGAP proteins. Numerous RhoGAP proteins have been identified and characterized in a variety of species. Shown here is an unrooted tree based on the homology within the GAP domain of characterized RhoGAPs from a number of species. Colored dots are used to identify the species in which the GAP protein is found: Red, Homo sapiens; blue, Mus musculus; green, Rattus norvegicus; pink, S. cerevisiae; yellow, D. melanogaster; turquoise, C. elegans; orange, D. discoideum; purple, Gallus gallus; grey, Xenopus laevis. This figure is reproduced from Tcherkezian and Lamarche-Vane, 2006 (230).


Rgd2p
Bem3p
Rga2p
Rga1p
3BP-1
Nadrin
RICH-1
RICH-2
FilGAP
p73RhoGAP
p68RacGAP
Vilse
DdRacGAP
p50RhoGAP
BPGAP-1
RARhoGAP
tGAP1
DLC-1
p122
DLC-2
RhoGAP88C
ArhGAP6
Sac7p
Bag7p
Lrg1p
RLIP76
RIP1
RalBP1
MgcRacGAP
RnRacGAP
DRacGAP
CYK-4
ARAP-1
ARAP-3
ARAP-2
srGAP1
srGAP3
srGAP2
p115
p190-A
p190-B
p190
Abr
Bcr
CAMGAP1
ArhGAP15
ArhGAP12
ArhGAP9
Rgd1p
CeGAP
XrGAP
ArhGAP10
mCdGAP
hCdGAP
TCGAP
Grit
d-CdGAPr
N-chimaerin
B-chimaerin
Oligophrenin-1
PSGAP
Graf2
Graf
OCRL-1
PARG-1
GMIP
Myr5
Myosin-IXb
Myr7
p85-a
p85-b
SYD-1
Bem2p

50 kDa protein that is ubiquitously expressed in humans (234, 235). p50RhoGAP is able to bind equally well to the GTP and GDP-bound forms of RhoA, Rac1 and Cdc42 (235). In addition to its RhoGAP domain, p50RhoGAP contains a proline-rich sequence which can interact with the p85α subunit of PI3 kinase (235), and with c-Src (236). p50RhoGAP is inhibited by interactions between its N-terminal and its C-terminal GAP domain. The N-terminal region is also involved in recognizing the prenyl group of small GTPases, and, when in contact with this group, it is no longer able to inhibit the C-terminal GAP domain. Indeed, removal of this N-terminal region greatly increases that ability of p50RhoGAP to act as a GAP for non-prenylated Rac-1, whereas there is no effect on prenylated Rac1 (237). Analysis of p50RhoGAP-deficient mice implicates p50RhoGAP in apoptosis. The mice had 25 to 40% decreases in body and organ sizes compared to wild-type mice, and this was due to decreased cell number, due in turn to increased apoptosis (238). The MEFs of p50RhoGAP deficient cells have elevated Cdc42-GTP levels, but no increase in RhoA-GTP or Rac1-GTP. The increased Cdc42 activity leads to an increase in PAK1 activity, and JNK activity, but not ERK1/2, or P38 MAPK. The elevated Cdc42 and JNK activity is also observed in various tissues of the p50RhoGAP deficient mice, and, since hyperactive JNK can lead to apoptosis, this is likely the cause of the phenotype (238). It has also been shown that p50RhoGAP is involved in aging, as a deficiency of p50RhoGAP leads to reduced population doubling of cells, less repair of DNA damage, and an accumulation of genomic abnormalities, with an increased activity of p53 (239). These phenotypes are mirrored by activation of Cdc42, and thus, it is likely that the effects of a lack of p50RhoGAP on these processes is due to the rise of active Cdc42 upon the loss of p50RhoGAP (239). p50RhoGAP may

also be involved in Waldenström Macroglobulinemia, a lymphoproliferative disorder, as p50RhoGAP protein expression is up-regulated greater than two-fold in Waldenström Macroglobulinemia tumor samples (240).

p190RhoGAP

P190RhoGAP is a ubiquitously expressed protein first identified as a binding partner of RasGAP (241, 242) that contains an N-terminal GTP-binding domain, a central region that is highly homologous with the glucocorticoid receptor repression factor (a transcriptional repressor), and a C-terminal GAP domain (242). P190RhoGAP is localized both in the cytoplasm and in the nucleus (242). P190RhoGAP is a GAP for RhoA, RhoB, Rac1, Rac2 and Cdc42, although it seems it is by far a stronger GAP for RhoA (233, 243). The GTP-binding domain of p190RhoGAP binds to GTP; however, binding of GTP does not seem to affect RhoGAP activities (244).

EGF stimulation of murine fibroblasts leads to phosphorylation of p190RhoGAP by c-Src (245-247), and this causes a change of p190RhoGAP location, from the perinuclear region to concentric arcs radiating away from the nucleus (245). This redistribution coincides with the dissolution of stress fibres and disruption of the actin cytoskeleton, and actin depolymerizing agents such as cytochalasin D lead to a similar redistribution of p190RhoGAP (245). Both before and during this relocalization, p190RhoGAP co-localizes with p120RasGAP (245). P190RhoGAP can interact with p120RasGAP, and this is regulated by tyrosine phosphorylation of p190RhoGAP, which is necessary for the interaction to occur (248). Binding of p190RhoGAP to p120RasGAP does not affect the activity of p190RhoGAP, and this interaction may

therefore be involved in localizing the activity of p190RhoGAP to particular locations in the cell (249). P190RhoGAP is a substrate of Src family kinases in both the developing and mature nervous system, and mice lacking p190RhoGAP have axon guidance and fasciculation defects (250), whereas overexpression of p190RhoGAP in N1E-115 cells leads to increased neurite outgrowth (250). Phosphorylation of p190RhoGAP can be dynamically regulated, and p190RhoGAP is a substrate for the protein tyrosine phosphatase PTP20 (251).

Overexpression of p190RhoGAP, or simply the GAP domain alone, can suppress v-Ras induced transformation, through suppression of c-Fos, which is upregulated by the Rho GTPases, and is an essential part of Ras-mediated transformation (252). Knockdown of p190RhoGAP or expression of a dominant negative GAP mutant leads to transformation of NIH 3T3 cells (252). P190RhoGAP may also have other roles in tumorigenesis, or metastasis, as has been shown with the activation of integrins in adherent LOX melanoma cells which leads to increased phosphorylation of p190RhoGAP co-incident with increased invasion of these cells. The phosphorylated p190 RhoGAP co-localizes with F-actin in the newly formed invadopodia (253). The p190RhoGAP gene resides in a region that is frequently deleted in oligodendrogliomas, and expression of p190RhoGAP in an inducible glioma model system leads to a decreased incidence of gliomas (254). Overexpression of p190RhoGAP in pancreatic cancer cells was able to reduce the invasive capacity of these cells, and when these cells were injected intrasplenically into the mouse, there was a reduced incidence of metastasis, further strengthening the notion of p190 as a tumor suppressor (255).

The level of p190RhoGAP can be regulated by ubiquitin-mediated degradation, and this occurs during late mitosis (256). P190RhoGAP overexpression results in multi-nucleated cells, abnormal positioning of the cleavage furrow, and unequal daughter cell partitioning (256).

P190RhoGAP is regulated by phospholipids. In the presence of phosphatidylserine, the GAP activity of p190RhoGAP loses activity towards RhoA, but, p190RhoGAP becomes a potent activator of Rac1 (257). Phosphatidylcholine and phosphatidylethanolamine both have no effect on the GAP activity of p190RhoGAP, whereas phosphatidylinositide and PIP_2 both affect the GAP activity in the same fashion as phosphatidylserine (257). Phosphatidic acid lowers activity towards RhoA, but does not have any effect on GAP activity towards Rac1 (257).

P190RhoGAP regulates transcription by binding to the serum-responsive transcriptional factor TFII-I and sequestering it in the cytosol. PDGF-stimulated phosphorylation of p190RhoGAP leads to the dissociation of TFII-I, freeing it to move to the nucleus where it leads to the upregulation of genes, such as c-Fos (258).

P190 RhoGAP, aside from mediating cross-talk between Ras and Rho pathways, can also mediate crosstalk within the Rho subfamily itself. The Rnd proteins activate P190 RhoGAP, increasing its activity towards RhoA (259). Tiam1/Rac1 signaling is also able to downregulate RhoA activity through activation of p190RhoGAP (260)

RhoGAPs are often thought to be potential targets to modulate activation states of GTPases without affecting the GTPases directly, and, in this regard, it been shown that chemically inactivating p190RhoGAP can lead to activation of the Rho GTPases it inhibits (261).

N-chimaerin

N-chimaerin was identified as a protein with C-terminal homology to the C-terminal region of BCR (the GAP domain), and N-terminal homology to the C1 regulatory domain of PKC (262). This latter domain is involved in binding to diacylglycerol and phorbol esters, and, indeed, n-chimaerin binds phorbol esters through this domain (263). N-chimaerin is specifically expressed in neurons (262) and functions as a GAP for Rac1 (232). The GAP activity of n-chimaerin is negatively regulated by the N-terminal region and removal of this region results in increased GAP activity (264). The presence of phosphatidylcholine and phosphatidylethanolamine has no effect on the GAP activity of n-chimaerin; however, both phosphatidylserine and phosphatidic acid are able to increase the GAP activity. The presence of lysophosphatidic acid (LPA) leads to a decrease in GAP activity (264). Interestingly, n-chimaerin is able to interact with all of the aforementioned phospholipids except for phosphatidylethanolamine, and thus binding alone does not lead to modulation of the GAP activity. N-chimaerin may also act downstream of GTPases, rather than solely upstream. Microinjection of N-chimaerin into fibroblasts and neuroblastoma cells induces lamellipodia as well as filopodia, and these can be blocked by dominant negative Rac1 and dominant negative Cdc42, respectively. The GAP activity of n-chimaerin is not necessary to induce these changes; however, their ability to bind Rac1 through the GAP domain must be intact (265).

GRAF

GRAF (GTPase regulator associated with FAK) was identified as a potential Focal adhesion kinase (FAK) binding partner. It is ubiquitously expressed with the highest expression in the brain, heart, liver and testis (266, 267). It is a GAP for RhoA and Cdc42 (266). GRAF also contains an SH3 domain through which it is able to bind FAK. GRAF is generally cytoplasmic; however, it does have some enrichment at both stress fibres and cortical actin (266). GRAF is phosphorylated downstream of EGF and NGF signaling, and, at least in the case of EGF, this may be mediated by ERK (267). Overexpression of GRAF in Swiss 3T3 cells causes dismantling of stress fibres and formation of long filopodia-like extensions; this is dependant on GAP activity. There is some evidence that GRAF can be point mutated in the GAP domain or contain an insertion leading to a premature stop codon, which leads to a lack of a GAP domain, in juvenile myelomonocytic leukemia (268). PKNβ is able to bind to the SH3 domains of GRAF through its proline-rich sequences, and, is able to phosphorylate GRAF *in vitro*. This could possibly modulate GRAF function, particularly downstream of active Rho, which has been shown to bind and activate the closely related kinase PKNα (269). GRAF expression is downregulated in acute myelogenous leukemia and in myelodysplastic syndrome through methylation of its promoter (270).

MgcRacGAP

MgcRacGAP (Male Germ Cell RacGAP), a GAP for Rac1 and Cdc42, was found in a search for binding partners for activated Rac2 (271, 272). MgcRacGAP is composed of an N-terminal myosin like coiled-coil domain, a cysteine-rich region, and an N-

terminal GAP domain (273). Unlike most GAPs which are generally cytosolic, MgcRacGAP is localized to the nucleus in interphase cells. The mRNA of MgcRacGAP is regulated by the cell cycle, and increases gradually as the cells progress through S phase, and peaks during the G2/M phase (273). During mitosis, MgcRacGAP accumulates at the mitotic spindle during metaphase, and then at the midbody during cytokinesis (273). The coiled-coil domain is necessary for localizing the MgcRacGAP to the midbody of cells (273). MgcRacGAP at the midbody is phosphorylated on serine by AuroraB, which also localizes to the midbody and is critical for proper cytokinesis (274) (275). Aurora B phosphorylates MgcRacGAP at multiple sites in the N-terminal basic region, the cysteine-rich domain, and in the GAP domain, but not in the coiled-coil region. Interestingly, this phosphorylation turns MgcRacGAP into a potent RhoA GAP (275, 276). Mutants of MgcRacGAP that are unable to be phosphorylated by Aurora B cause cytokinesis arrest (275). GAP defective mutants of MgcRacGAP also lead to cytokinesis arrest (194).

The GAP activity of MgcRacGAP is also regulated by protein-protein interactions. PRC1 (protein-regulating cytokinesis 1) co-localizes with MgcRacGAP throughout the cell cycle (276), and binds to the GAP domain of MgcRacGAP, as well as to an N-terminal basic region. PRC-1 binding to MgcRacGAP blocks GAP activity to Cdc42 (276). MgcRacGAP that has been phosphorylated by Aurora B is insensitive to PRC1, thus there is no inhibition of GAP activity towards Cdc42 (276). MgcRacGAP is able to interact with Ect2, a GEF for RhoA, Rac1 and Cdc42 (277, 278). This interaction occurs during early anaphase, and it seems that the binding of MgcRacGAP with Ect2 activates Ect2, allowing it to activate RhoA, which is necessary for cytokinesis to begin

(278). MgcRacGAP is essential to localize Ect2 at the central spindle and the cleavage furrow, and knockdowns of MgcRacGAP and Ect2 phenocopy each other (279).

MgcRacGAP has functions independant of its GAP activity, as MgcRacGAP overexpression leads to growth suppression and macrophage differentiation in a GAP activity independant fashion (271). MgcRacGAP also has anti-apoptotic roles in B-cells, that are GAP-activity independant (280).

MgcRacGAP is also involved in the regulation of transcription, as in the case of STAT5A, which upon IL-3 stimulation is translocated to the nucleus. This is dependant on its ability to bind MgcRacGAP, which co-translocates with STAT5A to the nucleus (281). Regulation of transcription factors by MgcRacGAP may be a common role, as it also interacts with and inhibits the activity of HIF-1α, the inducible subunit of the transcription factor HIF-1. MgcRacGAP binds HIF-1α, but does not alter its subcellular localization, nor does it affect protein levels; however, it does block its transcriptional activity (282).

FilGAP

FilGAP (Filamin A-associated RhoGAP) was identified as an interacting partner of the carboxyl terminus of Filamin A, a protein which crosslinks actin filaments and also binds to RhoA, Rac1 and Cdc42, as well as activators and effectors of these GTPases, such as Trio and PAK (283). It contains an N-terminal PH domain, a RhoGAP domain and a C-terminal coiled-coil domain. The mRNA of FilGAP is ubiquitously expressed, with the highest levels present in the kidney (283). FilGAP is a GAP for Rac1 and Cdc42, but not RhoG, RalA nor RhoA (283). FilGAP expression leads to decreased

activity of Rac1, and co-expression of FilGAP and Filamin A increase FilGAP's activity (283). Following EGF stimulation of HEK cells, both Filamin A and FilGAP accumulate at the lamellipodia, and this accumulation is dependant on FilGAP binding to Filamin A (283). Depletion of FilGAP or expression of GAP-deficient FilGAP leads to the formation of lamellipodia (283). FilGAP expression leads to decreased spreading on fibronectin (283). FilGAP is also involved in cross-talk between Rho subfamily members, and is phosphorylated by ROCK on 5 serines and 1 threonine, leading to enhanced activity of FilGAP towards Rac1 (283). Inactivation of Rac1 downstream of ROCK-activated FilGAP leads to circumferential blebbing (a consequence of inactivation of Rac), and this is reversible by addition of ROCK inhibitors (283).

DLC

DLC-1 (Deleted in Liver Cancer 1) was identified as a GAP for RhoA that was also able to bind to PLC-δ and activate its PIP2 hydrolyzing activity (284). It is frequently homozygously deleted in liver cancer (285). The mRNA of DLC-1 is ubiquitously expressed, as are the mRNAs of DLC-2 and DLC-3 (285-287). The three DLC proteins have a RhoGAP domain, along with a SAM domain and a START domain, and DLC-2 in addition has an ATP/GTP binding site (286-288). DLC-2 is a GAP for Rac1 and Cdc42 (286).

All three DLCs are downregulated in various cancers. DLC-1 is deleted in liver cancer and is downregulated in renal, uterine, prostatic and rectal cancers (285, 289). DLC-2 is also frequently deleted in liver cancer and is downregulated in lung, ovarian, renal, breast, uterine, gastric, colon and rectal tumors (286) (290). DLC-3 is

downregulated in kidney, lung, uterine, ovarian and prostatic cancers (287). Interestingly, DLC-3 is not downregulated in benign prostatic hyperplasia, and thus its downregulation may function as a marker for prostatic cancer (287). DLC-1 is also silenced in prostatic carcinomas where it is silenced by histone deacetylation and DNA methylation (289). DLC-2 expression suppresses Ras-induced transformation, and this effect is dependant on DLC-2 GAP activity (286). DLC-1 is also down-regulated during breast cancer metastasis, and expression of DLC-1 in transformed cells normally capable of efficient metastasis to the lung greatly reduces the metastatic capacity of these cells, bringing it down to the level of non-lung metastatic cells (291). Expression of DLC-1 in tumorigenic cell lines lacking DLC-1 leads to a decrease in tumorigenic capacity, and this is dependant on a functional GAP domain (292). The DLC proteins may have different roles in the cell, as some cancer cell lines are lacking DLC-1 but still express DLC-2, and forced expression of DLC-1 leads to reduced tumorigenicity (290).

p115RhoGAP

p115RhoGAP mRNA is found in hematopoietic cells, placenta, and lung, and at lower levels in the spleen, thymus and leukocytes (293). It contains a RhoGAP domain and an SH3 domain (293), and expression of p115RhoGAP leads to an abolition of stress fibres (293). p115RhoGAP is a GAP for RhoA (294). p115RhoGAP interacts with MEKK1 via p115RhoGAPs SH3 domain, but is not a substrate for MEKK1 (294), and the association of these two proteins does not seem to affect the catalytic activity of either (294). MEKK1 normally activates NF-κB, AP-1 and SRE, and the interaction of

MEKK1 and p115 RhoGAP leads to a reduction in activation of AP-1, and to a slight increase in activation of NF-κB, with no effect on SRE (294).

GMIP

GMIP (Gem interacting protein) is a GAP for RhoA (295). In addition to its GAP domain, GMIP also contains a cysteine-rich domain (295). A single nucleotide polymorphism in the promoter region of GMIP that leads to a decrease in promoter activity is significantly associated with major depressive disorder (296). Gem is a member of the Ras subfamily of small G proteins and regulates voltage gated calcium channels, and active Gem leads to an elongation of cells and a dismantling of stress fibres, due to Gem inactivating RhoA (297). This inactivation of Rho is mediated by GMIP (297).

The ARAPs

The ARAPs (ArfGAP, RhoGAP, Ankyrin repeat, Ras-associating (RA), and five PH domains) comprise three members, ARAP1, ARAP2 and ARAP3. ARAP1 and 2 mRNA is expressed ubiquitously in humans and ARAP1 is an ArfGAP towards Arf1 and Arf5 in a phospholipid dependant manner, and a RhoGAP towards RhoA and Cdc42 in a phospholipid independant manner (298). ARAP2 and ARAP3 are ArfGAPs for Arf6, and ARAP2 has no RhoGAP activity, while ARAP3 has RhoGAP activity towards RhoA (299-301). ARAP3 can bind to Rap1B and 2B, and Rheb via its Ras-associating domain, and GTP-bound Rap1B or 2B increase ARAP3 activity towards RhoA, while GDP-

bound forms have no effect. The presence of Rap has no effect on ArfGAP activity (299).

ARAP1 is localized to the Golgi apparatus, and during cell spreading on fibronectin, ARAP1 becomes diffusely spread throughout the cytoplasm (at about 4 hours), and after 12 hours is at the cell surface protrusions. By 24 hours, ARAP 1 is localized to the perinuclear region (298). ARAP1 expression negatively affects cell spreading, and this is dependant on both Arf and Rho GAP domains (298).

Overexpression of ARAP1 also induces peripheral membrane blebs which contain actin and a Golgi marker, and ARAP1 localizes to these blebs. The formation of these blebs is not dependant on RhoGAP activity, but is blocked by dominant negative Cdc42 (298). ARAP1 also causes an increase in filopodia formation, and this requires a functional ARFGAP domain (298). The expression of ARAP1 in NIH 3T3 cells also leads to decreased stress fibres and increased cell rounding, these effects being dependant on the RhoGAP domain.

ARAP3 is mostly localized to the cytosol, with a small proportion at the plasma membrane, and stimulation of PC12 cells with EGF leads to a translocation of some of the ARAP3 from the cytosol to the plasma membrane, whereas stimulation with PDGF leads to a translocation of a small proportion of ARAP3 from the cytosol to membrane ruffles. These translocations are dependant on active PI3K, and need the intact N-terminal PH domain (301). ARAP3 can block Arf6 induced membrane ruffling, and cause translocation of Arf6 from the cell periphery to an intracellular particulate compartment (301). This ability to block Arf6 is dependant on the ability to bind

phosphoinositides through its N-terminal PH domain, as well as on ArfGAP activity (301).

ARAP2 is able to interact with GTP-bound RhoA through its RhoGAP domain, (300), and ARAP2 may act as a RhoA effector. Expression of constitutively active RhoA leads to stress fibres, however, when ARAP2 is knocked-down, stress fibres do not form. Expression of ARAP2 can rescue this, but only if the RhoGAP domain is present (300).

ARAP3 is phosphorylated by Src and the Src family kinase Lyn (302). Both Src and Lyn can stably interact with ARAP3, and the sites of tyrosine phosphorylation between Lyn and Src seem to be different (302). Stimulation of cells with PDGF leads to ARAP3 phosphorylation, and EGF stimulation of LNCaP prostate cancer cells also leads to increased phosphorylation of ARAP3. Phosphorylation also increases during cell attachment to fibronectin (302). PI3K is involved in attachment-mediated phosphorylation, as are Src family kinases (302).

RA-RhoGAP

RA-RhoGAP (Rap-activated RhoGAP), was identified as a binding partner for active Rap1b (303). Expression of Rap1 leads to neurite outgrowth in a manner that is dependant on the downregulation of RhoA (303). In rat, RA-RhoGAP protein is expressed in the brain, liver and testis, and is localized in the cell body and at the growth cone of neurites in differentiated NG108 cells (303). RA-RhoGAP is a GAP for RhoA, and, very weakly, towards Rac1 and Cdc42. Ra-RhoGAP contains a PH domain, a Ras Association domain, a RhoGAP domain, and two ANXL repeats. When active Rap1B is added to GAP activity assays, the GAP activity of RA-RhoGAP towards RhoA is greatly

increased; inactive GDP-bound Rap1B increases GAP activity only very slightly (303). Removal of the RA-domain of RA-RhoGAP leads to a great increase in GAP activity, and thus, it is probable that binding of Rap1b to RA-RhoGAP relieves an intramolecular inhibition (303). Expression of RA-RhoGAP in undifferentiated NG108 cells leads to increased neurite outgrowth, and this is enhanced by co-expression with constitutively active Rap1b. These effects are dependant on RhoGAP activity. There is no effect on differentiated cells already bearing neurites (303).

Oligophrenin

Oligophrenin was identified as a gene involved in mental retardation (304). Oligophrenin mRNA is expressed in human fetal brain, kidney and lung, and in the adult placenta, pancreas, kidney, skeletal muscle and brain (304). Oligophrenin contains a RhoGAP domain, which has activity towards RhoA, Rac1 and Cdc42 (304). Oligophrenin-1 expression is absent in cells from patients with an X:12 translocation which causes mental retardation. Mutations in oligophrenin-1 are also linked to X-linked mental retardation with epilepsy, rostral ventricular enlargement, cerebellar hypoplasia (305), and to X-linked congenital cerebellar hypoplasia (306).

Oligophrenin is upregulated in colorectal tumors (307), and is also upregulated in gastric cancer cells that have developed resistance to vincristine, but not in non-resistant cells (308).

Oligophrenin contains a nuclear localization signal, and can be found in both the nucleus and the cytoplasm, (309). Oligophrenin is able to interact with F-actin directly,

and in neurons, it co-localizes with F-actin at the tips of neurites (310). In glial cells, oligophrenin co-localizes with cortical F-actin and with stress fibres (310).

In cultured mature hippocampal neurons, that have formed synapses, oligophrenin is localized in the cell body, and in puncta in axons, dendrites and dendritic spines (311). Expression of siRNAs for oligophrenin leads to a reduced number and length of spines on primary and secondary dendrites, but does not affect the density of spines, nor filopodia (311). Expression of constitutively active RhoA in hippocampal neurons leads to a similar phenotype as siRNA of oligophrenin, while constitutively active Rac1 has a different phenotype, of numerous lamellipodia-like protrusions. Constitutively active Cdc42 had no effect on spine morphology. Thus, it would seem that the effects of an absence of oligophrenin in dendritic spine morphology is probably due to a loss of its activity as a GAP for RhoA (311). Treatment of hippocampal neurons expressing oligophrenin siRNA with ROCK inhibitors leads to a rescue of the siRNA effects (311).

Grit

Grit (GTPase regulator interacting with TrkA) was identified in a yeast two-hybrid screen looking for interacting partners of n-Shc that has been activated by TrkA (312). Grit was also identified as p200RhoGAP (313), RICS (RhoGAP involved in the β-catenin-N-cadherin and NMDA receptor signaling) (314), and as p250GAP (315). Grit is a large protein, consisting of 1,783 amino acids, with an N-terminal RhoGAP domain, and a C-terminal proline-rich region. Grit mRNA has 3 transcripts, and it is expressed in human brain, testis, pancreas and colon, with the highest levels in testis and brain (312).

Grit is a GAP for RhoA, Rac1 and Cdc42; its activity being stronger towards RhoA and Cdc42 (312).

In neurite-bearing neuronal cells, Grit is distributed throughout the cytoplasm, and at the cell periphery, as well as at the tips of neurites (312). The C-terminal region interacts directly with the receptor TrkA, independantly of the phosphorylation of the receptor (312). Overexpression of either the GAP domain of Grit or of the C-terminal TrkA binding region blocks NGF-induced neurite formation in PC12 cells. Interestingly though, overexpression of the entire Grit protein does not block neurite formation (312). Expression of a GAP-activity deficient RhoGAP domain also has no effect on neurite outgrowth.

In unstimulated cells, there is a small association of Grit with the EGF receptor, and this is increased upon EGF stimulation. This is dependant on the ability of Grit to bind to N-Shc (312). EGF stimulation leads to increased tyrosine-phosphorylation of Grit; a process enhanced by N-Shc. This phosphorylation does not seem to affect the GAP activity of Grit (312). Grit is also phosphorylated by CaMKII, and this phosphorylation reduces the activity of Grit towards Cdc42 (314). Both Src and Fyn are able to phosphorylate Grit, and in the latter case this occurs during oligodendrocyte differentiation, although the effects of Src and Fyn phosphorylation on Grit GAP activity are unknown (313, 315). Grit can also be dephosphorylated, as is the case after NMDA stimulation (316).

Expression of Grit in NIH 3T3 cells leads to transformation. NIH 3T3 cells expressing Grit form foci, display anchorage independant growth, and cell clones expressing Grit in NIH 3T3 cells form secondary foci among NIH 3T3 cells (317). Grit

expressing cells also grow better in low serum conditions, reaching a saturation density almost twice that of control cells and these cells also have increased mitotic cell populations (317). The foci induced are similar to those induced by v-Ras, and, indeed, expression of Grit leads to an increase in Ras activity (317). During Grit-induced transformation, ERK activation is necessary, but PI3K does not seem to be needed beyond basal levels (317). Like p190RhoGAP, Grit is also capable of binding to P120RasGAP through the C-terminal region of Grit and the SH3 domain of p120RasGAP. This interaction is critical for the transforming activities of Grit (317). The RhoGAP domain of Grit is not necessary for transformation; however, Grit lacking the GAP domain, or Grit with a GAP-activity deficient mutation has reduced transforming ability (317). Both dominant negative RhoA and dominant negative Ras are able to greatly reduce Grit's transforming ability (317).

Grit is also regulated at the translational level by the microRNA miR132, whose expression is induced by CREB activation. miR132 inhibits the translation of Grit mRNA (318).

TCGAP

TCGAP (TC10/Cdc42 GTPase-activating protein) is a GAP for Cdc42 and Rac1, and to a lesser extent RhoA and TC10 (319). Interestingly, this activity seems to be exclusively *in vitro* (319). TCGAP can however co-immunoprecipitate with both active Cdc42 and TC10b, but not Rac1 or RhoA (319), and this is through the GAP domain. TCGAP binds Cdc42 in a GTP-dependant manner, and to TC10 in a GTP independant manner. TCGAP is normally found cytoplasmically and is translocated to the plasma

membrane in response to insulin stimulation (319). The N-terminal region contains a PX, an SH3 and the RhoGAP domain, and the C-terminal region contains most of the multiple PXXP (22 in total) motifs. TCGAP also contains 5 PEST motifs, which are generally found in proteins with short half-lives (319). The PX domain binds to phospholipids and this is necessary for the translocation of TCGAP to the plasma membrane. CrkII interacts with the C-terminal of TCGAP via CrkII's N-terminal SH3 domain, and stimulation of cells with insulin leads to increased interaction of CrkII and TCGAP (319). This interaction is important for the translocation of TCGAP to the plasma membrane (319).

Overexpression of TCGAP blocks glucose transport, thus, blocking the effect of insulin stimulation; this is mediated by blocking the translocation of GLUT4 to the plasma membrane (319).

NGF stimulation of PC12 cells leads to TCGAP phosphorylation (320), which is mediated by Src family kinases. TCGAP interacts with Fyn, through Fyn's SH3 domain (320), and Fyn can phosphorylate TCGAP leading to a suppression of GAP activity (320). TCGAP expression in PC12 cells suppresses NGF-induced neurite outgrowth, and the ability to do this is dependant on the GAP activity. A Fyn phosphorylation site mutant also blocks NGF induced neurite outgrowth (320).

As evidenced by the aforementioned GAPs, which constitute only a small fraction of all the RhoGAP proteins encoded in the human genome, these proteins form a diverse family of RhoGTPase regulators. Their commonality lies in the RhoGAP domain, but beyond that there is a great variety to the proteins and the domains they contain. This

allows for a great variety of regulatory mechanisms for the RhoGAP proteins, such as protein-protein interactions, ubiquitination, altered localization, phosphorylation, and phospholipid regulation to summarize a few, and some RhoGAPs act not only as regulators of GTPase signaling, but as effectors of GTPases as well. All of this allows the GAPs to function in particular contexts, and thus allows for regulation of the GTPases in specific contexts, without necessarily affecting the more general contexts of the GTPases.

1.6 CdGAP

CdGAP (Cdc42 GTPase-Activating Protein) is a RhoGAP protein that was identified in a yeast-two hybrid screen looking for interacting partners capable of binding to Cdc42 Y40C L61(constitutively active) Cdc42 (321), which has a mutation in the effector binding domain that prevents binding to PAK, and to WASP, but does not block filopodia formation (81). CdGAP is an 820 amino acid protein that has an N-terminal RhoGAP domain, followed by a serine-rich central region and C-terminal proline-rich region. The C-terminal third of the protein contains multiple proline rich motifs that are putative SH3 binding sites (321). CdGAP mRNA is ubiquitously expressed in the mouse, with the highest expression in heart and lung (321), and in the human fetus it is also ubiquitously expressed, with the highest levels in heart and muscle (322). In both humans and mice, CdGAP is a GAP for Cdc42 and Rac1 (321, 322). Downstream of EGF stimulation, CdGAP acts preferentially as a GAP towards Cdc42, although expression of CdGAP does seem to reduce active Rac1 in the absence of EGF stimulation (323, 324). CdGAP expression in cells leads to disruption of cortical actin, and cell

rounding, and eventual detachment from the plate, as well as to pseudopodial protrusions and cell blebbing (321-323).

CdGAP interacts with intersectin, an endocytic scaffolding protein. This interaction is particularly interesting, as although CdGAP interacts with intersectin via three of intersectin's five SH3 domains, SH3A, SH3B, and SH3D, these domains do not bind to the proline-rich region of CdGAP, and instead bind to the central region (325), which lacks classical putative SH3 binding sites. Both SH3A and SH3D are able to interact with CdGAP directly (325), and co-expression of CdGAP and intersectin prevents CdGAP from inhibiting PDGF-induced lamellipodia formation. Intersectin and CdGAP, when co-expressed, co-localize in the forming lamellipodia, whereas CdGAP when expressed alone is cytoplasmic/perinuclear (325). Intersectin's SH3D and SH3A compete for binding to CdGAP; however, only intersectin's SH3D domain is able to reduce CdGAP's activity (325). Interestingly, CdGAP lacking the PRD (which is not necessary for Intersectin binding) was still able to block PDGF induced lamellipodia formation, and the presence of intersectin does not affect this, indicating the importance of the PRD for intersectin-mediated regulation of CdGAP (325).

CdGAP is localized to focal adhesion sites in U2OS cells spread on collagen in the presence of serum, and co-localizes with paxillin. The localization of CdGAP at focal adhesions is more prominent in actively spreading cells, as opposed to cells that have been plated for an extended period of time (326). Stimulation of integrins by binding to collagen increases the GAP activity of CdGAP, with stimulation of CdGAP activity starting at 30 minutes and growing stronger until 150 minutes after plating of cells on collagen. Expression of wild-type CdGAP in U2OS cells leads to decreased cell

spreading at all time points (15, 30, 60 and 120 minutes) (326). Expression of CdGAP also inhibits the U2OS cells from becoming polarized and also reduces the level of active Rac in spreading cells, and this is GAP activity-dependant (326).

CdGAP can bind to actopaxin, a focal-adhesion protein which also binds F-actin, integrin-linked kinase and paxillin (326). Interestingly, actopaxin also binds to PIX, a Rac/Cdc42 GEF (326). Actopaxin binds to the central region of the short form of CdGAP, but it cannot bind the central region alone, and needs either the N-terminal region or the C-terminal region in addition to the central region, probably to stabilize the conformation of the central region (326). Reduction of actopaxin leads to increased cell spreading, and increased lamellipodia formation, and this can reverse the decreased spreading caused by overexpression of CdGAP (326). Actopaxin has no effect on CdGAP's GAP activity during cell spreading. Actopaxin does change the localization of CdGAP, and in cells overexpressing CdGAP, or CdGAP and actopaxin, CdGAP would localize to the cytoskeletal/focal-adhesion fraction one hour after the cells were plated on collagen. When CdGAP was co-expressed with actopaxin that could not bind CdGAP, CdGAP did not localize with the cytoskeletal fraction (326). Co-expression of CdGAP with actopaxin unable to bind CdGAP blocks the CdGAP induced phenotype, indicating a need for localized CdGAP activity (326). CdGAP expression also inhibits random motility of U2OS cells, and this also is in part mediated by the interaction of CdGAP with actopaxin (326).

1.7 The ERK Kinases and their involvement with the Rho Subfamily

1.7.1 The ERK Kinases

The ERK (extracellular-signal regulated kinases), also known as the MAP (microtubule associated protein-2) kinases, were originally identified as proteins that were phosphorylated after mitogen stimulation of cells (327). ERK1 and ERK2 are able to autophosphorylate on both tyrosine and threonine residues, and thus, are both tyrosine and serine/threonine kinases. This autophosphorylation does not seem to be sufficient to mediate full activation of the kinases, and other factors may be necessary (328). There are eight members of the ERK family, with ERK1 and ERK2 being the most studied. ERK1 and ERK2 (p44MAPK and p42MAPK, respectively) share 90% identity, and are both activated by dual phosphorylation of a tyrosine and threonine in their activation loops (329). ERK3-8 are less identical to ERK1 and 2 and less is known about their function (329).

ERK1/2 are proline-directed kinases that recognize substrates with the sequence Pro-X-Ser/Thr-Pro, where the first proline (-2 from the s/t) is somewhat conserved, and the proline (+1 from the s/t) is well conserved (330). There are more than 150 known substrates for ERK1/2, including transcription factors, other kinases, phosphatases, cytoskeletal proteins, signaling proteins, apoptotic proteins, and proteases (331). ERK1/2 activation is necessary for growth factor induced cell proliferation (332).

Classical activation of ERK downstream of growth factor receptors begins with the receptor becoming activated and auto-phosphorylated. These phosphorylated residues serve as docks for adapters which ultimately lead to the recruitment of Sos, a

GEF for Ras. Ras becomes activated by Sos, and then activates RAF (MAPKKK), which activates MEK1/2 (MAPKK), ultimately activating ERK (MAPK) (333). Various cell stimuli that act through G-protein coupled receptors are also capable of activating ERK, such as bombesin, endothelin-1, somatostatin and LPA (333). They can activate ERK through a variety of mechanisms, such as by activating Ras-GRF (Ras guanine nucleotide releasing factor) which stimulates Ras activation, or by activating growth hormone tyrosine kinase receptors which subsequently lead to Ras activation (333).

1.7.2 Crosstalk between ERK1/2 and the Rho Subfamily

Activation of Rac1 leads to activation of PAK, which can activate Raf, and can also lead to increased interaction of Raf and MAPKK. Through this mechanism, Rac1 leads to increased ERK activity (334). Mice lacking Rac1 have decreased levels of phospho-ERK1/2, with no changes in total ERK1/2 levels (335).

RhoA has been shown to prevent apoptosis through activation of ERK, and suppression of RhoA levels in zebrafish embryos leads to extensive apoptosis due to decreased ERK activation (336).

Activation of ERK downstream of ROCK activation seems to occur quite commonly, as is seen in migrating human aortic smooth muscle cells, whose velocity is increased by serotonin stimulation, and this is mediated through RhoA and ROCK, leading to ERK activation (337). ROCK is also involved in the activation of ERK downstream of CXCR4 receptor signaling after ligation of stromal-cell derived factor 1 (SDF-1) (338). RhoA activation also leads to activation of ERK in a ROCK-dependant fashion in glomerular epithelial cells stimulated with complement (339, 340). Likewise,

inhibition of ERK1/2 in rat intestinal epithelium overexpressing constitutively active RhoA attenuates the upregulation of COX-2 normally caused by overexpression of constitutively active RhoA (341).

Polyamine depletion of IEC-6 cells leads to decreased migration in a scratch-wound closure assay, and this is through inhibition of ERK (342). The decreased migration could be rescued by expression of constitutively active MAPKK. Polyamine depletion alters the actin cytoskeleton, and constitutively active MAPKK expression also rescues these defects. Control polyamine depleted cells have short stress fibres, and do not spread well, whereas polyamine depleted cells expressing MAPKK have normal lamellipodia and stress fibres, probably due to the activation of Rac1 and RhoA induced by MAPKK expression in these cells (342).

ERK is also activated by mechanical stretch, leading to Elk-1 activation, a transcription factor downstream of ERK1. Dominant negative Rac1, inhibition of RhoA by C3, or inhibition of ROCK all prevented activation of Elk-1 through modulation of the ERK pathway (343, 344).

Interestingly, active RhoA is able to directly bind to the N-terminal scaffolding region of MAPKKK, and this leads to as much as a 10-fold increase in MAPKKK activity. Aside from activating MAPKKK, RhoA binding to MAPKKK may also block MAPKKK from ubiquitinating other targets to signal them for degradation. RhoA binding to MAPKKK does not result in increased ubiquitination of RhoA (345).

RhoA activity can also inhibit ERK, as in cells treated with doxorubicin (which normally induces senescence), where RhoA activity activates the Ser/Thr phosphatase

PP2A, which dephosphorylates and inactivates ERK, leading to protection against doxorubicin induced cellular senescence (346).

Mouse ES cells lacking Cdc42 are defective in G1/S phase transition, and there is a reduced activation of ERK1/2 in response to serum (347). PPARgamma (peroxisome proliferator-activated receptor gamma) causes EMT in intestinal epithelial cells through activation of Cdc42, which activates PAK which activates ERK1/2 through MEK1 and MEK2 (348). Cdc42 can also inhibit ERK1/2 activity, as in human skin fibroblasts where Cdc42 represses MMP-1 expression by inhibiting ERK1/2 activity. It seems however that Rac1 may act antagonistically to Cdc42, and may activate ERK1/2 in these cells (349). In keratinocytes of the basal epidermal, layer Rac1 activity is also important for ERK1/2 phosphorylation (350). Rac1 is also upstream of ERK1/2 in cell proliferation of intestinal epithelial cells, which, when stimulated by cyclic strain, activate Rac1 leading to ERK activation (351).

1.8 The GSK-3 Kinases and their involvement with the Rho Subfamily

1.8.1 GSK-3

GSK-3 (Glycogen synthase kinase 3) exists as two major isoforms, α and β, as well as a third isoform, GSK-3β2, which is a splice variant of GSK-3β. GSK-3 has over 40 known substrates *in vivo*, and these proteins range in function from metabolic and signaling proteins, to structural proteins and transcription factors (352). GSK-3α and β have 85% overall sequence identity and 93% identity within the kinase domain (353). GSK-3 is well conserved; *Dictyostelium* GSK-3 is 70% identical to GSK-3β, and sea

urchin GSK-3 is 88% similar overall and 94% similar in the catalytic domain (353). GSK-3β2 contains a 13-amino acid insert within the kinase domain, which seems to reduce the activity of GSK-3 towards tau protein (354).

GSK-3 is a Ser/Thr kinase which recognizes the motif Ser/Thr-X-X-X-SerP/ThrP, whereby the first Ser/Thr is the GSK-3 target phosphorylation residue, and the +4 Ser/Thr must be phosphorylated for GSK-3 to recognize the motif and phosphorylate its target residue (355). Although X can be any amino acid, often one of these is a proline residue. This pre-phosphorylation is normally carried out by other kinases, such as casein kinase in the case of glycogen synthase, a substrate for GSK-3; however, the GSK-3 target Ser/Thr residue, once phosphorylated, could also serve as a priming phosphorylation for a closely located GSK-3 phosphorylation site (355). Also, it is possible for charged amino acid residues, such as glutamic acid, located at the +4 position to mimic a primed phosphorylation site, thus creating a "self-primed" substrate (356). This requirement for a primed substrate creates an interesting regulation mechanism for GSK-3, where, at its N-terminal region, it has a phosphorylation site, (Ser-21 for GSK-3α, Ser-9 for GSK-3β), which, when phosphorylated, occupies the site which would normally be occupied by the priming phosphorylation of the substrate, in such a fashion that it blocks substrate access to GSK-3 (355). This inhibition is competitive, and a high enough concentration of primed substrate can out-compete the autoinhibition (355).

GSK-3 is involved in many different signaling pathways in cells. One such pathway is the Wnt/β-catenin pathway, which results in the activation of the transcription factors T-cell factor/lymphoid enhancer factor (TCF/LEF). Wnt receptor activation leads to inactivation of GSK-3 which normally phosphorylates β-catenin and targets it for

degradation. This is due to a complex formed between GSK-3, axin, APC, and β-catenin. which allows for efficient phosphorylation of β-catenin. When Wnt receptor is activated, this complex is disassembled, possibly through the binding of FRAT1 to GSK-3, and this prevents the phosphorylation and degradation of β-catenin, which can then activate the TCF/LEF transcription factors leading to cell proliferation. Many cancers, such as skin, colon, prostate, liver, endometrial and ovarian have mutations in β-catenin that prevent it from being phosphorylated and degraded by GSK-3 (353, 355).

In resting cells, GSK-3 is generally active, and stimulation of cells with mitogens leads to inactivation of GSK-3 through the activation of PI3K, which in turn activates AKT. AKT then phosphorylates GSK-3 at Ser-21 or Ser9 (GSK-3α and β, respectively), leading to inactivation of GSK-3 (357). This pathway seems to be in isolation from the Wnt/β-catenin pathway as inhibition of GSK-3 by PI3K-mediated signaling has no effect on transcriptional activation of LEF-1 (357, 358). Other signaling pathways downstream of mitogens and hormones also lead to inhibition of GSK-3 by serine phosphorylation as well, and activation of P70 S6 kinase, Rsk1 and cAMP-dependant protein kinase (PKA) all lead to inhibitory serine phosphorylation of GSK-3 (357).

GSK-3 is also involved in microtubule dynamics, and has been shown to phosphorylate several microtubule associated proteins, such as APC, Tau and MAP2c, and this leads to decreased affinity, and thus decreased stabilizing activity of all of these proteins towards microtubules (359). GSK-3β is often found to be serine phosphorylated at the tips of growing axons, and general inhibition of GSK-3 in neuronal cells can lead to the formation of multiple axons (359).

GSK-3 is also known to regulate transcriptional activity through a diverse array of mechanisms, such as c-myc, which it phosphorylates and targets for degradation, c-jun, whose phosphorylation leads to decreased affinity for its target DNA sequence, and NFATc, whose phosphorylation by GSK-3 leads to nuclear export (359).

GSK-3 is also involved in Alzheimer's disease, by hyperphosphorylating tau protein, leading to its incorporation into neurofibrillary tangles. Amyloid β-peptide, which is accumulated in senile plaques in Alzheimer's disease, and is toxic to neurons, activates GSK-3 causing increased phosphorylation of tau. Presenilins, proteins that are associated with autosomal dominant Alzheimer's disease, are also involved in tau phosphorylation, by linking GSK-3 and tau together by binding to both of them (360).

GSK-3 is also a promoter of apoptosis downstream of growth factor withdrawal or inhibition of PI3K/AKT signaling, DNA damage, ER stress, hypoxia and oxidative stress. GSK-3 is also located within the mitochondria, and mitochondrial GSK-3 activity is increased during apoptosis. GSK-3 can phosphorylate and activate Bax, and it can phosphorylate and enhance the degradation of the anti-apoptotic MCL-1 protein, a member of the Bcl-2 family. In fact, overexpression of GSK-3β in various cell lines is sufficient to promote apoptosis (361).

1.8.2 Crosstalk between GSK-3 and the Rho subfamily

Inhibition of Ser/Thr kinases in keratinocytes leads to formation of E-lams, which are long straight thin lamellipodia that are similar to those that are extended by wound-edge keratinocytes. This process is dependant on relocalization of Rac1 from the cytosol to the cell periphery, at the site of the membrane ruffling, and this Rac1 relocalization is

dependant on GSK-3 activity (362). Rac1 can also function upstream of GSK-3, as is the case of CLASP binding to microtubules. CLASP is a microtubule binding protein which binds to the plus ends of microtubules and stabilizes the microtubules. Rac1 activity leads to enhanced CLASP binding of microtubules, through inhibition of GSK-3 (363). Rac1 activity is also upregulated by HGF, and this is critical for HGF induced cell scatter. Inhibition of GSK-3 can lessen the increase of active Rac1 upon HGF stimulation (364).

Polyamine depletion of IEC-6 cells leads to increased AKT activation, and increased GSK-3β Ser-9 phosphorylation (365). Polyamine depletion leads to decreased migration of IEC-6 cells during scratch-wound closure, due to a downregulation of Rac1 activity. Treatment of polyamine depleted cells with PI3K inhibitors leads to increased GSK-3 activity which leads to increased Rac1 activity and migration. Inhibition of GSK-3 is sufficient to block the activation of Rac1 after PI3K inhibition (365).

When α2β1 integrin binds to collagen, AKT is dephosphorylated, and thus inactivated, in human primary fibroblasts (366). This lower phosphorylation level of AKT is not due to a change in PI3K activity. The dephosphorylation is through activation of the serine/threonine phosphatase PP2A, which is activated downstream of α2β1 integrin binding to collagen (366). This also leads to a decrease in GSK-3β Ser-9 phosphorylation, and thus, presumably, activation of GSK-3. Integrins are able to activate Cdc42, and dominant negative Cdc42 blocks integrin induced activation of PP2A, while dominant negative Rac1 and RhoA have no effect. Constitutively active Cdc42 slightly increases PP2A activity. Thus, integrins, by means of activation of Cdc42, can lead to increased activity of GSK-3 (366).

When astrocyte monolayers are scratch-wounded, GSK-3β is quickly inactivated by phosphorylation of serine-9 (367). Prior to scratching, GSK-3β is in a complex with PKCζ and Par6, and GSK-3 dissociates from this complex when it is Ser-9 phosphorylated (367). Inhibition of either Cdc42 or PKCζ prevents phosphorylation of GSK-3β. Thus, Cdc42 regulates GSK-3β phosphorylation through the Par6-PKCζ complex. Expression of constitutively active GSK-3β or dominant negative Cdc42 leads to a block of centrosome re-orientation following scratch-wounding of the astrocyte monolayer. Inhibitors of GSK-3 also block centrosome polarity. Scratch-wounding of astrocytes leads to β-catenin stabilization and accumulation at the leading edge and this is dependant on Cdc42 and PKCζ activities leading to inhibition of GSK-3 activity (367). Post-scratch, APC associates with the plus-ends of microtubules, and this also is dependant on inhibition of GSK-3 in a Cdc42 and PKCζ dependant manner (367).

Cdc42 also regulates GSK-3 activity through Par3, Par6 and PKCζ in keratinocytes. Knock-out of Cdc42 in these cells leads to altered β-catenin signaling, and defective differentiation into hair follicles (368). Active Cdc42 is able to interact with the Par3, Par6, PKCζ complex, and activate PKCζ, and this leads to GSK-3 inactivation. In Cdc42 null keratinocytes, the increase of GSK-3 activity leads to increased axin phosphorylation and thus increased stability of the GSK-3/APC/axin complex, which enhances the ability of this complex to bind and phosphorylate β-catenin, targeting it for degradation (368).

CTGF stimulation of mesangial cells activates cell migration, and this also involves activation of PKCζ which then phosphorylates and inactivates GSK-3β, leading

to an increase in cellular β-catenin levels, and this may also be in a Cdc42-dependant fashion, as Cdc42 is activated downstream of CTGF stimulation (369).

Large G-proteins activate GSK-3 downstream of LPA stimulation in cerebellar granule neurons (370). In these cells, LPA stimulation leads to neurite-retraction (370). LPA stimulation also leads to increased GSK-3 activity, and to tau hyperphosphorylation. Inhibition of GSK-3 prevents the hyperphosphorylation of tau downstream of LPA stimulation (370). In Neuro2A mouse neuroblastoma cells, LPA stimulation leads to RhoA activation which is important for subsequent GSK-3 activation (370).

1.9 Final Thoughts on the Rho Subfamily

As can clearly be seen, the Rho GTPases constitute a diverse family of proteins that are involved in multiple aspects of cell function. They control the actin cytoskeleton, and thus control cell shape, cell adherence and cell motility. They also act as switches in signal transduction cascades and are able to affect cell growth and proliferation both positively and negatively, depending on the particular GTPase. They are also intertwined in major cell signaling pathways, such as those involving ERK and GSK-3. These proteins are highly and variously regulated including through their presence in particular cell types, altered location within cells, silencing of their genes, and of course, by their three main classes of regulatory proteins, the GEFs, the GDIs and the GAPs. These three classes of proteins are also highly and variously regulated, including through the presence of their protein, as with the GTPases themselves, as well as other mechanisms, such as degradation, phosphorylation, phospholipid regulation and regulation by protein-protein interactions. A greater understanding of how these regulators are controlled has beneficial practical implications, as it would be conceivable to turn on or turn off a particular GTPase within a specific context while leaving it unaffected in other, more general contexts. This is particularly important as many of the Rho subfamily GTPases are involved in multiple signaling pathways; activation or inactivation of the GTPase could be curative within one context, while pathologic in another. In the following chapters, the regulation of CdGAP, a GAP for Rac1 and Cdc42, is examined in further detail.

Rationale and Objectives:

Due to their involvement in numerous pathologies, such as cancer, there is much interest in the regulation of the RhoGTPases. The challenge is that the RhoGTPases are involved in a wide variety of cellular processes in a wide variety of cell types. Broadly altering their activation could lead to unanticipated problems. It is widely held that the regulators of the GTPases, such as the RhoGAP proteins which outnumber the RhoGTPases roughly three to one, may be more specific in their roles, and thus may control the GTPases in a temporal and spatial fashion. As such, these regulators become ideal targets for therapies, as their (in)activation could make it possible to control GTPase activity in specific contexts, and thus one could conceivably affect GTPase activity in the desired place and time without affecting it globally. The goal of this thesis is to gain a better understanding of how CdGAP, a negative regulator of the RhoGTPases Rac1 and Cdc42, is regulated. We accomplish this through three objectives:

1. To characterize the phosphorylation of CdGAP and the effect of this phosphorylation on CdGAP function.

2. To identify binding partners for the PRD of CdGAP, a region that is known to be important in the regulation of GAP activity, and characterize their interaction with CdGAP.

3. To characterize the signaling pathways involved in regulation of CdGAP mRNA levels.

Preface To Chapter 2

Many proteins are regulated by phosphorylation. This can directly affect a protein by altering its conformation, or affecting its ability to interact with other proteins. CdGAP harbors consensus phosphorylation motifs for a number of kinases, and in order to better understand the regulation of CdGAP we investigated the phosphorylation of CdGAP. We characterize which domains of CdGAP are phosphorylated, and, we screen for kinases that interact with CdGAP and are able to phosphorylate its PRD. Having identified ERK1 as a kinase for CdGAP we then go on to characterize its phosphorylation of CdGAP and how it regulates GAP activity.

CHAPTER 2

Extracellular Signal-regulated Kinase-1 (ERK-1) interacts with and phosphorylates CdGAP at an important regulatory site

Extracellular Signal-regulated Kinase-1 (ERK-1) interacts with and phosphorylates CdGAP at an important regulatory site

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ABSTRACT

Rho GTPases regulate multiple cellular processes affecting both cell proliferation and cytoskeletal dynamics. Their cycling between inactive GDP- and active GTP-bound states is tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). We have previously identified CdGAP (Cdc42 GTPase-activating protein) as a specific GAP for Rac1 and Cdc42. CdGAP consists of an N-terminal RhoGAP domain and a C-terminal proline-rich region. In addition, CdGAP is a member of the impressively large number of mammalian RhoGAP proteins that is well conserved among both vertebrates and invertebrates. In mouse, we find two predominant isoforms of CdGAP differentially expressed in specific tissues. Here, we report that CdGAP is highly phosphorylated *in vivo* on serine and threonine residues. We find that CdGAP is phosphorylated downstream of the MEK-ERK pathway in response to serum or PDGF stimulation. Furthermore, CdGAP interacts with and is phosphorylated by ERK-1 and RSK-1 *in vitro*. A putative DEF (docking for ERK FXFP) domain located in the proline-rich region of CdGAP is required for efficient binding and phosphorylation by ERK1/2. We identify Thr^{776} as an *in vivo* target site of ERK1/2 and as an important regulatory site of CdGAP activity. Together, these data suggest that CdGAP is a novel substrate of ERK1/2 and mediates cross talk between the Ras/MAPK pathway and regulation of Rac1 activity.

INTRODUCTION

RhoA, Rac1, and Cdc42, the best-characterized members of the Rho family of small GTPases are critical regulators of many cellular activities such as cell dynamics, cell growth, intracellular membrane trafficking, gene transcription, cell cycle progression and apoptosis (371, 372). The Rho proteins operate as molecular switches that cycle between an active GTP-bound and an inactive GDP-bound state. This GDP/GTP cycle is tightly regulated by three families of proteins: the guanine nucleotide exchange factors (GEFs) which activate Rho GTPases by inducing the exchange of GDP for GTP (373); the GTPase-activating proteins (GAPs) which enhance the intrinsic GTPase activity, leading to the inactive state of the GTPase (374); and the guanine nucleotide dissociation inhibitors (GDIs) which sequester Rho GTPases in their GDP-bound, inactive state (375).

Over 40 RhoGAP family members have been characterized in eucaryotes ranging from yeast to human (376). Recent analysis of the human genome unraveled 66 different genes encoding potential RhoGAP domain-containing proteins, far outnumbering the existing 23 mammalian Rho GTPases (377, 378). In most cases, GAP proteins are large proteins containing multiple signaling modules that mediate the cross talk between GAPs and other signaling pathways or serve to regulate the GAP activity (374). GAP proteins act not only as negative regulators but also function as downstream effectors of Rho GTPases. For instance, n-chimaerin can induce actin reorganization independently of its RhoGAP domain (379) and TCGAP plays a direct role in insulin-stimulated glucose transport (319). Given that Rho GTPases are implicated in a large number of biological responses, each GAP protein may selectively regulate a specific Rho GTPase signaling pathway. In addition, the overabundance of GAP proteins strongly suggests a tight

regulation of their activity in a spatial and temporal fashion. Indeed, accumulating evidence reveal that GAPs are regulated by lipid interaction, protein-protein interaction, phosphorylation, and proteolytic degradation (264, 325, 380, 381).

CdGAP (Cdc42 GTPase-activating protein) is a serine- and proline-rich RhoGAP protein showing GAP activity against both Cdc42 and Rac1 but not Rho A (321). In addition to its N-terminal GAP domain, CdGAP contains a central domain and a C-terminal proline-rich domain (PRD) harboring five consensus Src homology 3 (SH3)-binding sites whose functions are still unclear. We have recently shown that the endocytic protein intersectin interacts with CdGAP through a subset of its SH3 domains and negatively regulates CdGAP's activity, providing evidence of a direct regulation through protein-protein interaction (325). Here we report that CdGAP is highly phosphorylated on serine and threonine residues in the proline-rich region. We found that CdGAP interacts with members of the MAP kinase signaling pathway, RSK-1 and ERK1/2, and is phosphorylated *in vitro* by these Ser/Thr kinases. Mutation of key residues in the ERK docking site of CdGAP reduces both ERK binding and phosphorylation of CdGAP. In Swiss 3T3 fibroblasts, endogenous CdGAP is phosphorylated in response to PDGF and this *in vivo* phosphorylation of CdGAP is reduced in the presence of the MEK1 inhibitor PD98059. We identified Thr^{776} in the proline-rich domain of CdGAP as a major *in vivo* phosphorylation site of ERK-1, and amino acid substitution of this threonine for alanine significantly affects the GAP activity of CdGAP. We propose that CdGAP mediates cross talk between the MAP kinase and Rac1 signaling pathways and that phosphorylation of CdGAP by ERK1/2 participates to negatively regulate CdGAP activity.

MATERIALS AND METHODS

Reagents and Antibodies

Recombinant activated rat RSK-1 and human ERK-1 proteins, PD98059, Myelin Basic Protein (MBP) and Long S6 kinase Substrate Peptide (KRQEQIAKRRR LSSLRASTAKSGGSQK) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Lipofectamine was obtained from Invitrogen (Carlsbad, CA). Human recombinant PDGF-BB was obtained from Calbiochem. Protease inhibitor cocktail tablets were from Roche Applied Science (Indianapolis, IN). A protein assay Kit (micro BCA) was from Pierce Chemical (Rockford, IL). Trypsin-TPCK was purchased from Sigma. Protein G- and A-sepharose were obtained from Pharmacia Biotech. [γ^{32}P]-ATP (3000 Ci/mmol) and [^{32}P]-orthophosphate (3000 mci/ml) were purchased from Perkin Elmer. Anti-Rsk-1 and –ERK1/2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Ca) and phospho-specific antibodies recognizing RSK-1 and ERK1/2 were from Upstate Biotechnology. Anti-CdGAP antibodies were obtained by immunization of rabbits with CdGAP proline-rich domain (aa 515-820) fused to GST and affinity-purified on a CH-sepharose column (Pharmacia Biotech) covalently coupled to the CdGAP peptide ESQGASQPKPSTPQESLGAG (aa 601-620). Anti-Rac1 antibodies were purchased from Upstate Biotechnology. Myc-tagged proteins were detected using the 9E10 anti-Myc mAb, which was kindly provided by Dr. Nicole Beauchemin (McGill University, Montreal, Canada).

DNA constructs and site-directed mutagenesis

The threonine 612, 769, 776 and phenylalanine 677 and 679 residues located in the proline-rich region of CdGAP in pRK5myc vector (325) were substituted for alanine residues using a PCR oligonucleotide-directed mutagenesis approach, according to standard protocols. The following primers were used: T612A (5'-GAAGCCAGCGCGCCACAAGAGAGCCTCGGG-3', 3'-TCGGTCGGCTTCGGGT CGCGCGGTGTTCTC-5'), T769A (5'-CTCTCTCCCCCTCTTGCTCCTGCTCCTC CTCCC-3', 3'-CCTT AGAGAGAGGGGGAGAACGAGGACGAGG-5'), T776A (5'-CCTCCTCCCCCCGCTCCTCTGGAGGAGGAGCCT -3', 3'-GAATGAGGACGA GGAGGGGGGCGAGGAGACCTC-5'), T769AT776A (5'-CCCCCTCTTGCTCCT CCTCCCCCCGCTCCTCTGGAGGAGGAG-3', 3'-GAGAGAGGGGGAGAACGA GGACGAGGAGGAGGGGGGCGAGGAGACCTC-5') and FFAA (5'-CAGCCCCA GCTCCCGCTCCAGAAGCCCCTGG-3', 3'-GGACTCAGTTCGGGTCGGGGTCG AGGGCGAGGTCTT-5'). The PCR products were digested using NheI/XbaI restriction enzymes and inserted into pRK5mycCdGAP-s, digested with NheI/XbaI and dephosphorylated using calf intestine phosphatase (NEB). The proline-rich domain of CdGAP (aa 516-820) wild type or threonine and phenylalanine mutations were subcloned into pTrcHisA vector by PCR using pRK5mycCdGAP-s, -T612A, -T769A, -T776A, -T769AT776A or –FFAA as templates and 5'-CGGATCCCAAGGTTCAGAGAGTGG-3' and 3'-CGGGTTGAACAAATAACG-5' as forward and reverse primers, respectively. Both PCR products and pTrcHisA vector were digested with BamH1/EcoRI and ligated together. The pRK5myc CdGAP deletion mutants: CdGAP-ΔGAP, CdGAP-GAP,

CdGAP-ΔPRD and CdGAP-PRD were produced as described previously (325). To produce the pRK5mycCdGAP-l, the mouse cDNA clone mpf00743 containing the longer C-terminal tail of mouse CdGAP was obtained from the Kazuka DNA Research Institute (Chiba, Japan) and was cloned into pRK5mycCdGAP-s by PCR using 5'-CATGCCATGGCACAAGGTTCAGAGAGT

GG-3' and 3'AGTGGGAGAGCAGATAGAATGATCTAGAG-5' as forward and reverse primers. The resulting PCR product and pRK5mycCdGAP-s were digested with NheI and XbaI and ligated together.

Expression of recombinant proteins

Recombinant Rac1 and PAK (amino acids 56-272) were produced in *Escherichia coli* strain DH5α as GST fusion proteins and purified on glutathione-Sepharose beads as described previously (325, 382). pTrcHisA containing hexahistidine fusion proteins: CdGAP-PRD, CdGAP-PRD-T612A, CdGAP-PRD-T769A, CdGAP-PRD-T776A, CdGAP-PRD-T769AT776A and CdGAP-PRD-FFAA, were transformed into *Escherichia coli* DH5α strain, and grown in 100 ml of LB medium at 30 ^{0}C overnight. The next day, 900 ml of M9 medium 1X was added before induction with 1mM isopropyl-β-thiogalactopyranoside (IPTG), (Sigma-Aldrich) for 1 h. Bacteria pellets were lysed and sonicated in 10 ml of lysis buffer (50 mM Hepes, 300 mM NaCl, protease inhibitor cocktail (Sigma-Aldrich). Followed by the addition of 10 % Triton X-100 and centrifugation for 45 min at 4000 x g. 20 mM Imidazole pH 8.0 was added to the supernatant and His-tagged recombinant proteins were purified by adding 500 μl (50:50) of Ni-NTA beads (Amersham) per liter of culture and rotating for 3 h. After quick spin,

the beads were washed twice with buffer A (50 mM Hepes, 300 mM NaCl, 1 % Triton X-100, 20mM Imidazole, pH 8.0) and twice in buffer B (50 mM Hepes, 300 mM NaCl, 1 % Triton X-100, 20mM Imidazole, pH 6.5). The proteins were eluted by incubating beads three times in 3 ml of buffer C (Hepes 50 Mm, NaCl, 300 mM, 1 % Triton X-100, 200 mM Imidazole, pH 6.5) for 30 min each. Eluates were pooled and concentrated in Centricon (Millipore) and washed twice with 4 ml of cold PBS 1X to remove Imidazole. Protein concentration and purity were assessed by SDS-PAGE followed by Coomassie Blue-staining.

Preparation of mouse tissues

Murine tissue samples were obtained from adult male Balb/C mice, aged 7-9 weeks. Tissues were collected and homogenized in RIPA buffer (PBS 1X pH 7.4, 0.1 % SDS, 1 % Triton X-100, 12 mM deoxycholic acid, and protease inhibitor cocktail). Total tissue lysates were centrifuged at 5000 x g for 10 min. The protein concentration in the resulting supernatant was determined using the BCA Protein Assay Kit (Pierce Chemical) and 200 µg of proteins from each tissue were loaded on a 7.5 % SDS-PAGE and transferred onto a nitrocellulose membrane. CdGAP was detected using affinity-purified polyclonal anti-CdGAP antibodies. Competition assay was performed by incubating the membrane with 10 µg of GST-tagged CdGAP-PRD or GST alone as a control.

Cell transfection, immunoprecipitation and immunoblotting

COS-7, HEK293 and Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics, and

maintained at an atmosphere of 10 % CO_2 at 37 °C. COS-7 cells were transfected by DEAE-dextran as described previously (383). Briefly, 5 μg of pRK5myc-CdGAPs, -CdGAP-l, -CdGAP-ΔPRD, -CdGAP-PRD, -CdGAP-GAP, -CdGAP-ΔGAP, -CdGAPT769A, -CdGAPT769AT776A, -CdGAPT612A, -CdGAPT776A or -CdGAPFFAA were used per 100-mm dish. Then, 48 h post-transfection, cells were lysed in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 % Triton X-100) containing 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM NaF, 1 mM sodium orthovanadate followed by centrifugation for 15 min at 1000 x g_{max}. 1 mg of the resulting postnuclear supernatant was incubated overnight at 4 ^{0}C with 5 μg of anti-Myc antibodies and 20 μl of 50 % protein G-Sepharose. Samples were washed three times in lysis buffer and submitted to SDS-PAGE followed by immunoblotting analysis using anti-Myc, -ERK1/2 or -RSK-1 antibodies and revealed by ECL (Perkin Elmer). Endogenous CdGAP-l was immunoprecipitated from protein lysates obtained from Swiss 3T3 cells treated or not with PDGF (5 ng/ml) or FBS (20 %) for 5 and 30 min as described above using anti-CdGAP antibodies and protein A-Sepharose. Western Blotting was performed using anti-CdGAP antibodies.

In vivo [^{32}P]-orthophosphate labeling

Confluent Swiss 3T3 fibroblasts grown onto 150mm- dishes were serum-starved for 24 h. Then, cells were washed once in phosphate-free medium and then incubated in the same medium for 1 h prior to labeling. Cells were incubated for 3 h in the presence of 0.5 mCi/ml of [^{32}P]-orthophosphate. The cells were either unstimulated or stimulated with dialyzed FBS (20 % for 30 min) or PDGF (5 ng/ml for 5 min) or pre-treated with

PD98059 (50 μM for 1 h) prior to stimulation with PDGF. Endogenous CdGAP was immunoprecipitated from cell lysates, and proteins were separated by SDS-PAGE. Radiolabeled proteins were detected by autoradiography with an enhancing screen and Biomax MS film (Sigma-Aldrich) at -80 ^{0}C. COS-7 cells grown onto 100 mm- dishes were transfected with Myc-tagged CdGAP or different protein mutants as described above. 48 h post-transfection, cells were washed once in phosphate-free medium supplemented with 1% serum and incubated for 1h in the same medium prior to labeling as described above.

In Gel kinase assay

Following immunoprecipitation of Myc-tagged CdGAP from COS-7 cells, proteins were resolved by SDS-PAGE using 10 % acrylamide resolving gel containing 0.5 mg/ml of recombinant His-tagged CdGAP-PRD. Following electrophoresis, the gel was washed twice with 30 mM Tris-HCl pH 7.5, 20 % isopropanol. Then, the gel was washed twice (30 min each) in 30 mM Tris-HCl, 2 mM DTT, pH 7.5 and incubated for 45 min in the same buffer containing 6 M urea. The proteins were then subjected to renaturation by three washes of 45 min each in 30 mM Tris-HCl, 2 mM DTT, pH 7.5, containing 0.05 % Tween-20 and respectively 3, 1.5 and 0.75 M urea, then washed for 2 h in 30 mM Tris-HCl, 2 mM DTT, pH 7.5, 0.05 % Tween-20. The gel was then incubated for 30 min in the kinase reaction buffer (30 mM Tris-HCl, 2 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$, pH 7.5. Phosphorylation was carried out by incubating the gel in the same buffer containing 10 μCi/ml [γ-^{32}P]-ATP and 100 μM ATP for 45 min at room temperature. The gel was

washed extensively in 5 % (v/v) trichloroacetic acid, 1% (w/v) Na-pyrophosphate for 24 h. The gel was dried and the radiolabeled bands were visualized by autoradiography.

In Vitro kinase Assays

COS-7 cells were transfected with pRK5myc or pRK5mycCdGAP as described above. Proteins were immunoprecipitated and the pellets were washed three times with lysis buffer and twice with kinase buffer (20 mM Mops, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1mM sodium orthovanadate, 1mM DTT). Immunoprecipitates were incubated at 30 °C for 10 min in 50 µl of kinase reaction buffer (Kinase buffer + 10 mM $MgCl_2$, 100 µM ATP, 10 µCi/ml [γ-^{32}P]-ATP). The reaction was stopped by addition of Laemmli sample buffer and the phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. His-tagged CdGAP-PRD wild type, threonine and phenylalanine protein mutants were phosphorylated with 20 ng of recombinant activated ERK-1 (Upstate) or 20 ng recombinant activated RSK-1 (Upstate) in reaction buffer. Myelin basic protein (0.1 mg/ml, Sigma) or Long S6 substrate peptide (20µm, Upstate) were used as positive controls for ERK-1 and RSK-1 respectively.

Phospho amino Acid Analysis

Phosphoamino acid analysis was performed as described (384). Briefly, CdGAP wild-type or protein mutants overexpressed in COS-7 cells were radiolabeled and immunoprecipitated as described above. His-tagged CdGAP-PRD was phosphorylated *in vitro* by recombinant activated ERK-1 or RSK-1. Samples were resolved by SDS-PAGE

and electroblotted onto PVDF membrane. Bands corresponding to ^{32}P-labeled CdGAP proteins were excised and placed in a 1.5 ml screw cap tube and washed extensively with distilled water. Acid hydrolysis was performed by incubating samples in 200 µl of 6 N HCl followed by heating at 110°C for 60 min, lyophilization and dissolved in 10 µl of pH 1.9 buffer [88 % formic acid, glacial acetic acid, H_2O; 2.5:7.8:89.7 (v/v/v)] containing 0.5 µl of 2 mg/ml of combined unlabelled phospho-amino acid standards (phospho-serine, -threonine and -tyrosine). The phospho-amino acids were first separated by high voltage (1,5 kV) electrophoresis at pH 1.9 for 20 min using a Hunter thin-layer electrophoresis system (CBS Scientific, Del Mar, CA), followed by a second dimension electrophoresis in pH 3.5 buffer [glacial acetic acid, pyridine, H2O; 5:0.5:94.5(v/v/v)]. The standards were visualized by spraying a 0.25 % (w/v) ninhydrin acetone solution followed by incubation at 65 ^{0}C for 10 min. The radiolabeled amino acids were detected by autoradiography with an enhancing screen and Kodak Biomax film at -80 ^{0}C.

Tryptic phosphopeptide mapping

Radiolabeled proteins were resolved by SDS-PAGE and electroblotted onto nitrocellulose membrane. Corresponding CdGAP bands were excised and digested with 10 µg of TPCK-treated trypsin for 4 h at 37 ^{0}C. Peptides were diluted in 500 µl of water and lyophilized by Speed-Vac. The peptides were then oxydized in 50 µl performic acid for 60 min on ice, diluted to 500 µl with deoinized water before lyophilization. Pellets were dissolved in pH 1.9 buffer [88% formic acid, glacial acetic acid, H_2O; 2.5:7.8:89.7 (v/v/v)] and were first separated by electrophoresis in pH 1.9 buffer 25 min at 1.0 kV employing a Hunter thin-layer electrophoresis system (CBS Scientific, Del Mar, CA)

followed by a second dimension separation by ascending chromatography in phospho-chromatography buffer [glacial acetic acid, pyridine, n-butanol, H_2O; 7.5:25:37.5:30(v/v/v/v)]. The radiolabeled phosphopeptides were detected by autoradiography.

In vitro GAP Assay

COS-7 cells were transfected with pRK5myc, pRK5mycCdGAP, pRK5mycCdGAP-T769A, pRK5mycCdGAP-T776A and pRK5mycCdGAP-T769AT776A as described above. 48 h post-transfection, Myc-tagged proteins were immunoprecipitated as described above using anti-Myc antibodies. Then, samples were washed three times in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Triton X-100 and twice in 20 mM Tris-HCl (pH 7.5) and used for the *in vitro* GAP assay as follows. The amount of immunoprecipitated CdGAP is estimated on Coomassie Blue-staining by comparison with different amounts of purified bovine serum albumin. According to this estimation immune complexes corresponding to 1 µg of immunoprecipitated CdGAP were resuspended in 24 µl of 20 mM Tris-HCl (pH 7.5), 0.1 mM DTT, 1 mM GTP, 0.86 mg/ml bovine serum albumin. At the same time, 2 µg of recombinant Rac1 was incubated with 5 µCi of [γ-^{32}P]-GTP (30 Ci/mmol) in 20 µl of 20 mM Tris-HCl (pH 7.5), 25 mM NaCl, 0.1 mM DTT, and 5 mM EDTA for 10 min at 30 °C. GTP-loaded Rac1 was kept on ice after addition of 20 mM $MgCl_2$. 3 µl of [γ^{32}P]-GTP-loaded Rac1 was incubated at 20 °C with the immune complexes. After 0 and 6 min incubation, 4 µl mixtures were diluted in 1 ml of cold buffer A (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5

mM $MgCl_2$), and filtered through pre-wetted nitrocellulose filters. Filters were washed with 10 ml of cold buffer A, dried, and counted.

Rac activation assay

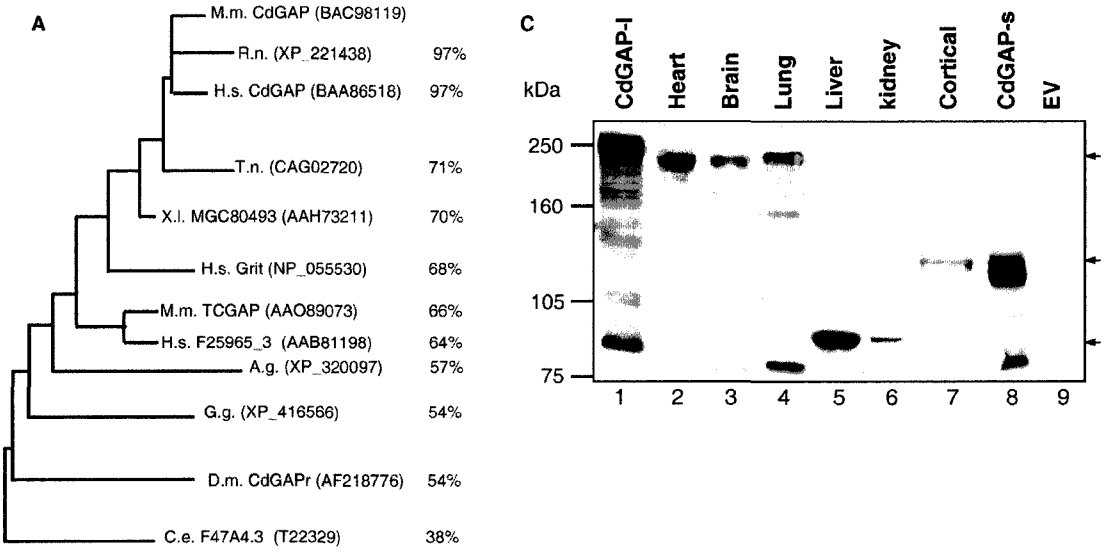
HEK293 cells grown onto 100mm- dishes were transfected with lipofectamine according to the manufacturer's procedures. Briefly, cells were transfected with 2 μg of pRK5myc, pRK5mycCdGAP, pRK5mycCdGAP-T769A, pRK5mycCdGAP-T776A and pRK5mycCdGAP-T769AT776A, together with 1.5 μg of pRK5mycRac1 and 1 μg of pRK5mycRasV12. Then, cells were serum-starved overnight and the next day lysed in lysis buffer B (25 mM Hepes pH 7.5, 100 mM NaCl, 1% NP-40 and 5% glycerol) containing 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 20 mM NaF, 1 mM sodium orthovanadate followed by centrifugation for 15 min at 1000 x g_{max}. The amounts of GTP-loaded Rac1 in the supernatant were measured using a pull-down assay with GST-CRIB domain of PAK as described previously (382).

RESULTS

CdGAP isoforms are differentially expressed in mouse tissues

We have previously identified and characterized mouse CdGAP (mCdGAP), a protein of 820 amino acids. Since then, CdGAP-related genes have been found in both vertebrate and invertebrate organisms. In addition to the previously characterized orthologue of mCdGAP in *D.melanogaster (385),* a single gene encoding a putative protein with high degree of homology to CdGAP within the RhoGAP domain is found in at least six other organisms including rat (R.n.), fish (T.n.), frog (X.l.), chicken (G.g.), and worm (C.e.) (Fig. 2.1A). In *H. sapiens*, there are at least three different genes encoding proteins that are highly homologous to mCdGAP. One encodes a previously characterized protein named Grit (also known as p200RhoGAP, RICS, p250GAP and GC-GAP) (312, 314, 315, 386), which shares 68 % homology to mCdGAP within the rhoGAP domain and one uncharacterized cDNA, F25965_3 which is the human orthologue of mouse TCGAP protein (319). The cDNA KIAA1204 is predicted to encode a protein of 1,444 amino acids that shares 97 % homology to mCdGAP within the rhoGAP domain and 84 % homology within the entire protein sequence. The longer C-terminal tail of human CdGAP suggested that a similar protein might also exist in *M. musculus*. Indeed, BLASTP analysis at the National Center for Biotechnology Information (NCBI) Genbank databases revealed a novel mouse cDNA identical to that of CdGAP but with an additional 1,815 bp at the 3' end (Gen Bank^{TM} accession number BAC98119). This cDNA is predicted to encode a protein of 1,425 amino acids with a predicted molecular mass of 155 724 Da (Fig. 2.1B). We have named these two isoforms: CdGAP long (-l) and CdGAP short (-s). When overexpressed into fibroblasts,

Figure 2.1: Tissue distribution of CdGAP proteins. (A) Phylogenetic analysis of the RhoGAP domain of CdGAP and CdGAP-related proteins generated with Treeview following ClustalW. Gen Bank™ accession numbers are indicated in parenthesis. The percent identities to mouse CdGAP are shown at the right. M.m., *Mus musculus*; H.s., *Homo sapiens*; R.n. *Rattus norvegicus*; X.l., *Xenopus laevis*; C.e., *Caenorhabditis elegans*; D.m., *Drosophila melanogaster*; G.g., *Gallus gallus*; A.g., *Anopheles gambiae* and T.n., *Tetraodon nigroviridis*. (B) Structure of short and long mCdGAP protein. (C) Total protein cell lysates from mouse tissues, primary cortical neurons, or COS-7 cells overexpressing CdGAP-s and -l were resolved by SDS-PAGE and CdGAP was revealed by immunoblotting analysis using affinity-purified polyclonal anti-CdGAP antibodies. Lanes 1, 8, 9: 5 μg; lanes 2, 3: 150 μg; lanes 4-6: 50 μg. Arrows indicate the three major CdGAP proteins. EV: empty vector.


A
M.m. CdGAP (BAC98119)
R.n. (XP_221438) 97%
H.s. CdGAP (BAA86518) 97%
T.n. (CAG02720) 71%
X.l. MGC80493 (AAH73211) 70%
H.s. Grit (NP_055530) 68%
M.m. TCGAP (AAO89073) 66%
H.s. F25965_3 (AAB81198) 64%
A.g. (XP_320097) 57%
G.g. (XP_416566) 54%
D.m. CdGAPr (AF218776) 54%
C.e. F47A4.3 (T22329) 38%
C
kDa
CdGAP-l
Heart
Brain
Lung
Liver
kidney
Cortical
CdGAP-s
EV
250
160
105
75
1 2 3 4 5 6 7 8 9
B
GAP
Central Domain
PRD
CdGAP-s
1 2 3 4 5
CdGAP-l
1 2 3 4 5

we found that both CdGAP-s and CdGAP-l migrate higher than their expected molecular weights of 90 kDa and 155 kDa, respectively (Fig. 2.1C, lanes 1 and 8). In fact, CdGAP-s migrates at 125 kDa and CdGAP-l at 250 kDa. These mobility shifts may be in part due to post-translational modifications such as phosphorylation in addition to unidentified modifications of the proteins. Using affinity-purified polyclonal antibodies against the third proline-rich sequence of CdGAP, three major bands of 250 kDa, 125 kDa and 90 kDa are detected in lysates of different mouse tissues and primary mouse cortical neurons (Fig. 2.1C). These bands were not recognized in western-blots using preimmune sera (data not shown). The band of 250 kDa, which migrates at the same level as overexpressed CdGAP-l (lane 1), is present in the heart, brain and lung tissues (lanes 2, 3 and 4). The 90 kDa band which corresponds to the expected molecular weight of CdGAP-s, is very abundant in liver and present in kidney tissues (lanes 5 and 6). We hypothesized that in these tissues, post-translational modifications of CdGAP-s are absent or different than the overexpressed CdGAP-s protein migrating at a higher molecular weight of 125 kDa. Interestingly, cortical neurons express a CdGAP protein of 125 kDa (lane 7) corresponding to the overexpressed CdGAP-s (lane 8). All three bands of 250 kDa, 125 kDa, and 90 kDa were also detected using affinity-purified polyclonal antibodies against the first proline-rich sequence of CdGAP (data not shown). In addition, preadsorption of antibodies with GST-tagged CdGAP-PRD reduced significantly their ability to recognize all three bands (data not shown). We conclude that at least three abundant endogenous CdGAP proteins exist in mouse.

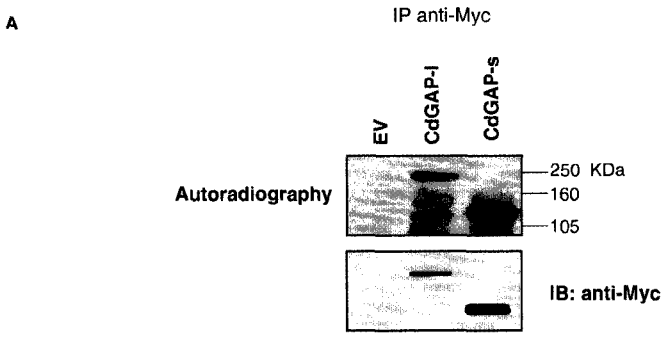
CdGAP-s and –l are highly phosphorylated in fibroblast cells

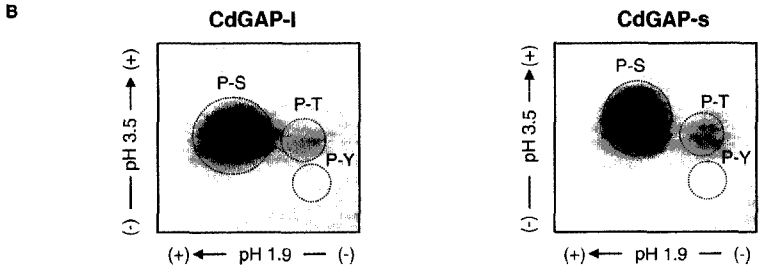
To examine whether CdGAP-s and –l are phosphorylated *in vivo*, Myc-tagged CdGAP-s and –l were expressed in COS-7 fibroblasts that were then incubated in phosphate-free medium supplemented with [^{32}P]-orthophosphate for 2 h prior to lysis. As shown in Fig. 2.2A, both immunoprecipitated CdGAP proteins are phosphorylated *in vivo* to a similar extent in COS-7 cells. To assess the content of phosphorylated residues on CdGAP-s and -l, a phosphoamino-acid analysis of immunoprecipitated CdGAP-s and –l was performed and revealed that both CdGAP proteins are highly phosphorylated on serine residues and to a lesser extent on threonine and not on tyrosine (Fig. 2.2B). The absence of tyrosine phosphorylation was also confirmed by immunoblotting using anti-phosphotyrosine antibodies (data not shown). The tryptic phosphopeptide maps of both immunoprecipitated CdGAP-s and –l show a similar pattern of phosphorylation with six major phosphopeptides (Fig. 2.2C), suggesting that most of the phosphorylation sites are present in the short form of CdGAP. Thus, these results show that CdGAP-s and –l are phosphorylated on serine and threonine residues in fibroblasts.

CdGAP is predominantly phosphorylated in the proline-rich domain

CdGAP-s consists of an N-terminal GAP domain, a central domain, and a C-terminal proline-rich domain (PRD) harboring five consensus SH3-binding motifs. To determine which region of CdGAP-s is phosphorylated *in vivo*, we have investigated the phosphorylation status of various deletion mutants of CdGAP (Fig. 2.3A) expressed in COS-7 cells. As shown in Fig. 2.3B, CdGAP lacking the GAP domain (CdGAP-ΔGAP, lane 3) is as well phosphorylated as the wild type protein (lane 2). Indeed, the GAP

Figure 2.2: CdGAP-s and CdGAP-l have similar phosphorylation profiles. (A) COS-7 cells expressing Myc-tagged CdGAP long (l) or short (s) forms were metabolically labeled with 0.5 mCi/ml of [^{32}P]-orthophosphate. The proteins were immunoprecipitated (IP) using anti-Myc antibodies. The samples were resolved by SDS-PAGE and radiolabeled proteins were identified by autoradiography (upper panel). The membrane was immunoblotted (IB) with anti-Myc antibodies to show the total amount of immunoprecipitated CdGAP (bottom panel). EV: empty vector. (B and C) The phosphorylated protein bands corresponding to CdGAP-l and CdGAP-s were cut and subjected to phosphoaminoacid analysis (B) or tryptic phosphopeptide mapping (C). Phosphopeptides or phosphoamino acids were resolved by thin layer chromatography (TLC) and detected by autoradiography. Migration of phosphoamino-acid standards is indicated with dashed circles: phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y). Numbers 1 to 6 on phosphopeptide maps represent the six most abundant tryptic phosphopeptides. (●) indicates origin of migration.


A
IP anti-Myc
EV
CdGAP-I
CdGAP-s
Autoradiography
250 KDa
160
105
IB: anti-Myc


B
CdGAP-I
CdGAP-s
P-S
P-T
P-Y
(-) — pH 3.5 — (+)
(+) ← pH 1.9 — (-)

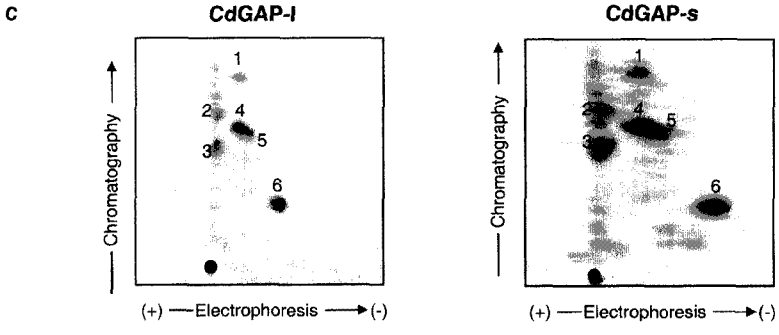
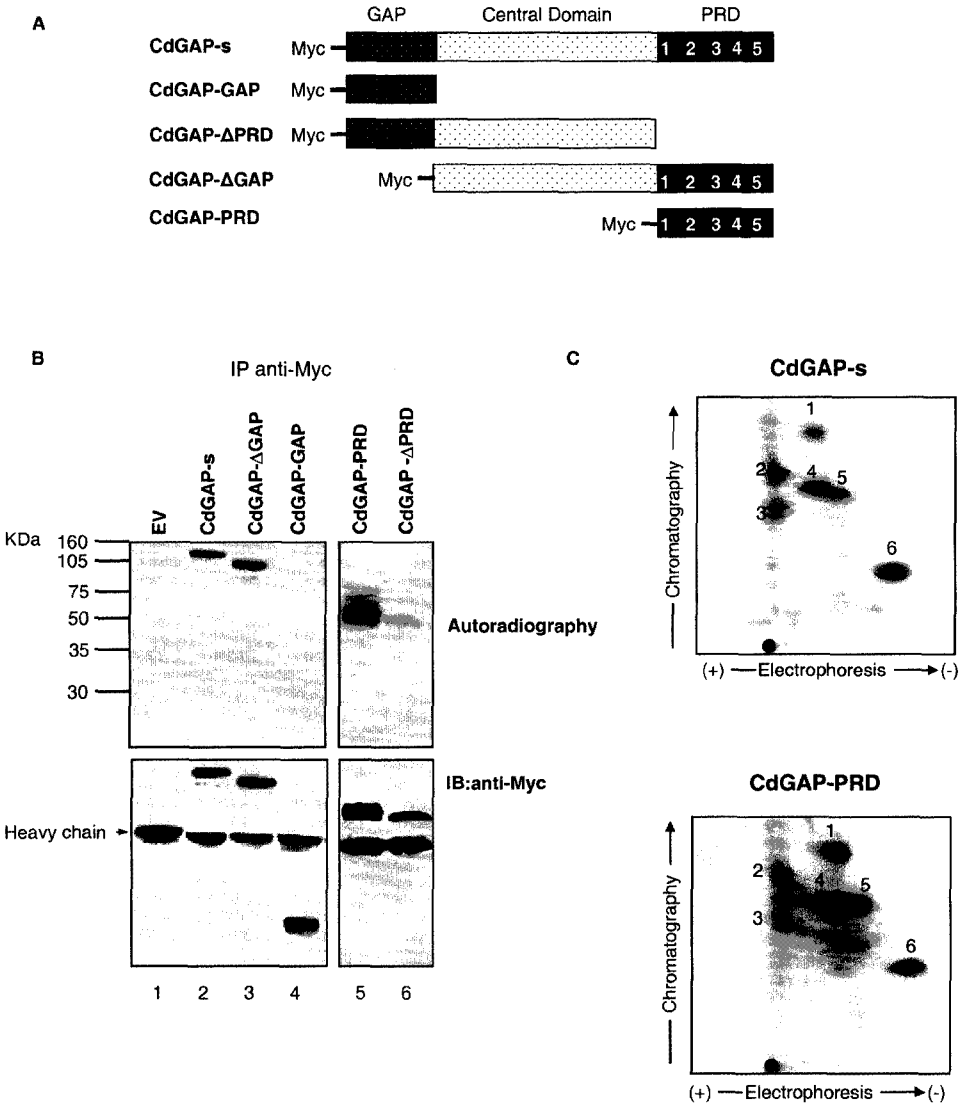

C
CdGAP-I
CdGAP-s
1
2
3
4
5
6
Chromatography
(+) —Electrophoresis —→(-)

Figure 2.3: Phosphorylation analysis of CdGAP deletion mutants. (A) Schematic representation of CdGAP-s, CdGAP-GAP, CdGAP-ΔPRD, CdGAP-ΔGAP and CdGAP-PRD constructs. (B) COS-7 cells transfected with empty vector (EV) or pRK5myc encoding CdGAP-s or various protein mutants were labeled with [^{32}P]-orthophosphate for 3h. Proteins were immunoprecipitated (IP) from cell lysates using anti-Myc antibodies. The samples were resolved by SDS-PAGE and the radiolabeled proteins were identified by autoradiography (upper panel). The membrane was immunoblotted (IB) with anti-Myc antibodies to show the total amount of immunoprecipitated CdGAP proteins (bottom panel). (C) Comparison of tryptic phosphopeptide patterns between CdGAP-s and CdGAP-PRD. The phosphorylated protein bands corresponding to CdGAP-s and CdGAP-PRD were cut and subjected to two-dimensional tryptic phosphopeptide mapping. (•) indicates origin of migration.

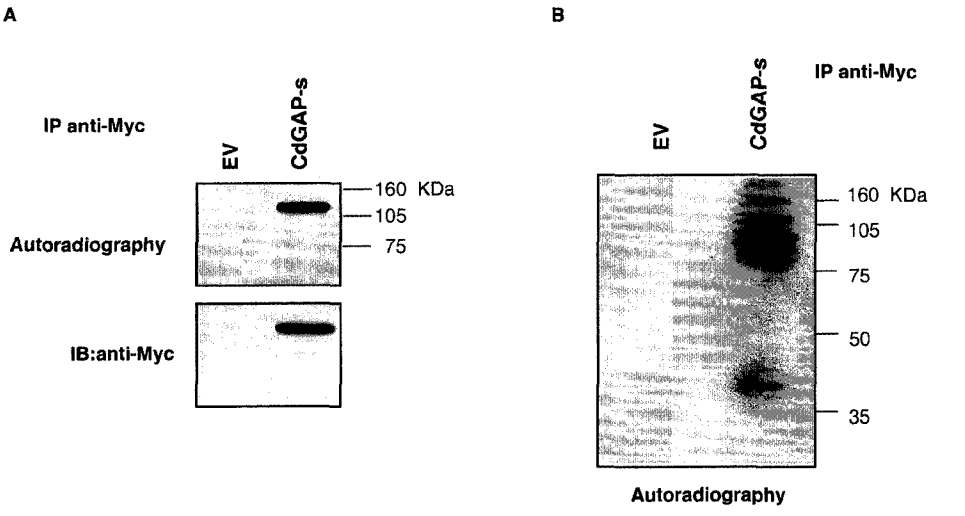

A
GAP
Central Domain
PRD
CdGAP-s
Myc
1 2 3 4 5
CdGAP-GAP
Myc
CdGAP-ΔPRD
Myc
CdGAP-ΔGAP
Myc
1 2 3 4 5
CdGAP-PRD
Myc
1 2 3 4 5
B
IP anti-Myc
EV
CdGAP-s
CdGAP-ΔGAP
CdGAP-GAP
CdGAP-PRD
CdGAP -ΔPRD
KDa
160
105
75
50
35
30
Autoradiography
IB:anti-Myc
Heavy chain
1 2 3 4 5 6
C
CdGAP-s
Chromatography
(+) Electrophoresis (-)
CdGAP-PRD
Chromatography
(+) Electrophoresis (-)

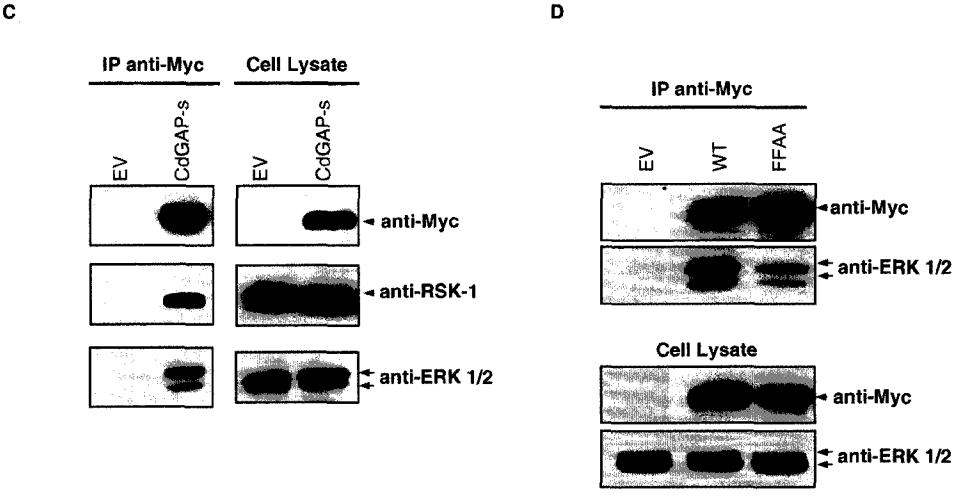
domain by itself is not phosphorylated *in vivo* (CdGAP-GAP, lane 4). Interestingly, the CdGAP-PRD protein (lane 5) is phosphorylated at similar levels to that of the wild type CdGAP and removing the PRD significantly decreases the phosphorylation levels of the mutant protein CdGAP-ΔPRD (lane 6). To confirm that the PRD contains the majority of the phosphorylation sites, we performed a 2-D tryptic phosphopeptide mapping of immunoprecipiated CdGAP and CdGAP-PRD. We found that the proline-rich domain comprises the six major phosphopeptides also found in the wild type CdGAP protein (Fig. 2.3C). These findings indicate that the majority of the phosphorylation sites are present in the proline-rich domain of CdGAP.

CdGAP-s associates with ERK1/2 and RSK-1

To identify the putative kinase (s) involved in CdGAP phosphorylation, we searched for kinase activities present in CdGAP immunoprecipitates. For this purpose, Myc-tagged CdGAP-s was immunoprecipitated from COS-7 cell lysates and incubated in kinase buffer containing [γ-^{32}P]-ATP. Interestingly, CdGAP-s was phosphorylated even without addition of exogenous kinases (Fig. 2.4A), suggesting that kinases co-immunoprecipitate with CdGAP-s. To further characterize the kinases associated with CdGAP-s, we performed an in-gel kinase assay in which purified His-tagged CdGAP-PRD was used as a substrate embedded in the polyacrylamide gel. The proteins were renatured and the gel was incubated with [γ-^{32}P]-ATP. As shown in Fig. 2.4B, two major kinases corresponding to the molecular weights of 90 kDa and 40 kDa were able to renature efficiently and to phosphorylate CdGAP-PRD in the polyacrylamide gel. Curiously, this in-gel phosphorylation pattern is similar to those obtained by at least two

Figure 2.4: ERK1/2 and RSK interact with CdGAP-s. (A) COS-7 cells were transfected with empty vector (EV) or pRK5-myc CdGAP-s. Proteins were immunoprecipitated (IP) using anti-Myc antibodies. *In vitro* kinase assay was performed by incubating immunoprecipitates in kinase buffer containing [γ-^{32}P]-ATP. The samples were resolved by SDS-PAGE and radiolabeled proteins were identified by autoradiography (upper panel). The membrane was immunoblotted (IB) with anti-Myc antibodies to show the total amount of immunoprecipitated CdGAP (bottom panel). (B) In gel kinase assay was performed by resolving immunoprecipitated Myc-CdGAP on SDS-PAGE containing purified His-tagged CdGAP-PRD protein. The kinases were renatured and the acrylamide gel was incubated in kinase buffer containing [γ-^{32}P]-ATP. (C and D) Immunoprecipitated CdGAP-s and CdGAP-s-FFAA from COS-7 cell lysates was resolved by SDS-PAGE and proteins were transferred on nitrocellulose membrane for immunoblotting (IB) using anti-Myc, polyclonal anti-ERK1/2 and RSK-1 antibodies. Protein expression levels are shown as input controls (cell lysate) in the right panel (C) and bottom panel (D).


A
IP anti-Myc
EV
CdGAP-s
160 KDa
105
75
Autoradiography
IB:anti-Myc
B
IP anti-Myc
EV
CdGAP-s
160 KDa
105
75
50
35
Autoradiography


C
IP anti-Myc
Cell Lysate
EV
CdGAP-s
EV
CdGAP-s
anti-Myc
anti-RSK-1
anti-ERK 1/2
D
IP anti-Myc
EV
WT
FFAA
anti-Myc
anti-ERK 1/2
Cell Lysate
anti-Myc
anti-ERK 1/2

other groups using different proteins as substrates (387, 388). In both cases, they showed that the 40 kDa kinases represented p42 ERK-2 and p44 ERK-1 whereas the 90 kDa kinase appeared to be p90 RSK-1. We have also investigated this possibility since the protein sequence of CdGAP contains a significant number of ERK1/2 and RSK-1 consensus phosphorylation sites PX(S/T)P and RXXS, respectively (Fig. 2.6A). As shown in Fig. 2.4C, both ERK1/2 and RSK-1 co-immunoprecipitate with CdGAP expressed in COS-7 cells. Members of the MAPK family of proteins are known to interact with substrates through consensus docking motifs (12). In the case of ERK1/2, two docking sequences have been identified, the D domain and the DEF domain (12). The D domain consists of a cluster of basic amino acids adjacent to a cluster of hydrophobic residues. Many MAPK family members are able to bind to the D domain whereas the DEF domain comprising the amino acid sequence FXFP is specific to ERK1/2 (12). The proline-rich sequence of CdGAP-s contains a consensus DEF domain (FPFP). To determine whether this DEF domain is required for the interaction between ERK1/2 and CdGAP-s, we generated a FPFP to APAP mutant in which the phenylalanine residues at positions 677 and 679 were substituted with alanines. As shown in Fig. 2.4 D, the ability of the FFAA mutant to bind ERK1/2 was significantly reduced compared to the wild type protein, suggesting that the FPFP sequence of CdGAP is required for interacting with ERK1/2.

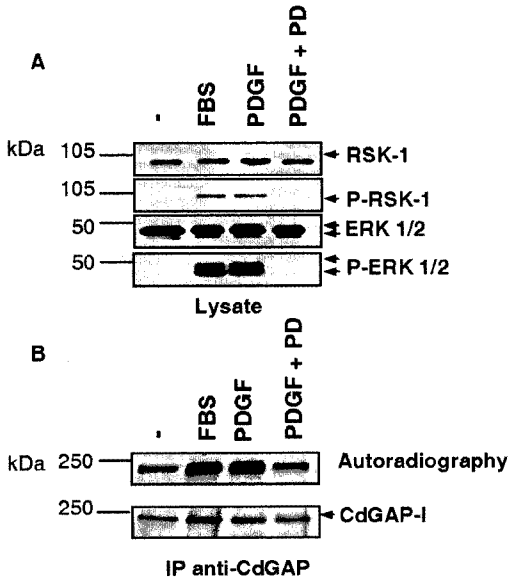
CdGAP-l is phosphorylated *in vivo* in response to serum and PDGF-stimulation via the MAPK pathway

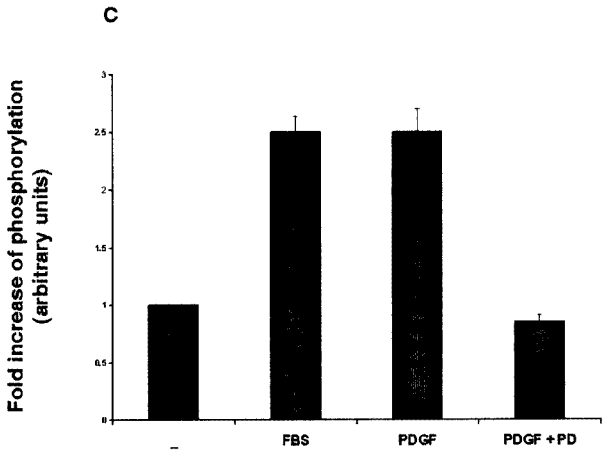
To examine whether endogenous CdGAP is phosphorylated in response to activation of the MAPK pathway, Swiss 3T3 fibroblasts were serum-starved and then incubated in phosphate-free medium supplemented with [^{32}P]-orthophosphate for 2 hours prior to stimulation with Fetal Bovine Serum (FBS) or Platelet Derived Growth Factor (PDGF) for 30 and 5 min, respectively. As expected, both RSK-1 and ERK1/2 are activated after stimulation of the fibroblasts by serum or PDGF (Fig. 2.5A). The activation of these kinases is inhibited in cells incubated with the MEK-1 inhibitor PD98059 prior to stimulation with PDGF (Fig. 2.5A). CdGAP-l, the most abundant CdGAP isoform in Swiss 3T3 cells, shows a 2.5-fold increase in the level of phosphorylation in response to FBS and PDGF and this is inhibited in the presence of the MEK-1 inhibitor PD98059 (Fig. 2.5B and 2.5C). These results suggest that endogenous CdGAP is phosphorylated *in vivo* in response to activation of the MAPK pathway.

The proline-rich domain of CdGAP-s is phosphorylated *in vitro* by ERK-1 and RSK-1

ERK1/2 is a proline-directed kinase that phosphorylates serine and threonine residues that precede proline residues within the Pro-X-(Ser/Thr)-Pro consensus motif. On the other hand, RSK-1 phosphorylates serines and threonines within the Arg-X-X-Ser/Thr consensus motif (389). The amino acid sequence of CdGAP contains three consensus phosphorylation motifs for ERK1/2, located within the proline-rich sequence

Figure 2.5: The MEK-1 inhibitor PD98059 blocks PDGF-stimulated CdGAP phosphorylation. Serum-starved Swiss 3T3 cells were labeled with 0.5 mCi/ml of [^{32}P]-orthophosphate for 2 h and then were either left unstimulated (-) or stimulated with 20% FBS, PDGF (5 ng/mL) alone or PDGF (5 ng/mL) after treatment with 50 μM PD98059 for 1h. (A) Protein cell lysates resolved by SDS-PAGE were immunoblotted with anti-ERK1/2 and anti-RSK-1 antibodies or with polyclonal anti-P-ERK1/2 and anti-P-RSK1 antibodies to show MAP Kinase pathway activation during similar conditions to CdGAP phosphorylation. (B) CdGAP was immunoprecipitated (IP) using anti-CdGAP antibodies and subjected to SDS-PAGE and the radiolabeled proteins were identified by autoradiography (upper panel). The membrane was immunoblotted with anti-CdGAP antibodies to show the total amount of immunoprecipitated CdGAP (lower panel). (C) Quantitative analysis of CdGAP phosphorylation. Fold increase in CdGAP phosphorylation was determined by densitometry and the values correspond to the average of at least three independent experiments.


A
-
FBS
PDGF
PDGF + PD
kDa
105
RSK-1
105
P-RSK-1
50
ERK 1/2
50
P-ERK 1/2
Lysate
B
-
FBS
PDGF
PDGF + PD
kDa
250
Autoradiography
250
CdGAP-I
IP anti-CdGAP


C
Fold increase of phosphorylation
(arbitrary units)
3
2.5
2
1.5
1
0.5
0
-
FBS
PDGF
PDGF +PD

and three consensus RSK-1 phosphorylation sites, two in the central domain and one in the proline-rich sequence (Fig. 2.6A). To examine the ability of ERK-1 and RSK-1 to directly phosphorylate CdGAP, *in vitro* kinase assays were performed with purified His-tagged CdGAP-PRD incubated with recombinant activated ERK-1 or RSK-1. Figure 2.6B shows that CdGAP-PRD is efficiently phosphorylated by ERK-1 *in vitro*. In fact, we found that at similar protein concentrations, MBP and CdGAP are equally good substrates for ERK-1. RSK-1 is also able to phosphorylate CdGAP-PRD although to a lower extent than ERK-1. To determine the content of phosphorylated residues on CdGAP-PRD, we performed a phosphoamino acid analysis on His-tagged CdGAP-PRD, phosphorylated *in vitro* by either ERK-1 or RSK-1. As expected from the consensus phosphorylation motifs (Fig. 2.6A), ERK-1 phosphorylates CdGAP mainly on threonine residues whereas CdGAP is predominantly phosphorylated on serine residues by RSK-1 (Fig. 2.6C). We have also investigated if the FFAA mutant is phosphorylated by ERK1/2 *in vitro*. As shown in Fig. 2.6D, ERK-1 fails to phosphorylate the FFAA mutant, indicating that the FPFP motif is not only important for ERK1/2 binding but also for efficient phosphorylation by ERK-1.

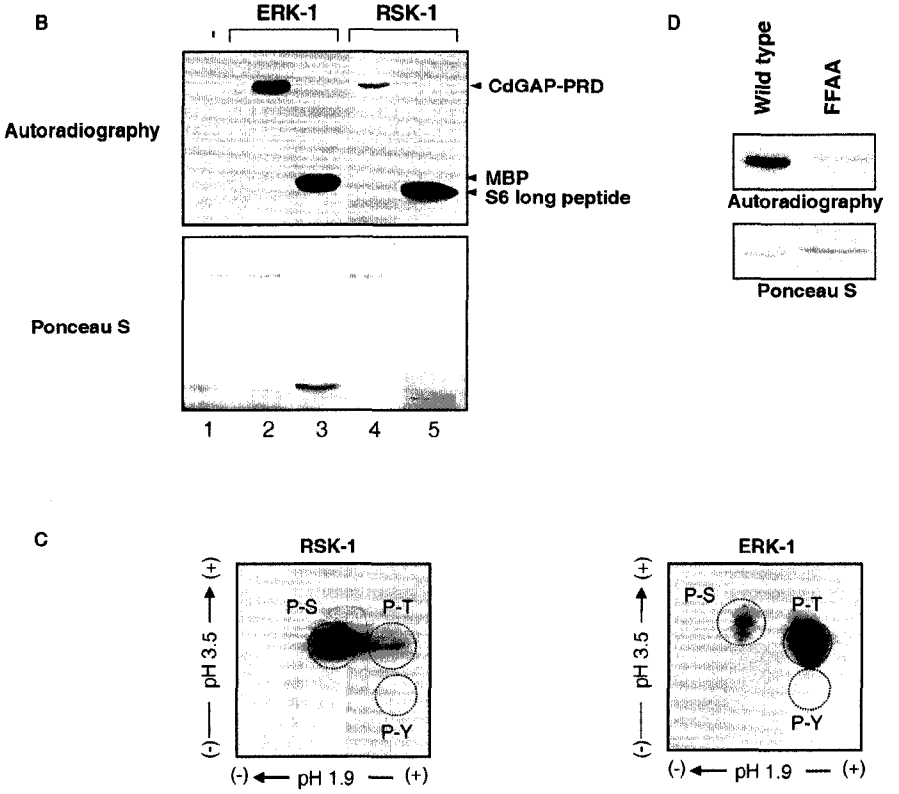
ERK-1 phosphorylates CdGAP on Thr^{769} and Thr^{776} *in vitro*

To identify the phosphorylation sites for ERK-1, each threonine of the three potential ERK-1 phosphorylation sites of CdGAP was substituted for an alanine residue. As shown in Fig. 2.7A, CdGAP-PRD containing the T612A amino acid substitution is phosphorylated by ERK-1 at comparable levels to those of the wild type protein and the tryptic phosphopeptide map does not show any noticeable differences (Fig. 2.7B).

Figure 2.6: RSK-1 and ERK-1 phosphorylate CdGAP *in vitro*. (A) Consensus phosphorylation sites of RSK and ERK in CdGAP protein sequence. The potential phosphorylation site within the consensus motif is shown in bold. (B) An *in vitro* kinase assay was performed by incubating recombinant His-tagged CdGAP-PRD without (lane 1) or with activated ERK-1 (lane 2) or activated RSK-1 (lane 4) in the presence of 10 μCi/ml [γ-^{32}P]-ATP. As positive controls, ERK-1 was incubated with MBP (lane 3) and RSK-1 with long S6 kinase substrate peptide (lane 5). The products were resolved by SDS-PAGE and phosphorylated substrates were detected by autoradiography (upper panel). Comparable amounts of substrate are shown by Ponseau S staining (lower panel). (C) His-tagged CdGAP-PRD proteins phosphorylated by either ERK-1 or RSK-1 were subjected to phosphoamino acid analysis. (D) *In vitro* kinase assay with His-tagged CdGAP-PRD and CdGAP-PRD-FFAA and activated ERK-1 (autoradiograph, upper panel). Protein loading was determined by Ponceau-S staining (bottom panel)

A

RSK consensus sequence RXXS	ERK Consensus sequence PX(S/T)P
Central: 267 ERRENSL 273	PRD-3: 608 PKPSTPQ 614
Central: 290 NKRKLSS 296	PRD-5: 765 SPPLTPA 771
PRD-5: 760 GPRNLSP 766	PRD-5: 772 PPPPTPL 778



However, the phosphorylation of CdGAP-PRD (T769A) and the double mutant CdGAP-PRD (T769AT776A) by ERK-1 are significantly reduced compared to wild type CdGAP-PRD (Fig. 2.7A). In fact, the tryptic phosphopeptide map of the T769A mutant protein reveals that phosphorylation of peptide a is absent and phosphorylation of peptide c is reduced compared to the wild type protein (Fig. 2.7B). In the phosphopeptide map of the double mutant T769AT776A, we find that phosphorylation of peptides a, b and c has disappeared (Fig. 2.7B). Since Thr^{769} and Thr^{776} are located on the same tryptic peptide, these results suggest that phosphopeptides a, b and c are the result of partial tryptic digestion. Although total phosphorylation of the T776A mutant protein by ERK-1 is slightly reduced (Fig. 2.7A), tryptic phosphopeptide mapping (Fig. 2.7B) clearly indicates that phosphopeptide a is missing and that phosphopeptide c is increased compared to the wild type protein. These data suggest that in the absence of threonine 776, threonine 769 becomes hyperphosphorylated by ERK-1. Altogether, these findings demonstrate that Thr^{769} and Thr^{776} are phosphorylation target sites of ERK-1 *in vitro*.

Thr^{776} is an *in vivo* phosphorylation site of CdGAP-s

To determine if CdGAP is a physiological substrate for ERK-1, we mixed the phosphopeptides obtained by tryptic digestion of recombinant His-tagged CdGAP-PRD phosphorylated with ERK-1 *in vitro* with those obtained from immunoprecipitated CdGAP phosphorylated *in vivo* in COS-7 cells. As shown in figure 2.8A, phosphopeptides 1, 4 and 6 from the *in vivo* phosphopeptide map co-migrate with phosphopeptides c, d and e from the *in vitro* map, suggesting that ERK-1 phosphorylates CdGAP *in vitro* and *in vivo* on similar sites. We then compared the *in vivo*

Figure 2.7: Thr^{769} and Thr^{776} in the proline-rich domain of CdGAP are phosphorylated *in vitro* by ERK-1. (A) *In vitro* phosphorylation of His-tagged CdGAP-PRD wild type (WT) or the indicated alanine mutants (T612A, T769A, T776A and T769AT776A) by recombinant activated ERK-1 (upper panel). The bottom panel corresponds to Ponceau S-staining indicating that equal amounts of protein were used. WT and T776A are on a separate gel containing lower amounts of proteins. (B) Two-dimensional separation of tryptic phosphopeptides derived from wild type CdGAP-PRD, -T612A, T769A, -T776A or -T769AT776A. Letters a to f designate the six most abundant phosphopeptides present in CdGAP-PRD wild type. Equal counts of samples were applied onto the TLC plates and autoradiographs with the same exposure times are presented for each sample. Arrows in panel B indicate the reduction or absence of phosphopeptides a, b and c. (•) indicates origin of migration.

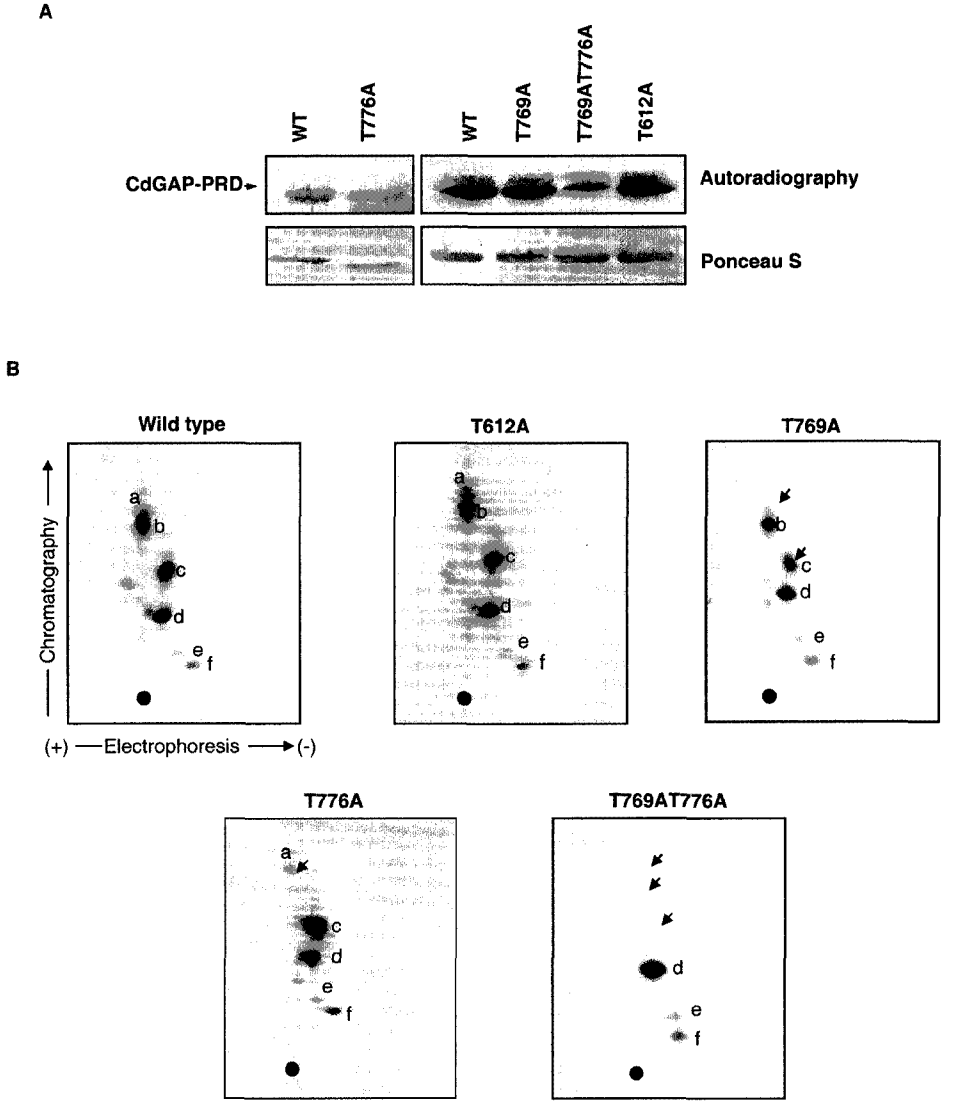
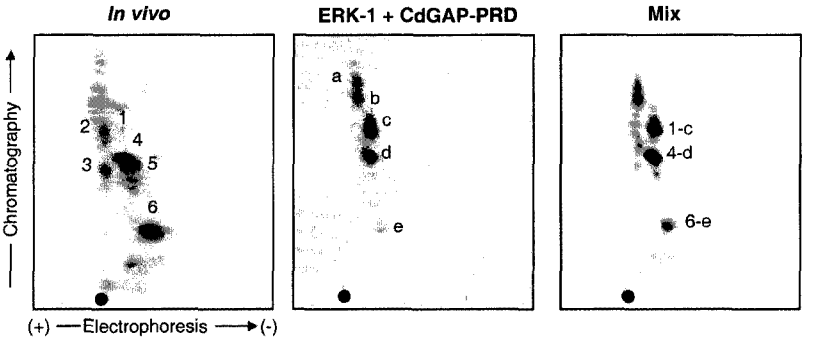
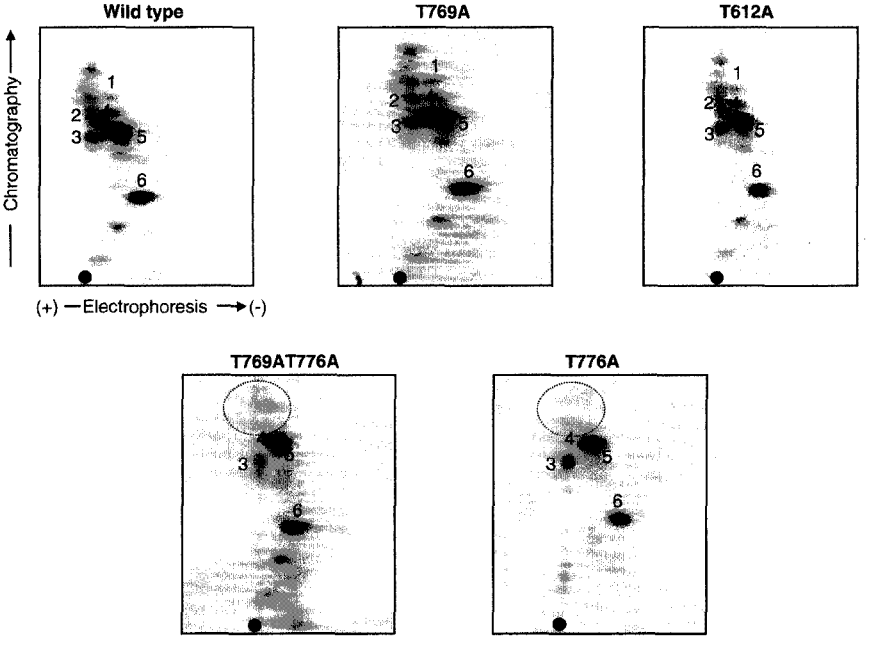

A
WT
T776A
WT
T769A
T769AT776A
T612A
CdGAP-PRD
Autoradiography
Ponceau S
B
Wild type
T612A
T769A
T776A
T769AT776A
Chromatography
(+) Electrophoresis (-)
a
b
c
d
e
f

Figure 2.8: Thr^{776} is an *in vivo* phosphorylation site of CdGAP. (A) Comparison of the tryptic phosphopeptide maps between *in vivo* and *in vitro* phosphorylated CdGAP by ERK-1. Tryptic phosphopeptide map derived from *in vivo* ^{32}P-labeled Myc-CdGAP-s immunoprecipitated from COS-7 cell lysate (left panel). Tryptic phosphopeptide map of His-tagged recombinant CdGAP-PRD phosphorylated *in vitro* by activated ERK-1 (middle panel). Tryptic phosphopeptide map derived from a mixture of the *in vitro* and *in vivo* phosphopeptides (right panel). Equal counts of samples were applied onto the TLC plates and autoradiographs with the same exposure times are presented for each sample. Phosphopeptides from the *in vitro* map that co-migrate with the *in vivo* phosphopeptides are indicated in right panel (1-c, 4-d, 6-e). (B) *In vivo* Phosphopeptide mapping of ^{32}P-labeled Myc-CdGAP wild type or the indicated protein mutants. Dashed circle indicates the missing phosphopeptides. (•) indicates origin of migration.

A


In vivo
ERK-1 + CdGAP-PRD
Mix
Chromatography
(+) —Electrophoresis —→(-)
1
2
3
4
5
6
a
b
c
d
e
1-c
4-d
6-e

B

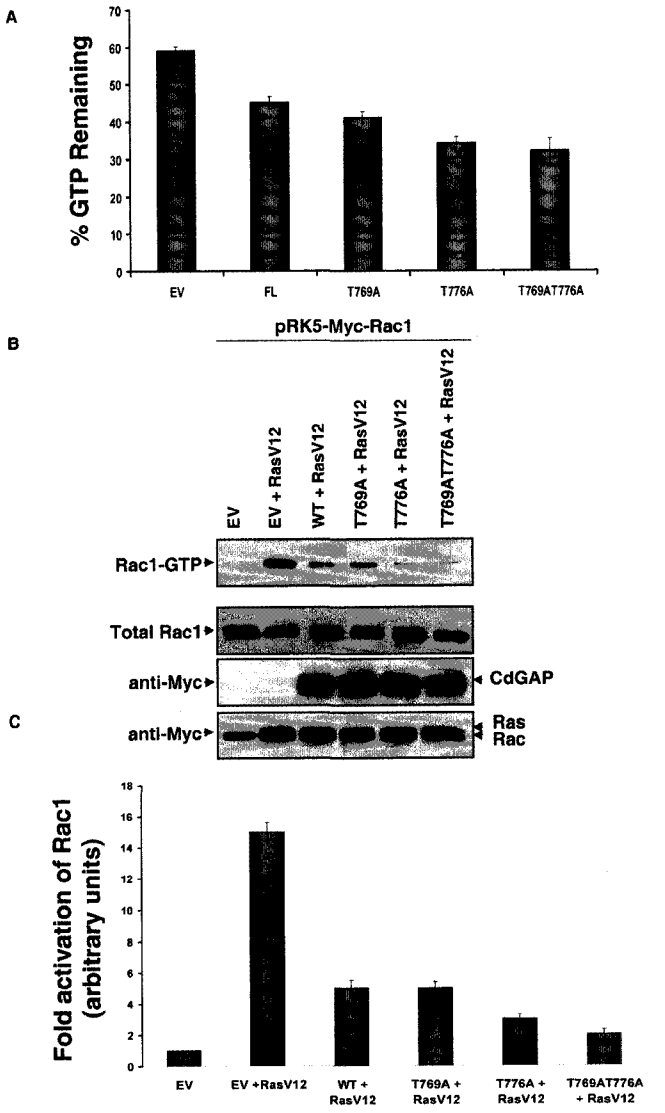

Wild type
T769A
T612A
T769AT776A
T776A
Chromatography
(+) —Electrophoresis —→(-)
1
2
3
4
5
6

phosphopeptide patterns of CdGAP mutants to that of wild type CdGAP expressed in COS-7 cells. We found that the *in vivo* phosphorylation patterns of both T769A and T612A CdGAP protein mutants are very similar to that of the wild type protein (Fig. 2.8B). However, phosphopeptides 1 and 2 are absent in the *in vivo* map of both T776A and the double mutant T769AT776A. Together, these results strongly suggest that CdGAP is an *in vivo* substrate of ERK-1 and that Thr^{776} is a phosphorylation site *in vivo*.

T776A and T769AT776A mutant proteins have increased GAP activity towards Rac1 in response to oncogenic Ras

To assess whether threonine phosphorylation of CdGAP is involved in the regulation of its GAP activity, [^{32}P]-GTP-loaded Rac1 was incubated with myc-tagged CdGAP or with myc-tagged CdGAP threonine mutant proteins immunoprecipitated from COS-7 cell lysates. The GTPase stimulating activity of CdGAP was measured by the estimation of the ratio of Rac1-bound GTP in the absence versus the presence of CdGAP as described previously (325). As shown in Fig. 2.9A, 45 % of GTP remains bound to Rac1 after a 6 min incubation in the presence of immunoprecipitated CdGAP compared to 59% of GTP bound to Rac1 incubated with immune complexes from lysates of COS-7 cells transfected with empty vector. However, the GAP activity of the T776A and T769AT776A mutant proteins shows a significant 1.4- and 1.5-fold increase compared to the wild type protein, respectively. The GAP activity of the T769A mutant protein appears to be only weakly affected compared to the wild type protein. To further support these findings, we investigated the *in vivo* GAP activity of CdGAP wild type and threonine mutant proteins towards Rac1 activated by RasV12 in serum- starved HEK 293

Figure 2.9: Phosphorylation of Thr^{776} reduces CdGAP activity *in vitro* and *in vivo.* (A) [γ-^{32}P]-GTP-loaded Rac1 was incubated at 20 ^{0}C with anti-Myc immune complexes from lysates of COS-7 cells transfected with empty vector (EV) or pRK5 encoding Myc-tagged CdGAP-s (FL), -T769A, -T776A or T769AT776A and a GAP assay was performed. Equal amount of immunoprecipitated CdGAP and CdGAP protein mutants was estimated in each sample by coomassie blue-stained SDS-PAGE by comparison with different amount of purified bovine serum albumin (B) HEK293 cells were transfected with empty vector (EV) or pRK5 encoding Myc-tagged CdGAP-s (WT), -T769A, -T776A or T769AT776A together with Myc-Rac1 and Myc-RasV12. GTP-loaded Rac1 proteins were pulled-down from cell lysates using GST-PAK1 (amino acids 56-272). GTP-bound Rac1 and total Rac1 in protein cell lysates were detected by western blotting using anti-Rac1 antibodies. The amount of CdGAP and myc-tagged RasV12 in protein cell lysates were revealed by western blotting using anti-myc antibodies. (C) Quantitative analysis of Rac1 activation by RasV12 in the absence or presence of CdGAP wild type and threonine protein mutants. Error bars represent standard deviations relative to three separate experiments.


A
% GTP Remaining
70
60
50
40
30
20
10
0
EV
FL
T769A
T776A
T769AT776A
B
pRK5-Myc-Rac1
EV
EV + RasV12
WT + RasV12
T769A + RasV12
T776A + RasV12
T769AT776A + RasV12
Rac1-GTP
Total Rac1
anti-Myc
CdGAP
C
anti-Myc
Ras
Rac
Fold activation of Rac1 (arbitrary units)
18
16
14
12
10
8
6
4
2
0
EV
EV +RasV12
WT + RasV12
T769A + RasV12
T776A + RasV12
T769AT776A + RasV12

cells. The GTPase stimulating activity of CdGAP was measured by the amount of GTP-bound Rac1 obtained in a pull-down assay using GST-CRIB domain of PAK. As shown in Fig. 2.9B and 2.9C, RasV12 stimulated a 15-fold increase in the level of activated Rac1. In the presence of wild type CdGAP or the T769A mutant proteins, the levels of activated Rac1 show a 3-fold reduction compared to RasV12 alone. However, both T776A and T769AT776A mutant proteins show a higher GAP activity resulting in a 5- and 7.5-fold reduction in the levels of activated Rac1 in response to RasV12, respectively. These results correlate well with the *in vitro* GAP assays and indicate that phosphorylation of at least one threonine residue in the CdGAP protein sequence downstream of the Ras/ERK signaling pathway is sufficient to modulate the intrinsic GTPase stimulating activity towards Rac1. We conclude that phosphorylation of Thr^{776} plays an important regulatory role in the GAP activity of CdGAP.

DISCUSSION

In the present study, we show that the proline-rich domain of CdGAP is phosphorylated *in vivo* at multiple sites containing serine and threonine residues. We also demonstrate that CdGAP is phosphorylated downstream of the MEK-ERK pathway in response to serum or PDGF stimulation of fibroblasts. In particular, we find that CdGAP interacts with both ERK1/2 and RSK-1 and is directly phosphorylated by ERK-1 and RSK-1 *in vitro*. Site-directed mutagenesis reveals that threonine 776 of CdGAP is a phosphorylation site for ERK-1 and is an important regulatory site of CdGAP activity.

The incredibly large number of RhoGAP proteins strongly suggests a tight regulation of their activities at specific sites within the cell. Indeed, accumulating evidence indicates that RhoGAP activities are regulated by a wide variety of mechanisms, including phosphorylation. For example, tyrosine phosphorylation of p190RhoGAP by Src is necessary for its association with p120RasGAP and activation of its rhoGAP activity *in vivo (380, 390)*. On the other hand, the *in vitro* GAP activity of RICS, a GTPase-activating protein for Cdc42 and Rac1, is inhibited by phosphorylation from Ca^{2+}/calmodulin-dependent protein kinase II (314). Interestingly, MgcRacGAP known to be involved in cytokinesis and a GAP for Rac1 and Cdc42, is functionally converted to a GAP for RhoA following serine phosphorylation by Aurora B kinase (275). In this paper, we found that the replacement of threonine 776 by an alanine within the proline-rich domain of CdGAP is sufficient to induce a 1.5- and 2.0-fold increase in the *in vitro* and *in vivo* CdGAP activity towards Rac1, respectively. These findings suggest that phosphorylation of threonine 776 is an important regulatory site of CdGAP activity and may lead to a conformational change affecting the enzymatic activity.

However, it is clear that CdGAP contains additional phosphorylation sites within the proline-rich domain that may affect the GAP activity as well. Future identification of these phosphorylation sites will help to understand better the mechanism of regulation of CdGAP activity. In addition, it is more than likely that phosphorylation of the proline-rich domain may affect the localization of the protein or alter protein-protein interactions. Indeed, CdGAP contains five consensus Src homology 3 (SH3)-binding sites. We however, have not yet been able to identify any SH3 domain-containing proteins binding to these motifs. Interestingly, Thr^{776} is located directly in the fifth proline-rich sequence PPLTPAPPPPTP. Therefore, it is possible that phosphorylation of serine or threonine residues within the proline-rich domain cause a conformational change that negatively regulates their ability to bind SH3 domains. Similarly, it has been reported that phosphorylation of the proline-rich sequence of SOS is important to modulate its interaction with the SH3 domain-containing adaptor molecule Grb2 (391).

To identify potential Ser/Thr kinases that interact with CdGAP, we have performed an in-gel kinase assay. The success of this technique depends greatly on the ability of kinases to renature in the polyacrylamide gel. Several lines of evidence have demonstrated that RSK and ERK1/2 are able to efficiently recover their kinase activity following in-gel renaturation (387, 388). We found striking similarities between the in-gel kinase profile reported in these previous studies and our own results. In fact, we have found that the two kinase activities recovered from the in-gel kinase are indeed ERK1/2 and RSK-1. Consistent with these results, we observed that CdGAP is present in a ternary protein complex including ERK1/2 and RSK-1. Moreover, we presented evidence that the proline-rich domain of CdGAP is directly phosphorylated by ERK-1 and RSK-1 *in*

vitro on distinct sites. These results suggest that both enzymes can phosphorylate CdGAP independently of each other. However, since RSK-1 phosphorylates CdGAP significantly less than ERK-1 *in vitro*, it remains to be determined whether pre-phosphorylation of CdGAP by ERK-1 or another kinase leads to a better substrate for RSK-1. It is also possible that efficient phosphorylation of CdGAP by RSK-1 requires the full-length protein and not only the proline-rich sequence. In fact, although the PRD domain of CdGAP contains a minimal RSK recognition phosphorylation site, the two additional ones in the central domain are preceded by arginine residues, which could make these sites more favorable for RSK-1. The levels of kinase activity obtained in the In Gel Kinase experiment suggested that RSK-1 is more efficient than ERK-1 to phosphorylate the PRD of CdGAP but the data obtained from the *in vitro* kinase assay using recombinant activated kinases suggests the opposite. These conflicting results can be explained by at least two possibilities. First, it is possible that RSK-1 recovers more efficiently its kinase activity after renaturation than ERK1/2. Second, RSK-1 seems to be more abundant than ERK-1/2 in co-immunoprecipitation assays with CdGAP.

In this study, we have shown that the majority of the *in vivo* phosphorylation sites of CdGAP are on serine residues. Since RSK-1 interacts with and phosphorylates CdGAP on serine residues, it is likely that RSK-1 is responsible for most of the *in vivo* serine phosphorylation. In particular, treatment of Swiss 3T3 cells with the MEK inhibitor which blocks both ERK1/2 and RSK-1 activation also completely abolishes PDGF-induced CdGAP phosphorylation. We are currently investigating the role of RSK-1 phosphorylation on CdGAP and future studies will provide valuable knowledge on this issue. Nevertheless, our studies clearly demonstrate that although the extent of threonine

phosphorylation *in vivo* is weak, the importance of this phosphorylation on CdGAP activity is significant.

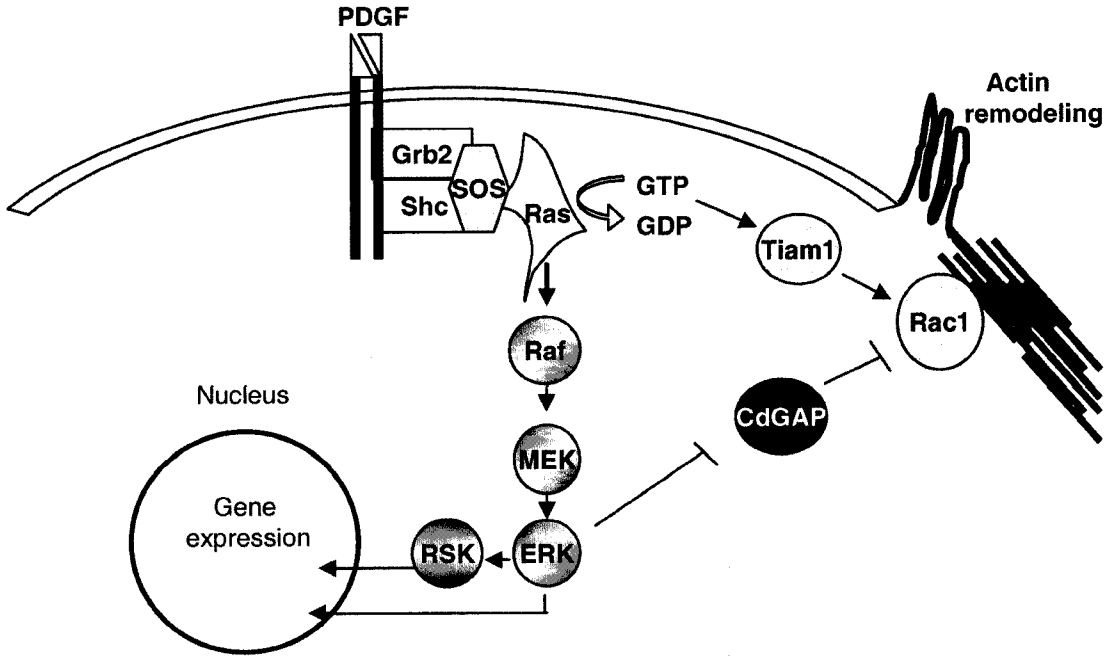
The carboxy terminal tail of CdGAP contains a number of putative ERK phosphorylation sites containing the consensus sequence P-X-S/T-P (392). The *in vitro* and *in vivo* phosphopeptide mapping of CdGAP protein mutants strongly support the conclusion that ERK-1 phosphorylates CdGAP on Thr^{776} *in vivo*. However, amino acid substitution of both Thr^{769} and Thr^{776} to alanine did not completely abolish ERK-1 phosphorylation of CdGAP *in vitro*. In addition to the three ERK putative phosphorylation sites mutated in this study, the proline-rich domain of CdGAP contains twelve S/T-P motifs containing the minimum consensus motif for ERK phosphorylation (330). Interestingly, two of these sites are adjacent to a putative DEF domain containing the FPFP motif known to be an ERK docking site (393). Indeed, mutation of this motif alters CdGAP binding to ERK1/2 and leads to a loss of CdGAP phosphorylation by ERK-1 *in vitro*.

CdGAP belongs to a novel family of RhoGAP proteins that are phylogenetically well conserved among different species. Up to three human genes encode for CdGAP-related proteins which consist of a rhoGAP domain at the N-terminus and multiple SH3-binding motifs at the C-terminus of the proteins. Interestingly, the DEF domain and the sequence surrounding Thr^{776} are found only in CdGAP and not in the closely related Grit or TCGAP. This suggests that ERK may exclusively interact with and phosphorylate CdGAP. In fact, among all the characterized RhoGAP proteins, only mouse CdGAP and human DLC-1 proteins contain a DEF domain. Interestingly, we observed the differential expression of at least two major isoforms of CdGAP in specific mouse tissues. CdGAP-1

(250 kDa) is predominantly expressed in the brain, lung and heart whereas CdGAP-s (90 kDa) is predominantly expressed in the liver and kidney. Whether the differential expression of CdGAP leads to a tissue specific function for each isoform will require further investigation. We also found that both overexpressed CdGAP-s and –l migrates higher than their expected molecular weights of 90 kDa and 155 kDa, respectively. It will be of great interest to investigate the post-translational modifications responsible for this impressive mobility shift.

Thus far, one of the most exciting roles attributed to RhoGAP proteins is their implication in the cross-talk between members of the Rho family of small GTPases. For example, p120RasGAP interacts with and regulates p190RhoGAP activity, suggesting a possible interplay between Ras and Rho GTPases (394). The connection between the Ras/MAP kinase pathway and the effects on cytoskeletal dynamics becomes more evident with the identification of a number of cytoskeleton-related proteins as ERK and RSK substrates (395-398). Here we have demonstrated that phosphorylation of Thr^{776} by ERK affects CdGAP activity both *in vitro* and *in vivo*. One possibility is that mitogenic signal regulates Rac1 through phosphorylation and downregulation of CdGAP activity by ERK, leading to Rac1 activation and cytoskeletal remodeling (Figure 2.10). In conclusion, we provide evidence that CdGAP is a novel ERK substrate and may play roles in the connection between the Ras/MAPK and Rac1 pathways.

Figure 2.10: CdGAP mediates cross-talk between the Ras/MAPK pathway and the regulation of Rac1 activity. Upon PDGF stimulation, Ras activates the MAPK pathway leading to gene expression and phosphorylation of many cytoplasmic and membrane proteins. In addition, Ras causes cytoskeleton remodeling by activating a GEF (Tiam-1) for Rac1. We propose that ERK downregulation of CdGAP activity is an additional mechanism by which Ras can maintain active Rac1 in response to growth factor stimulation.


PDGF
Actin
remodeling
Grb2
SOS
Shc
Ras
GTP
GDP
Tiam1
Rac1
Raf
Nucleus
CdGAP
MEK
Gene
expression
RSK
ERK

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Preface To Chapter 3

Previous to this study we had known that the PRD of CdGAP is important for the regulation of CdGAP by intersectin, however it was not necessary for the interaction of CdGAP and intersectin. In order to better understand how this region may be involved in regulation of GAP activity we undertook studies to identify binding partners of this region. We identify GSK-3 as a binding partner, and we characterize its interaction with CdGAP. We also characterize the phosphorylation of CdGAP by GSK-3, furthering the knowledge of phospho-regulation of CdGAP that we had gained in chapter 2. In addition to this we explore regulation of CdGAP by serum and look at the effect of serum stimulation on CdGAP mRNA and protein levels.

CHAPTER 3

GLYCOGEN SYNTHASE KINASE-3 PHOSPHORYLATES CDGAP AT A CONSENSUS ERK 1 REGULATORY SITE

GLYCOGEN SYNTHASE KINASE-3 PHOSPHORYLATES CDGAP AT A CONSENSUS ERK 1 REGULATORY SITE

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Running Title: GSK-3 regulates CdGAP

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ABSTRACT

Rho GTPases regulate a multitude of cellular processes from cytoskeletal reorganization to gene transcription and are negatively regulated by GTPase-activating proteins (GAPs). CdGAP is a ubiquitously expressed GAP for Rac1 and Cdc42. In this study, we set out to identify CdGAP binding partners and, using a yeast two-hybrid approach, GSK-3α was identified as a partner for CdGAP. GSK-3 exists in two isoforms, α and β, and is involved in regulating many cellular functions from insulin response to tumorigenesis. We show that GSK-3α and β interact with CdGAP in mammalian cells. We also demonstrate that GSK-3 phosphorylates CdGAP both *in vitro* and *in vivo* on Thr-776 which we have previously shown to be an ERK1/2 phosphorylation site involved in CdGAP regulation. We report that CdGAP's mRNA and protein levels are increased upon serum stimulation, and that GSK-3 activity is necessary for the up-regulation of CdGAP's protein levels, but not for the increase in mRNA. We conclude that GSK-3 is an important regulator of CdGAP, and that regulation of CdGAP protein levels by serum presents a novel mechanism for cells to control Cdc42/Rac1 GTPase signaling pathways.

INTRODUCTION

The Rho subfamily of small GTPases controls a wide variety of cellular functions. RhoA, Rac1, and Cdc42 are the best known members of this family, and they are most often associated with their roles as regulators of cytoskeleton re-modeling, and as key mediators of the activation of transcription of genes downstream of growth factor receptors (399). Much evidence exists linking Rho GTPases to transformation of cells, however, contrary to the Ras gene, activating mutations in Rho genes are rarely found in human cancers (65, 400). It seems instead that the expression of Rho GTPases, and expression and function of regulators of the Rho subfamily of GTPases is altered during cellular transformation (65, 400).

Rho GTPases act in a cycle as molecular switches with an active GTP-bound form and an inactive GDP-bound form (399). The GTPase-activating proteins (GAPs) negatively regulate the GTPases by enhancing the hydrolysis of GTP to GDP (399). To date, approximately 70 human genes are predicted to encode for potential RhoGAP proteins (401), which is roughly triple the number of Rho GTPases (399). This lends weight to the notion that regulators like the RhoGAPs tightly control Rho GTPases and lend context-dependant specificity to their processes. Thus, the activity of RhoGAPs must be highly regulated in both spatial and temporal fashions. RhoGAPs are regulated at the protein level by a variety of mechanisms ranging from protein-protein interactions to phosphorylation, lipid interactions, and proteolytic degradation (377).

Cdc42 GTPase-Activating-Protein (CdGAP) has been shown to regulate both Cdc42 and Rac1 *in vitro* and *in vivo*, and exists in two main isoforms, a short form of 820

amino acids containing an N-terminal RhoGAP domain, a central region, and a C-terminal proline-rich region (PRD) (321, 325); and a long isoform comprising the entire short form with an additional C-terminal region extending to 1425 amino acids total (402). Recently, we demonstrated that CdGAP activity is negatively controlled by protein-protein interactions via the endocytic protein intersectin (325) and by phosphorylation within its PRD (402).

In this study, we used a yeast two-hybrid approach to look for binding partners for the PRD of CdGAP and found Glycogen Synthase Kinase-3α (GSK-3α) as an interacting partner. GSK-3α and its closely related isoform GSK-3β are serine/threonine protein kinases initially identified as key enzymes in the regulation of glycogen metabolism by insulin and are now known to be implicated in many diverse cellular processes including tumorigenesis, cell survival, and developmental patterning (353, 355). We demonstrate that GSK-3β can also bind CdGAP. We further report that GSK-3 phosphorylates CdGAP *in vivo* under serum-starved conditions where GSK-3 is most active. GSK-3 phosphorylates CdGAP both *in vitro* and *in vivo* at Thr-776, which we have previously shown to be an ERK1/2 phosphorylation site involved in CdGAP regulation (402). We demonstrate that CdGAP's mRNA and protein levels are up-regulated in response to serum in a transcriptionally mediated manner, and that GSK-3 activity is critical in the upregulation of protein levels, but not mRNA levels.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies - Lithium Chloride, AR-A014418, SB 415286, Actinomycin D (ActD), and 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) were purchased from Sigma-Aldrich Canada, Sodium Chloride was from Fisher Scientific. Anti-GSK-3α antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Anti-GSK-3β antibody was from Cell Signaling Technologies, anti-GSK3 (both α and β) antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti-phospho-GSK-3 antibody, anti-phospho-Ser-641 Glycogen Synthase, and anti-Glycogen Synthase antibodies were from Cell Signaling Technologies. [^{32}P]ATP (3,000 Ci/mmol) and [^{32}P]orthophosphate (3,000 μCi/ml) were purchased from Perkin-Elmer. Recombinant GSK-3α and β, and phospho-glycogen synthase peptide were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal anti-CdGAP antibodies were produced and purified as previously described (322, 402). Polyclonal antibodies against phospho-Thr-776 of CdGAP were produced by immunizing rabbits with a peptide CXPPPPT*PLEEEPE (T* represents phosphorylated threonine, X represents hexanoic acid spacer). Serum from the immunized rabbits was taken and affinity purified against the same peptide. This fraction was taken and run through a column containing a non-phosphorylated peptide CXPPPPTPLEEEPE and the flow-through (antibody unable to bind the non-phosphorylated residue) was kept. Myc-tagged proteins were detected by using the 9E10 anti-Myc monoclonal antibody (MAb), which was kindly provided by Dr. Nicole Beauchemin (McGill University, Montreal, Canada).

DNA constructs - CdGAP constructs were as previously described (322, 325, 402). GSK-3α was obtained as a full clone in the pACTII vector (see yeast-two hybrid screen). It was subcloned into pRK5 vector using the XhoI/EcoRI restriction sites. GSK-3β cDNA was kindly provided by Dr. James Woodgett (Ontario Cancer Instititute, Toronto, Canada).

Yeast Two-Hybrid Screen - The PRD of CdGAP (aa 516-820) was fused to GAL4 DNA-binding domain (using a pYTH6 vector) and stably integrated into the genome of the Y190 strain of *S. cerevisiae*. This was used to screen a library of human brain cDNAs fused to the GAL4 Activation Domain (pACTII vector) (A kind gift from Dr. Alan Hall, Sloan-Kettering, New York, USA) (403), as previously described (404). Approximately $6x10^6$ clones were screened for their ability to grow on selective medium containing 25 mM 3-amino-1,2,4-triazole. The 80 fastest growing clones were replated, and plasmids were isolated using the Wizard clean-up kit (Promega), and re-transformed into the CdGAP-PRD yeast strain. The clones growing on selective medium and positive for β-Galactosidase activity were sequenced. Six clones corresponded to the entire coding sequence of GSK-3α.

Cell Transfection and Immunoprecipitation – HEK-293, NIH 3T3 and U2OS cells (the latter kindly provided by Dr. Christopher E. Turner, SUNY Upstate Medical University, Syracuse, New York, USA) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics and maintained at an atmosphere of 5% CO_2 at 37°C. HEK-293 cells were transfected using lipofectamine

(Invitrogen) according to the manufacturer's protocol. Briefly, 2 μg total of DNA (1 μg of either GSK-3α or GSK-3β along with 1 μg of either empty vector, pRK5-myc-CdGAP-s, pRK5myc-CdGAP-l, or pRK5myc-CdGAP-PRD) was transfected per 60mm-dish. Cells were lysed 24 hours post-transfection in (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, 10 μg of leupeptin/ml, 20 mM NaF, and 1 mM sodium orthovanadate, followed by centrifugation for 15 min at 1,000 x g. Then, 1 mg of the resulting postnuclear supernatant was incubated overnight at 4°C with 5 μg of anti-Myc antibodies and 25 μl of 50% protein G-Sepharose (Pharmacia Biotech). Samples were washed three times in lysis buffer and subjected to SDS-PAGE, followed by immunoblotting analysis with anti-Myc, anti-GSK-3α or GSK-3β antibodies. NIH 3T3 cells were plated at a density of $4x10^5$ cells per 100 mm dish. Twenty-four hours later, cells were serum-starved for twenty hours followed by stimulation with 15% serum for various periods of time. The cells were lysed as described above and the amount of protein was quantified using a Pierce BCA kit. Equal amounts of total protein from the various stimulation conditions were submitted to SDS-PAGE and Western blotting. U2OS cells were plated at a density of $2x10^6$ cells per 100mm dish. Twenty-four hours later, cells were serum-starved for twenty hours followed by a four hour stimulation with GSK-3 inhibitors. The cells were lysed as described above and the amount of protein was quantified using a Pierce BCA kit. Equal amounts of total protein were submitted to SDS-PAGE and Western blotting.

In vivo [^{32}P]-orthophosphate labeling - NIH 3T3 cells were serum-starved overnight. The cells were either left untreated or were treated with 50 mM LiCl, or 50mM NaCl, or 0.1% DMSO, or 100 μM SB 415286, or 100 μM AR-A014418 for one hour. This was followed by a one hour incubation in either phosphate-free medium or phosphate-free medium treated with the above-mentioned compounds for one hour. Cells were then incubated for two hours in phosphate-free medium supplemented with 0.5 mCi of [^{32}P]orthophosphate per ml, with or without treatment with the above-mentioned compounds. The cells were lysed, and CdGAP was immunoprecipitated using polyclonal anti-CdGAP antibodies (402) overnight at 4° C. The samples were submitted to SDS-PAGE followed by transfer to nitrocellulose and then autoradiography and Western blotting against CdGAP.

In Vitro Kinase Assay and Phospho-Amino Acid Analysis - Hexahistidine fusion proteins—CdGAP-PRD, or CdGAP-PRD-T776A were produced and purified as previously described (402). His-tagged CdGAP-PRD or CdGAP-PRD-T776A were incubated with 10 ng of active GSK-3α or GSK-3β (Upstate) in 8 mM MOPS pH 7.0, 200 nM EDTA, 10 mM $MgCl_2$, 100 μM ATP and 10 μCi/mL γ-[^{32}]P-ATP for 10 minutes at 30°C. The reaction was stopped by the addition of Laemmli buffer. The samples were submitted to SDS-PAGE, transferred to nitrocellulose or PVDF membrane and phosphorylated proteins were visualized by autoradiography. A range of CdGAP-PRD concentrations from 18.2nM to 9.32 μM were used to estimate the Km value using the Lineweaver-Burk equation (405). Phosphoamino acid analysis was performed as described previously (384, 402).

Quantitative RT-PCR - NIH 3T3 cells were serum-starved overnight. The cells were either left untreated or were treated with 15% serum, or 15% serum with tanscriptional or GSK-3 inhibitors for various times. Total RNA was extracted using a Qiagen RNeasy kit (Qiagen). mRNA was reverse-transcribed using enzymes from Invitrogen. The cDNA was then run in a quantitative real-time PCR reaction using a Roche Lightcycler, Qiagen Quantitect Sybr green reagents, and CdGAP primers obtained from Geneglobe. 18s ribosomal subunit primers (a kind gift from Dr. Simon Wing, McGill University, Montreal, Canada) were used as a loading control.

RESULTS

The proline-rich domain (PRD) of CdGAP harbours 5 consensus SH3-binding motifs and is known to be necessary in the regulation of CdGAP activity (325). To identify target proteins that interact with CdGAP through this region of the protein we used the PRD (aa 516-820) as bait in a yeast two-hybrid screen with a human brain cDNA library. GSK-3α was isolated as a positive clone growing on selective medium (Fig. 3.1A), and expressing β-galactosidase (Fig. 3.1B). To examine whether CdGAP interacts with both GSK-3 α and β in mammalian cells, myc-tagged CdGAP-long (CdGAP-l), short (CdGAP-s), or PRD (CdGAP-PRD) and GSK-3α (Fig. 3.2A) or β (Fig. 3.2B) were transfected into HEK-293 cells and CdGAP-s, CdGAP-l and CdGAP-PRD were immunoprecipitated using anti-myc antibodies. As shown in Fig. 3.2A and 3.2B, both GSK-3α and β co-immunoprecipitated with CdGAP-s and -l, as well as with the PRD of CdGAP in mammalian cells. Taken together, these data indicate that CdGAP interacts with both GSK-3α and β.

We have previously shown that both CdGAP-s and CdGAP-l are highly phosphorylated on serine and threonine residues in fibroblasts (322, 402). Moreover, we have demonstrated that CdGAP-l is *in vivo* phosphorylated at a basal level in serum-starved conditions where GSK-3 is mostly active (355, 402), and further phosphorylation is stimulated via the MAPK pathway in response to serum (402). To investigate whether endogenous CdGAP phosphorylation is mediated by GSK-3, NIH 3T3 cells endogenously expressing the long isoform of CdGAP (CdGAP-l) were serum-starved overnight and were then left untreated, or were treated with either the GSK-3 inhibitors

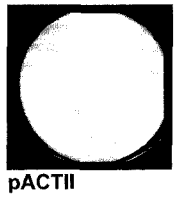
Figure 3.1: CdGAP interacts with GSK-3 in a Yeast Two-Hybrid Screen. A Y190 yeast strain stably expressing Gal4 DNA-binding domain fused to CdGAP-PRD was transformed with pACTII or pACTII-GSK-3α. **A.** Growth on 3-AT medium plates. **B.** β-galactosidase assay (shown after three hours incubation).

A

pACTII

pACTII-GSK-3α

B

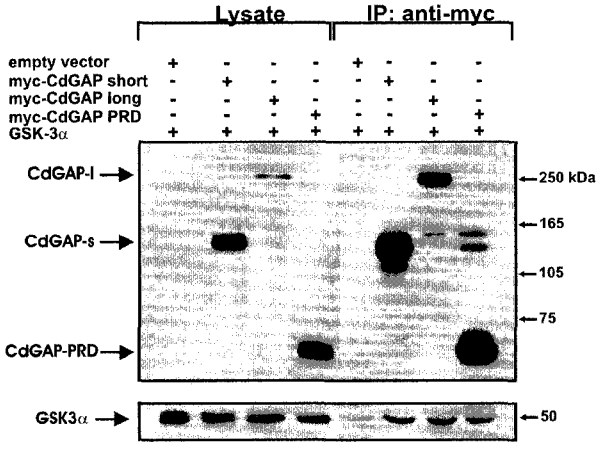


pACTII

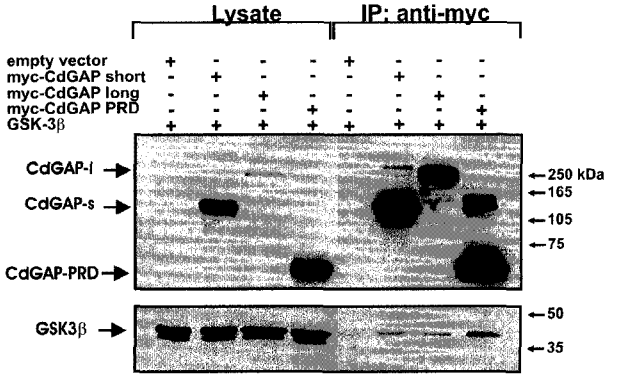
pACTII-GSK-3α

Figure 3.2: CdGAP interacts with GSK-3 in mammalian cells. GSK-3α (**A**) and β (**B**) were co-transfected with either pRK5myc vector, or pRK5myc vector containing CdGAP-s, CdGAP-l or CdGAP-PRD into HEK 293 cells. Anti-myc immunoprecipitations were carried out followed by SDS-PAGE and transfer to nitrocellulose membrane. Western blots were performed against the myc epitope tag and GSK-3α (**A**), or GSK-3β (**B**).

A


Lysate
IP: anti-myc
empty vector
myc-CdGAP short
myc-CdGAP long
myc-CdGAP PRD
GSK-3α
CdGAP-l
CdGAP-s
CdGAP-PRD
GSK3α
250 kDa
165
105
75
50

B


Lysate
IP: anti-myc
empty vector
myc-CdGAP short
myc-CdGAP long
myc-CdGAP PRD
GSK-3β
CdGAP-l
CdGAP-s
CdGAP-PRD
GSK3β
250 kDa
165
105
75
50
35

lithium chloride (LiCl) (353), SB 415286 (406) or AR-A014418 (407) for one hour prior to incubation in phosphate-free medium supplemented with [^{32}P]orthophosphate. As expected, GSK-3 activity was inhibited as indicated by Western blotting for phospho-Ser-641 of glycogen synthase, a known GSK-3 substrate (408) (Fig 3.3B). Additionally, inhibition of GSK-3 by LiCl is indicated by the increase in phospho-serine 9 of GSK-3, a site whose phosphorylation is affected by LiCl but not the SB 415286 or AR-A014418 inhibitors (409). CdGAP-l was phosphorylated under serum-starved conditions where GSK-3 was active, as well as when sodium chloride and DMSO were present as negative controls, however, its phosphorylation was significantly reduced by the three GSK-3 inhibitors (Fig 3.3A). Therefore, these results show that CdGAP-l is *in vivo* phosphorylated by GSK-3 in NIH 3T3 cells.

We next tested whether GSK-3 phosphorylates CdGAP *in vitro*. Since we know that most of the phosphorylation of CdGAP is in the PRD (402), we performed *in vitro* kinase assays with recombinant his-tagged CdGAP-PRD incubated with activated GSK-3α or β. As shown in Fig. 3.4A, both GSK-3α and β were able to phosphorylate CdGAP-PRD *in vitro*. Using a range of CdGAP-PRD concentrations from 18.2 nM to 9.32uM, we estimated a Km value of 0.5 μM, showing that CdGAP-PRD is a very good substrate for GSK-3 (Fig. 3.4B). To determine which type of residues are phosphorylated by GSK-3, CdGAP-PRD phosphorylated *in vitro* by GSK-3α was used to perform a phospho-amino acid analysis. We found that CdGAP-PRD is mainly phosphorylated on threonine residues (Fig. 3.4C). The predicted consensus phosphorylation motif for GSK-3 consists of (S/T)-X-X-X-(pS/pT) (410) in which a proline is a preferred residue adjacent to the phosphorylation site, and, in most cases, a "primed" phosphorylation site is

required at the +4 position prior to GSK-3 phosphorylation of its substrates (410). However, several targets of GSK-3 bypass the need for the +4 phosphorylation by substituting a charged residue at this site (356, 360). This is of particular interest since GSK-3 efficiently phosphorylates CdGAP-PRD *in vitro*, while targets of GSK-3 that need priming generally make poor *in vitro* substrates of GSK-3, indicating that it may not need to be primed. CdGAP contains one atypical motif, within the proline-rich domain, T^{776}-P-L-E-***E***. We mutated Thr-776 to alanine and determined by *in vitro* kinase assays that its phosphorylation by GSK-3α and β was significantly reduced compared to wild-type CdGAP-PRD (Fig. 3.4D). Interestingly, we have previously demonstrated that Thr-776 is a target site of ERK1/2 and acts as an important regulatory site of CdGAP activity *in vivo* (402).

We then developed a polyclonal antibody against CdGAP phospho-Thr-776. This antibody was able by Western blotting to recognize myc-tagged CdGAP-s protein overexpressed in COS-7 fibroblasts, but was unable to recognize both myc-tagged CdGAP-s T776A, and recombinant CdGAP-PRD expressed in *E. Coli* (data not shown). When myc-tagged CdGAP-s was expressed in serum-starved NIH 3T3 cells we found that CdGAP-s was phosphorylated on Thr-776 (Fig. 3.5A,B). However, when these cells were incubated with LiCl for five hours, the level of CdGAP-s with phosphorylated Thr-776 was markedly decreased relative to both serum-starved conditions, as well as cells that were incubated with NaCl for five hours (Fig. 3.5A,B). Likewise, when cells were incubated with the GSK-3 inhibitor SB 415286 (406) for five hours, the level of phospho-Thr-776 CdGAP-s was markedly decreased relative to both serum-starved conditions, and cells that were incubated instead with DMSO (Fig. 3.5A,B). To examine whether

Figure 3.3: CdGAP is phosphorylated *in vivo* by GSK-3. A. NIH 3T3 cells were serum-starved overnight and then incubated in serum free medium with or without 50 mM NaCl, 50mM LiCl, 0.1%DMSO, 100μM SB 415286, or 100μM AR-A014418 prior to and during metabolic labelling with 0.5 mCi of [^{32}P]-orthophosphate per ml. CdGAP was immunoprecipitated and submitted to SDS-PAGE followed by transfer to nitrocellulose and autoradiography (top panel) and Western blotting (WB) using anti-CdGAP antibodies (bottom panel). **B.** NIH 3T3 cells were treated under similar conditions as above, and protein lysates were submitted to SDS-PAGE followed by Western blotting (WB) against phospho-Ser-641 Glycogen Synthase (GS), GS, phospho-Ser-9 GSK-3β, GSK-3α and β, and β-actin.

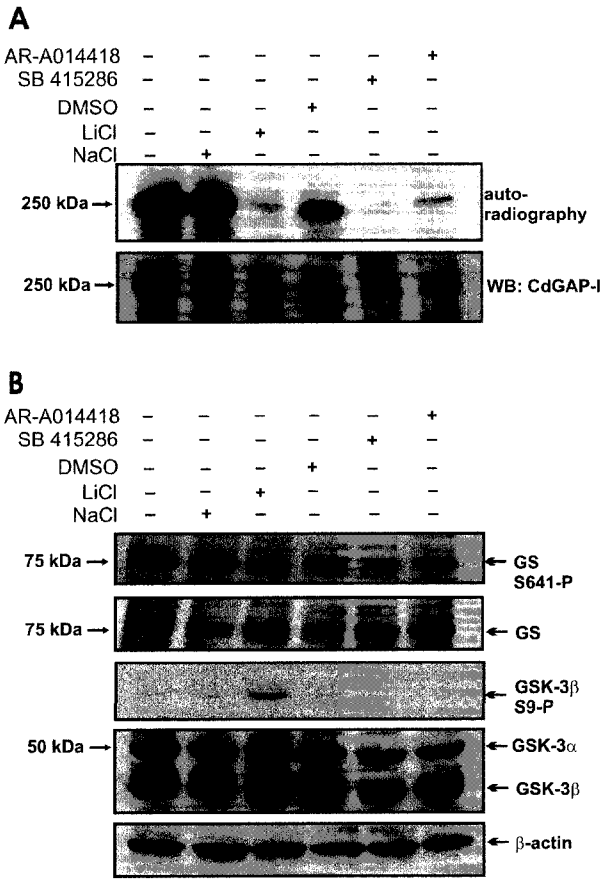

A
AR-A014418 – – – – – +
SB 415286 – – – – + –
DMSO – – – + – –
LiCl – – + – – –
NaCl – + – – – –
250 kDa
auto-radiography
250 kDa
WB: CdGAP-I
B
AR-A014418 – – – – – +
SB 415286 – – – – + –
DMSO – – – + – –
LiCl – – + – – –
NaCl – + – – – –
75 kDa
GS S641-P
75 kDa
GS
GSK-3β S9-P
50 kDa
GSK-3α
GSK-3β
β-actin

Figure 3.4: CdGAP is phosphorylated *in vitro* on Thr-776 by GSK-3. A. An *in vitro* kinase assay was performed using either active GSK-3α (left panel), GSK-3β (middle panel), or no kinase (right panel), and either his-CdGAP-PRD or phospho-Glycogen Synthase (p-GS), followed by SDS-PAGE, transfer to nitrocellulose, and autoradiography (top panels) or Ponceau-S staining (bottom panels). **B.** An *in vitro* kinase assay was performed using recombinant GSK-3α, and concentrations of His-CdGAP-PRD ranging from 18.2 nM to 9.32 uM. Incorporation of [^{32}P]-phosphate into CdGAP-PRD was determined by measuring the counts per minute (CPM). A Michaelis-Menten plot **(i)** was constructed by plotting V (V = CPM/minute) against [S] ([S] = concentration of CdGAP-PRD in moles/litre), and a Lineweaver-Burk plot **(ii)** was constructed by plotting 1/V against 1/[S]. **C.** *In vitro* phosphorylated his-CdGAP-PRD was hydrolyzed and submitted to phosphoamino acid analysis. Migration of phosphoamino acid standards is indicated with circles: phospho-serine (P-S), phospho-threonine (P-T), and phosphotyrosine (P-Y) **D.** *In vitro* kinase assay using either GSK-3α or β with His-CdGAP-PRD, and His-CdGAP-PRD T776A. Samples were resolved by SDS-PAGE and were transferred to nitrocellulose followed by autoradiography and Western blotting (WB) using anti-His antibodies.

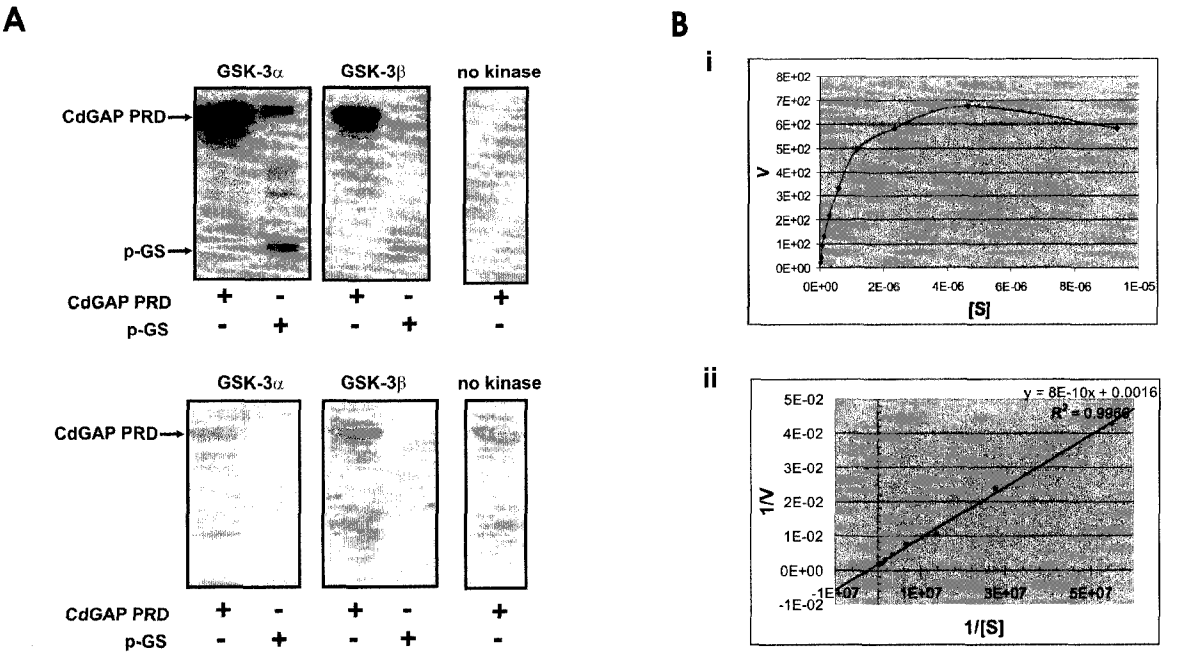
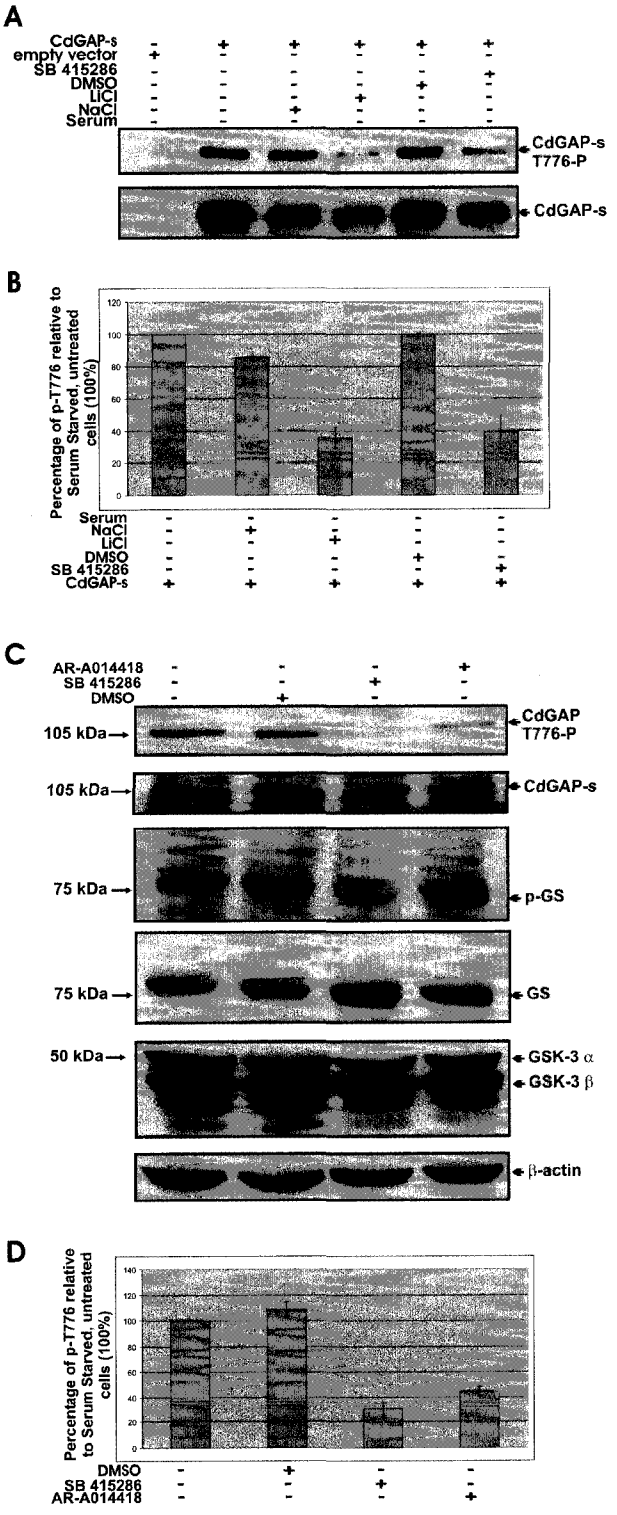

A
GSK-3α
GSK-3β
no kinase
CdGAP PRD
p-GS
CdGAP PRD + - + - +
p-GS - + - + -
GSK-3α
GSK-3β
no kinase
CdGAP PRD
CdGAP PRD + - + - +
p-GS - + - + -
B
i
V
[S]
ii
1/V
1/[S]
y = 8E-10x + 0.0016
C
PH 3.5
(-)
(+)
PH 1.9
D
GSK-3α
Autoradiography
WB: anti-HIS
PRD
PRD T776A
GSK-3β
Autoradiography
WB: anti-HIS
PRD
PRD T776A

Figure 3.5: CdGAP is phosphorylated *in vivo* on Thr-776 by GSK-3. A. NIH 3T3 cells transfected with empty vector or CdGAP-s were serum-starved overnight and were either left untreated, or were treated with 50 mM NaCl, 50 mM LiCl, 0.1%DMSO, or 100μM SB 415286 for five hours. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP phospho-Thr-776 (top panel). The membrane was stripped and re-probed with anti-CdGAP antibodies (bottom panel). **B.** Quantitative analysis of **A** representing CdGAP phospho-Thr-776 relative to serum-starved, untreated cells. **C.** U2OS cells were serum-starved overnight, and were either left untreated, or treated with 0.1% DMSO, 100 μM SB 415286 or 100 μM AR-A014418 for four hours. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP phospho-Thr-776 (top panel). The membrane was stripped and re-probed with anti-CdGAP antibodies (second panel). Lysates were also probed for levels of phospho-Ser-641 GS, GS, GSK-3, and β-actin. **D.** Quantitative analysis of **C** representing CdGAP phospho-Thr-776 relative to serum starved, untreated cells. Error bars represent standard errors of the mean relative to three independent experiments.

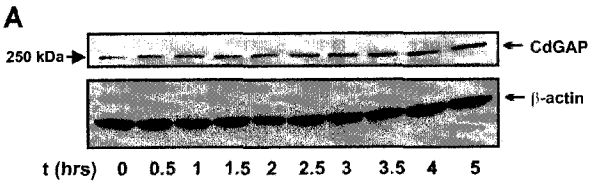

A
CdGAP-s
empty vector
SB 415286
DMSO
LiCl
NaCl
Serum
CdGAP-s T776-P
CdGAP-s
B
Percentage of p-T776 relative to Serum Starved, untreated cells (100%)
120
100
80
60
40
20
0
Serum
NaCl
LiCl
DMSO
SB 415286
CdGAP-s
C
AR-A014418
SB 415286
DMSO
105 kDa
CdGAP T776-P
105 kDa
CdGAP-s
75 kDa
p-GS
75 kDa
GS
50 kDa
GSK-3 α
GSK-3 β
β-actin
D
Percentage of p-T776 relative to Serum Starved, untreated cells (100%)
140
120
100
80
60
40
20
0
DMSO
SB 415286
AR-A014418

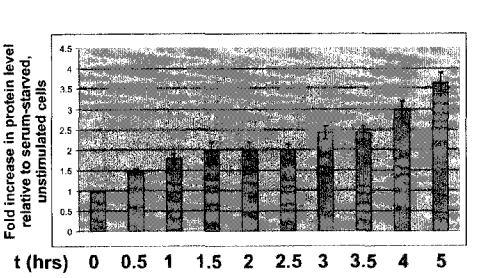
Thr-776 of endogenous CdGAP is phosphorylated *in vivo* by GSK-3, U2OS osteosarcoma cells endogenously expressing the short isoform of CdGAP (411) were serum-starved overnight and then left untreated or were treated with the GSK-3 inhibitors SB 415286 or AR-A014418. We found that CdGAP-s was phosphorylated on Thr-776 under serum-starved conditions, however, CdGAP-s from cells that were incubated with the GSK-3 inhibitors showed a dramatic decrease in phosphorylation at this site (Fig 3.5C,D). As expected, the decrease in the levels of phospho-Ser-641 Glycogen Synthase relative to total Glycogen Synthase confirmed that GSK-3 activity was inhibited (Fig 3.5C). Altogether, these results indicate that GSK-3 phosphorylates *in vivo* the residue Thr-776 of CdGAP-s.

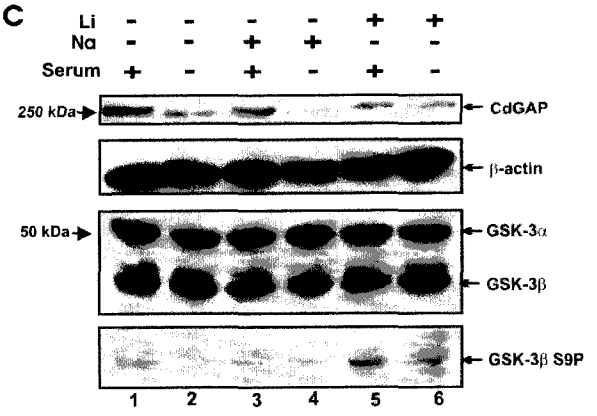
GSK-3 regulates a great deal of cellular functions, including gene expression and protein stability (355). To determine whether GSK-3 affects CdGAP protein levels, we first examined the levels of endogenous CdGAP-l proteins in subconfluent NIH 3T3 fibroblasts stimulated with serum for various periods of time. Interestingly, we observed that CdGAP-l protein levels were significantly augmented in response to serum (Fig 3.6A,B). This induction occurred as early as one hour post-stimulation and showed a 3.7-fold increase in CdGAP protein levels after five hours of serum stimulation (Fig. 3.6B). It has been reported that GSK-3 is only transiently inhibited by growth factors such as EGF and FGF-1, with GSK-3 regaining activity in as little as twenty minutes post-stimulation (412, 413). Indeed, after five hours of stimulation with serum, the levels of total GSK-3 were unchanged and inactive GSK-3 phosphorylated on Ser-9 was barely detectable (Fig. 3.6C, lanes 1 and 2). Under these conditions, we found that inhibition of GSK-3 by LiCl did not alter the levels of CdGAP in serum-starved cells (Fig. 3.6C, compare lanes 2 and

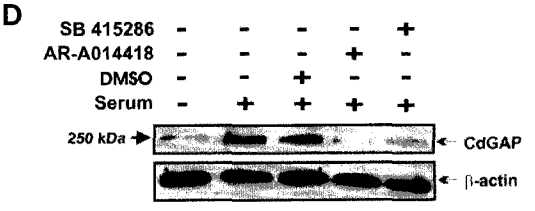
6; Fig. 3.6E), however, when GSK-3 activity was inhibited in cells stimulated with serum, the increase in CdGAP protein levels was blocked (Fig. 3.6C, compare lanes 1 and 5; Fig. 3.6E). NaCl was used as a negative control and had a slight effect on the levels of CdGAP in either serum-stimulated or serum-starved cells (Fig. 3.6C, compare lanes 3 and 4 with lanes 1 and 2, Fig. 3.6E). Consistent with the results obtained with LiCl inhibition of GSK-3, we found that both AR-A014418 and SB 415286 inhibited the increase in the levels of CdGAP proteins in response to serum (Fig. 3.6D and 3.6E). Thus, these findings demonstrate that GSK-3 activity is necessary to regulate the levels of CdGAP proteins in response to serum. To address whether the change in the levels of CdGAP protein resulted from a change in mRNA levels, we determined the mRNA levels of CdGAP in serum-stimulated NIH 3T3 fibroblasts by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). As shown in Fig. 3.7A, the mRNA levels of CdGAP-l increased over a period of 30 minutes to five hours of serum stimulation, with a peak at four hours (Fig. 3.7A). In order to determine whether this increase in CdGAP mRNA was mediated transcriptionally, serum-starved NIH 3T3 cells were pretreated with either Actinomycin D (ActD), or 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) prior to serum stimulation. These compounds inhibit transcription of RNA (414). In serum-starved cells treated with ActD or DRB, the mRNA levels of CdGAP appeared to be reduced compared to control cells (Fig. 3.7B lanes 5 and 7). Following serum stimulation, a slight increase in the levels of CdGAP mRNA was observed in the presence of ActD whereas there was no increase in CdGAP mRNA levels in DRB-treated cells (Fig. 3.7B lanes 6 and 8). This demonstrates that transcription is involved in the process of up-regulation of mRNA levels, however, it does not exclude the possibility

Figure 3.6: CdGAP protein levels are increased in response to serum in a GSK-3 dependant manner. **A.** NIH 3T3 cells were serum-starved overnight and were left unstimulated (t=0) or stimulated with 15% serum for the indicated times. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP and β-actin. **B.** Quantitative analysis of **A** representing the amounts of CdGAP protein relative to those in serum-starved conditions. Error bars represent standard errors of the mean relative to three independent experiments. **C.** NIH 3T3 cells were serum-starved overnight and were left unstimulated, or were stimulated for five hours with 15% serum in the presence or absence of 50 mM NaCl or 50 mM LiCl. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP, phospho-Ser-9 GSK-3β, GSK-3α and β, and β-actin. **D.** NIH 3T3 cells were serum-starved overnight and were left unstimulated, or were stimulated for five hours with 15% serum in the presence or absence of 0.1% DMSO, or 100μM AR-A014418 or 100μM SB 415286. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP and β-actin. **E.** Quantitative analysis of **C,D** was performed as above.


A
250 kDa
CdGAP
β-actin
t (hrs) 0 0.5 1 1.5 2 2.5 3 3.5 4 5


B
Fold increase in protein level relative to serum-starved, unstimulated cells
t (hrs) 0 0.5 1 1.5 2 2.5 3 3.5 4 5


C
Li - - - - + +
Na - - + + - -
Serum + - + - + -
250 kDa
CdGAP
β-actin
50 kDa
GSK-3α
GSK-3β
GSK-3β S9P
1 2 3 4 5 6


D
SB 415286 - - - - +
AR-A014418 - - - + -
DMSO - - + - -
Serum - + + + +
250 kDa
CdGAP
β-actin

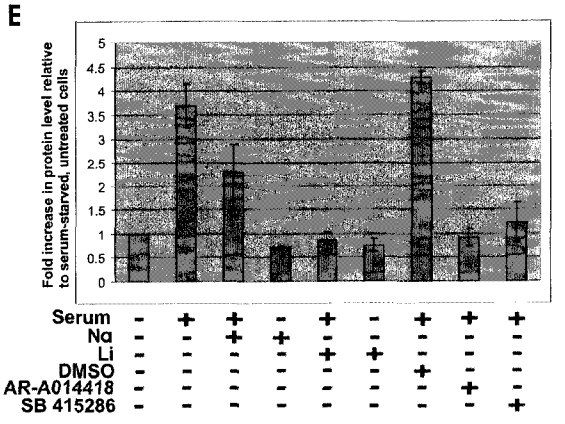
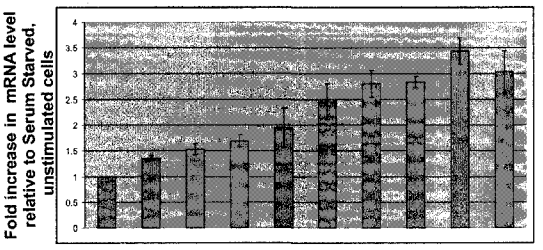
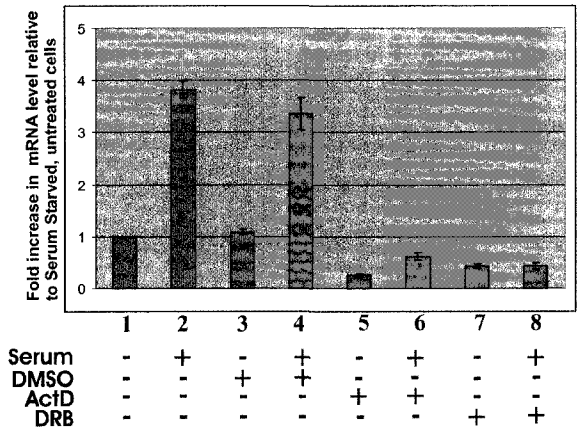

E
Fold increase in protein level relative to serum-starved, untreated cells
Serum - + + - + - + + +
Na - - + + - - - - -
Li - - - - + + - - -
DMSO - - - - - - + - -
AR-A014418 - - - - - - - + -
SB 415286 - - - - - - - - +

Figure 3.7: CdGAP mRNA levels are upregulated by serum. A. NIH 3T3 cells were serum-starved overnight and were left unstimulated (t=0), or were stimulated with 15% serum for the indicated times. Total RNA was extracted and submitted to reverse transcription followed by quantitative PCR to measure amounts of CdGAP and 18S ribosomal subunit mRNAs. Quantitative analysis represents the amounts of CdGAP mRNA relative to those in serum-starved conditions. Error bars represent standard errors of the mean relative to at least three independent experiments. **B.** NIH 3T3 cells were serum-starved overnight and were then incubated with 0.1% DMSO (lanes 3,4), 10 μg/mL ActD (lanes 5,6), or 100 μM DRB (lanes 7,8) for one hour before stimulation with 15% serum or 15% serum with either DMSO, ActD, or DRB. Quantitative analysis of CdGAP mRNA levels was done as above. **C.** NIH 3T3 cells were serum-starved overnight and were left unstimulated, or were stimulated for five hours with 15% serum in the presence or absence of 50 mM NaCl or 50 mM LiCl. Quantitative analysis of CdGAP mRNA levels was done as above.

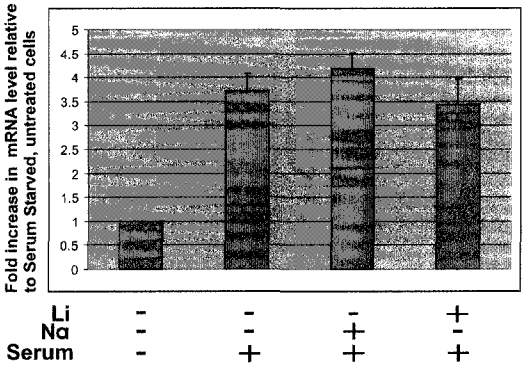
A


Fold increase in mRNA level relative to Serum Starved, unstimulated cells
0
0.5
1
1.5
2
2.5
3
3.5
4
t (hrs) 0 0.5 1 1.5 2 2.5 3 3.5 4 5

B


Fold increase in mRNA level relative to Serum Starved, untreated cells
0
1
2
3
4
5
1 2 3 4 5 6 7 8
Serum - + - + - + - +
DMSO - - + + - - - -
ActD - - - - + + - -
DRB - - - - - - + +

C


Fold increase in mRNA level relative to Serum Starved, untreated cells
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Li - - - +
Na - - + -
Serum - + + +

that there may be some alternative mechanisms, such as mRNA stabilization. To determine whether GSK-3 affects the levels of CdGAP mRNA, serum-starved cells were treated with LiCl or NaCl while being stimulated with serum. As shown in Fig. 3.7C, the mRNA levels of CdGAP showed a fourfold increase following a five hour stimulation with serum, similar to the increase in protein levels (Fig. 3.6B). Interestingly, the LiCl GSK-3 inhibitor did not inhibit the increase in CdGAP mRNA levels induced by serum (Fig. 3.7C). Taken together, these findings indicate that CdGAP expression is up-regulated by serum and that GSK-3 activity is necessary to regulate the levels of CdGAP proteins post-transcriptionally.

DISCUSSION

In order to gain insight into the cellular function of CdGAP, we undertook a search for binding partners for the proline-rich domain of CdGAP, a region known to be important for the regulation of CdGAP activity (325). Through a yeast-two hybrid screen, we identified GSK-3α as a binding partner for CdGAP-PRD, and then determined that both GSK-3α and β are able to interact with the short and long isoforms of CdGAP in mammalian cells. This coupled with our knowledge of CdGAP phosphorylation within the PRD led us to examine whether CdGAP is a physiological substrate for GSK-3. We found that inhibiting GSK-3 activity greatly attenuated the phosphorylation of endogenous CdGAP in both NIH 3T3 (Fig. 3.3) and Swiss 3T3 fibroblasts (data not shown). Subsequent analysis *in vitro* led us to discover that GSK-3 can phosphorylate CdGAP on Thr-776 within the proline-rich domain of CdGAP. Using a polyclonal antibody which recognized the phosphorylated residue Thr-776 of CdGAP, we demonstrated that this residue is an *in vivo* target site of GSK-3 in both NIH 3T3 cells and U2OS osteosarcoma cells. The identification of Thr-776 as a GSK-3 phosphorylation site is of great interest for at least two reasons. First, this site is an atypical GSK-3 phosphorylation site. The motif T^{776}-P-L-E-***E*** does not contain the usual +4 priming phospho-serine or phospho-threonine site that is typical for many GSK-3 substrates (360). Instead, it contains a negatively charged glutamic acid residue that can mimic the priming phosphorylation event required for the subsequent phosphorylation by GSK-3 (356). This may in part explain why GSK-3 efficiently phosphorylates recombinant CdGAP-PRD *in vitro*, as it would not require this substrate to be primed by another

kinase. Of note however, is that amino acid substitution of Thr-776 to alanine did not completely eliminate GSK-3 phosphorylation of CdGAP *in vitro*, suggesting that other residues are phosphorylated by GSK-3. Second, is the fact that this residue is an ERK1 phosphorylation site important in the negative regulation of the GAP activity of CdGAP (402). Although there are not many examples of substrate sites that function for both ERK and GSK-3, it has been reported that the transcription factor c/EBPβ and myelin basic protein (MBP) are phosphorylated at a consensus ERK/GSK-3 site (415) (416). In the case of c/EBPβ, it appears that its phosphorylation at a GSK-3/ERK consensus site is required for the induction of adiponectin gene expression during differentiation of mouse fibroblasts into adipocytes (415). For MBP, it is unclear what is the role of the consensus ERK/GSK-3 phosphorylation site, but this site is thought to play a role in changing the conformation of the protein, leading to changes in the interaction of MBP with the lipid bilayer in brain myelin (416). In the present study, our findings strongly suggest that phosphorylation of Thr-776 in the proline-rich domain of CdGAP is a consensus ERK-1/GSK-3 site required for a tight regulation of CdGAP activity under different cellular conditions.

This study also demonstrates that the cellular protein levels of CdGAP are serum-responsive. Five hours post-stimulation with serum, the protein levels of CdGAP within the cells are elevated, and we have shown that this is concomitant with an increase in the amount of CdGAP mRNA. The inhibition of CdGAP mRNA increase by ActD or DRB suggests that this process is transcriptionally mediated, however, it does not rule out the possibility that there may also be other mechanisms such as stabilization of the mRNA that contribute to this process as well. Interestingly, we found that GSK-3 activity is

required for the serum-dependent increase in the cellular protein levels of CdGAP, but not the mRNA levels. Thus, these findings indicate that GSK-3 is acting post-transcriptionally to regulate the protein levels of CdGAP. It remains to be determined whether GSK-3 stimulates CdGAP protein synthesis or regulates its stability. Although more studies have reported the converse situation, where GSK-3 phosphorylation leads to protein instability, there are a few examples of proteins being stabilized after phosphorylation by GSK-3, namely, the retinoblastoma-related pocket protein RBL2/p130 (417), the nuclear receptor Rev-erbα, a negative component of the circadian clock (418), and axin, a component of the ternary complex including β-catenin and APC (419).

As reported earlier by many studies, RhoGAPs are regulated post-translationally via various molecular mechanisms such as lipid interaction (420), protein-protein interaction (325), phosphorylation (402) (275), and proteolytic degradation (381). Clearly, CdGAP utilizes at least two of these mechanisms of regulation, including phosphorylation (402) and protein-protein interactions (325) to control its GAP activity. Here, we report for the first time the regulation of a RhoGAP protein at the transcriptional level in response to serum. The induction in mRNA levels occurred early (one hour) and peaked at four hours, suggesting that CdGAP expression may represent a novel mitogen-inducible early gene (421). Future studies will be required to determine the molecular pathways necessary to activate CdGAP gene expression and their consequences on cell proliferation, migration, and survival.

In conclusion, we have identified CdGAP as a novel GSK-3 substrate and stimulation of the cellular protein and mRNA levels of CdGAP by serum provides a novel mechanism to control Cdc42/Rac1 GTPase signaling pathways.

ACKNOWLEDGEMENTS

We are grateful to Nathalie Bedard for advice on qRT-PCR and to Dr. John F. Presley for critical reading of this manuscript. This work was supported by the Canadian Cancer Society through the National Cancer Institute of Canada. E.I.D. is the recipient of a Canada Graduate Scholarship administered by the CIHR, M.M. is the recipient of a Post-Doctoral Fellowship Award from the CIHR, and N.L-V. is the recipient of a CIHR New Investigator Award.

The abbreviations used are: GAP, GTPase-activating protein; CdGAP, Cdc42 GTPase-Activating-Protein; CdGAP-l, CdGAP long isoform; CdGAP-s, CdGAP short isoform; PRD, proline-rich domain; 3-AT, 3-amino-1,2,4-triazole; GSK-3, Glycogen Synthase Kinase 3; LiCl, Lithium Chloride; NaCl, Sodium Chloride; ActD, Actinomycin D; DRB, 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside; MBP, Myelin Basic Protein.

Preface To Chapter 4

Having identified a novel potential regulatory mechanism of RhoGAP proteins in the previous chapter, namely, serum-induced upregulation of CdGAP mRNA, we decided to further explore the mechanism by which this occurs. We look at components within the serum in an effort to identify the agents that could be responsible for this increase, and we further look at signal transduction pathways and their roles in the upregulation of CdGAP mRNA by serum. We also explore the promoter region of CdGAP and its role in this upregulation using a luciferase reporter system.

CHAPTER 4

Rho GTPases Regulate Serum-Induced Upregulation of CdGAP Expression

Rho GTPases Regulate Serum-Induced Upregulation of CdGAP Expression

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Running Title: Rho GTPases regulate CdGAP mRNA levels

ABSTRACT

The Rho subfamily of small GTPases regulates many cellular processes such as cell motility and gene transcription. GTPase-activating proteins (RhoGAPs) negatively regulate the small GTPases. Our previous studies have shown that CdGAP, a GAP for Rac1 and Cdc42, is transcriptionally upregulated upon serum stimulation. Here we use quantitative PCR approaches to further understand this process. We find that the intact protein component of serum is not necessary for the up-regulation of CdGAP mRNA. Using inhibitors, we demonstrate that Ser/Thr kinases are necessary for this process, and that the ERK MAP kinases, P38 MAPK and mTOR are not necessary for the up-regulation of CdGAP. Our results for PI3K are less clear, as one inhibitor, LY 294002 blocked the upregulation of CdGAP mRNA, whereas wortmannin had no effect. Finally, we show that RhoGTPases are necessary for the upregulation of CdGAP mRNA in response to serum stimulation. This gives greater insight into the mechanism by which serum upregulates CdGAP mRNA levels.

INTRODUCTION

Cells constantly monitor their external milieu and relay these signals inside the cell, directing the cell to take action as necessary. The Rho subfamily of small GTPases is implicated in the transduction of these signals. This subfamily is best known by three of its members, RhoA, Rac1 and Cdc42, which in turn are best known for their roles in actin reorganization (399). They are also highly involved in signal transduction leading to a variety of processes such as the activation of transcription of various genes involved in growth control and tumorigenesis (399). Contrary to the Ras subfamily of small GTPases, activating mutations are rare (65, 400), and it seems instead that these proteins are regulated by their expression, as well as by the expression and activity of positive and negative regulators within the cell (65, 400).

These small GTPases act in a cycle, where, when bound to GTP they are in an active conformation that allows them to interact with effectors, and when they are bound to GDP they are in an inactive conformation (399). To accelerate the change from a GTP to a GDP bound state, GTPase-activating proteins (GAPs) enhance the intrinsic GTPase activity to hydrolyze the GTP to GDP (399). Interestingly, there are approximately 70 RhoGAP proteins predicted in the human genome, which is approximately three-fold higher than the number of Rho GTPase subfamily members (230). This supports the notion that it is regulators of the Rho GTPases that control the GTPases in a temporal and spatial fashion.

Cdc42 GTPase-Activating-Protein (CdGAP) has been demonstrated to be a GAP for both Rac1 and Cdc42 *in vitro* and *in vivo*. It consists of two main isoforms in

mammals, a shorter, 820 amino acid form containing an N-terminal RhoGAP domain, a central region, and a C-terminal proline-rich region (PRD), and a long isoform comprising the entire short form with an additional C-terminal region extending to a total of 1425 amino acids (321, 325, 402). GAPs are controlled by a variety of mechanisms, such as protein-protein interactions, lipid interactions, proteolysis and phosphorylation (230). We have recently shown that CdGAP is regulated by protein-protein interactions with the endocytic protein intersectin (325), by phosphorylation by both ERK and GSK-3 (402, 422), and we have recently also identified a novel potential regulatory mechanism of RhoGAP proteins, transcriptional regulation of CdGAP induced by serum stimulation (422).

In this study, we further elucidate the mechanism by which serum upregulates CdGAP mRNA levels using quantitative PCR and luciferase reporter assays. We show that the intact protein component of serum is not necessary to increase CdGAP mRNA levels, and that Ser/Thr kinase activity is necessary. Neither, ERK, mTOR, nor P38 MAPK activity is necessary for the serum upregulation of CdGAP levels; however, Rho GTPases are involved in this process.

EXPERIMENTAL PROCEDURES:

Reagents and Antibodies - U0126, Staurosporine, LY 294002, wortmannin, and rapamycin were purchased from LC Labs (Woburn MA, USA). SB 239063 and LPA were purchased from Sigma-Aldrich Canada. EGF and PDGF were kind gifts from Dr. Barry Posner and Dr. Louise Larose, respectively (McGill University, Montreal, Canada). Anti-ERK1 antibody was from Santa Cruz Biotechnology (Santa Cruz CA, USA), Anti-phospho-ERK1/2 antibody was from Cell Signaling Technologies, anti-AKT antibody and anti-phospho-Ser-473-AKT antibody were a kind gift from Dr. Isabelle Royal (University of Montreal, Montreal, Canada).

Cell Culture and Lysis - NIH 3T3 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics and maintained at an atmosphere of 5% CO2 at 37 °C. Cells were lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride, 1X Complete protease inhibitor cocktail (Roche), 20 mM NaF, and 1 mM sodium orthovanadate, followed by centrifugation for 15 min at 8,000 x g and discarding of the pellet. Equal amounts of cell lysate were submitted to SDS-PAGE followed by Western blotting.

DNA constructs - The first 1497 bp of the promoter of *Mus musculus* CdGAP (the first 1497 bp upstream of the ATG start site of the CdGAP protein), and the ATG, named 1497ATG, cloned in the pGL3-basic vector was obtained from Bio S&T (Montreal, Canada). The cloning of 1137ATG, 907ATG, 677ATG, and 362ATG (the first 1137,

907, 532 and 362 bp upstream of the ATG respectively, as well as the ATG) was done using the forward primers 5'-GAGAGAGCTAGCGAGACTGGCAAAATGTCAGC, 5'- GAGAGAGCTAGCAGGAGACGTCTGGGCTTCAG, 5'GAGAGAGCTAGCCGCCGCCTCCTGCGCTGTGC, and GAGAGAGCTAGCAAGACAGGAAAAAAGGGACG respectively, with the reverse primer 5'-GGCGTCTTCCATGGTGGCTTTAC and the 1497ATG plasmid as a template (Fig. 4.6A). The forward primer contained an NheI restriction site, and the reverse primer was downstream of an XhoI restriction site, which were used to sub-clone the PCR fragments into the pGL3-basic vector (a kind gift from Dr. Jean-Jacques Lebrun, McGill University, Montreal, Canada).

Luciferase Assay - Cells were transfected using poly-ethylenimine (PEI) (Sigma-Aldrich Canada). 5 μg of luciferase-construct (PGL-3 empty vector or 1497ATG, 1137ATG, 907ATG, 677ATG or 362ATG) along with 1 μg of β-galactosidase vector (a kind gift from Dr. Jean-Jacques Lebrun, McGill University, Montreal, Canada) was mixed with 36 μg of PEI in 500 μL of serum-free medium. This mixture was left to stand at room temperature for 20 minutes. The medium from the cells was then removed and replaced with 5 mL of serum-free medium, and the transfection mix was added to this. The cells were left for 20 hrs, and were then stimulated with serum for 4 hrs. The cells were then lysed using Reporter Lysis Buffer (Promega), and the luciferase and β-galactosidase activities of the lysates were assayed.

Quantitative Reverse Transcription-PCR - NIH 3T3 cells were serum-starved for 20 hours. The cells were either left untreated or were treated with inhibitors of various mediators of signal transduction for 30 minutes, one hour or two hours depending on the inhibitor, followed by stimulation with serum, EGF, PDGF, LPA, or serum that had been boiled for 10 minutes, in the absence or presence of the various inhibitors for 4 hours. Total RNA was extracted using a Qiagen RNeasy kit (Qiagen). mRNA was reverse-transcribed using enzymes from Invitrogen. The cDNA was then run in a quantitative real-time PCR reaction using a Roche Applied Science Lightcycler, Qiagen Quantitect Sybr green reagents, and CdGAP primers obtained from Geneglobe. 18 S ribosomal subunit primers (a kind gift from Dr. Simon Wing, McGill University, Montreal, Canada) were used as a loading control.

RESULTS

The mRNA levels of CdGAP are increased upon stimulation of NIH 3T3 cells with serum (422). In order to further characterize this phenomenon, we set out to identify the agent(s) in the serum that is responsible for this effect. NIH 3T3 cells were stimulated with 100 ng/mL of EGF (Fig. 4.1A), 20 ng/mL PDGF (Fig. 4.1B) or 10 μM LPA (Fig. 4.1C). All the compounds were competent as indicated by their ability to stimulate phosphorylation of ERK (Fig. 4.1D); however, none of these compounds were able to significantly increase the levels of CdGAP mRNA. In an effort to narrow down the type of agent, the serum was boiled for 10 minutes, and then used to stimulate the cells. The boiled serum was as effective in stimulating an increase in CdGAP mRNA as the non-boiled serum (Fig. 4.1E). These results indicate that intact proteins in the serum are not necessary to induce an increase in CdGAP mRNA levels, and that EGF, PDGF and LPA are not able to induce CdGAP mRNA levels on their own.

Many signal transduction pathways depend on serine/threonine kinases to relay their signals. In order to determine whether Ser/Thr kinases are involved in the signal transduction mechanisms leading to an increase in CdGAP mRNA, NIH 3T3 cells were treated for 30 minutes with either 1μM staurosporine or control vehicle (0.05% DMSO). This was followed by a four hour stimulation with 20% serum in the presence or absence of staurosporine or its control vehicle. Staurosporine was active, as evidenced by cell shrinkage (data not shown) (423), and was able to block an increase in the mRNA levels of CdGAP (Fig. 4.2). This indicates that Ser/Thr kinases are involved in the signal transduction pathways leading to upregulation of CdGAP mRNA levels.

Figure 4.1: Intact proteins within serum are not necessary to stimulate an upregulation of CdGAP mRNA. **A.** NIH 3T3 cells were serum starved overnight and were then left unstimulated or were stimulated with 15% serum, control vehicle (0.1% PBS), or 100 ng/mL EGF for four hours. Total RNA was extracted and submitted to reverse transcription followed by quantitative PCR to measure amounts of CdGAP and 18S ribosomal subunit mRNAs. Quantitative analysis represents the amounts of CdGAP mRNA relative to those in serum-starved, unstimulated conditions. Error bars represent the standard deviations relative to three independent experiments. **B.** NIH 3T3 cells were serum starved overnight and were then left unstimulated or were stimulated with 15% serum, control vehicle (8 μM Tris HCl pH7.4, 280 μM NaCL), or 20 ng/mL PDGF for four hours. Quantitative analysis of CdGAP mRNA levels was performed as above. **C.** NIH 3T3 cells were serum starved overnight and were then left unstimulated or were stimulated with 15% serum, control vehicle (0.05% Chloroform:acetic acid:methanol 90%:5%:5% v:v:v), or 10 μM LPA for four hours. Quantitative analysis of CdGAP mRNA levels was performed as above. **D.** NIH 3T3 cells were serum starved overnight and were either left unstimulated (lane 1), or were stimulated with 15% serum (lane 2), control vehicles (0.1% PBS – lane 3, 8 μM Tris HCl pH7.4, 280 μM NaCL – lane 5, 0.05% Chloroform:acetic acid:methanol 90%:5%:5% v:v:v – lane7), or 100 ng/mL EGF (lane 4), 20 ng/mL PDGF (lane 6) or 10 μM LPA (lane 8) for 20 minutes. Protein lysates were subjected to SDS-PAGE followed by Western blotting against phospho-ERK1/2 (top panel). The membrane was stripped, and re-probed with an antibody against total ERK1 (bottom panel). **E.** NIH 3T3 cells were serum starved overnight and were then left unstimulated or were stimulated with 20% serum, or with 20% serum that had been

"boiled" (incubated at 100 ^{o}C) for 10 minutes. Quantitative analysis of CdGAP mRNA levels was performed as above.

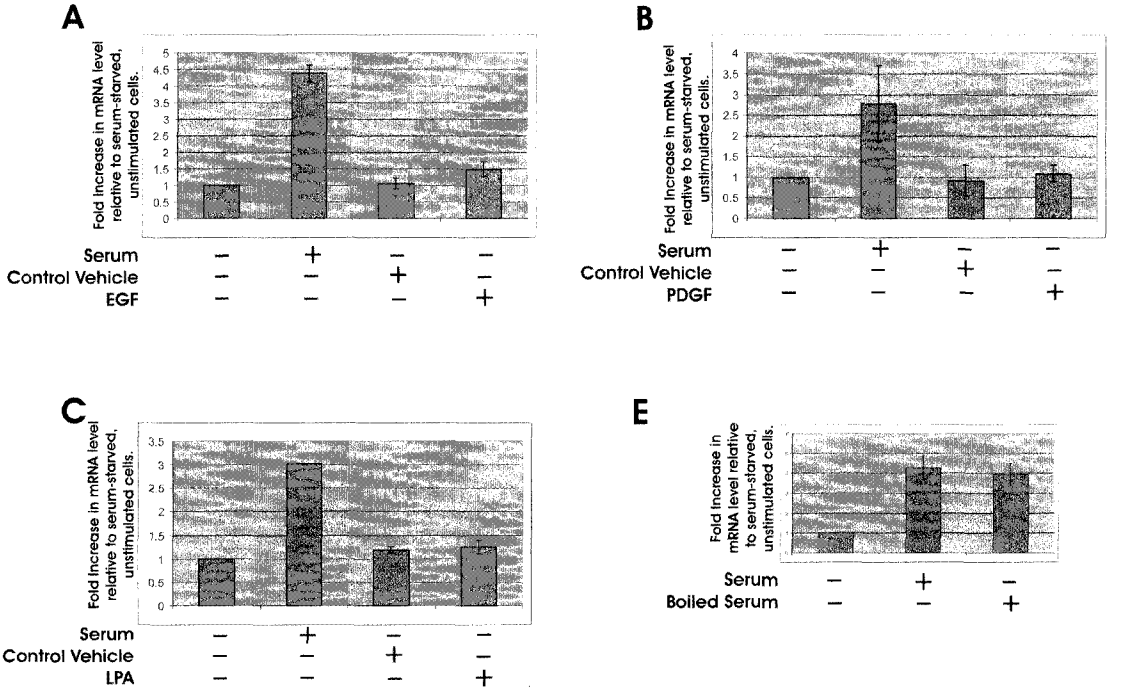
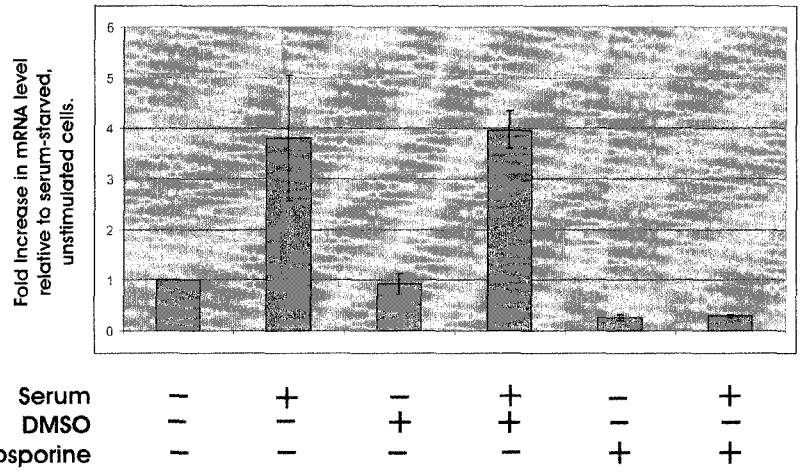

A
Fold increase in mRNA level relative to serum-starved, unstimulated cells.
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Serum – + – –
Control Vehicle – – + –
EGF – – – +
B
Fold increase in mRNA level relative to serum-starved, unstimulated cells.
4
3.5
3
2.5
2
1.5
1
0.5
0
Serum – + – –
Control Vehicle – – + –
PDGF – – – +
C
Fold increase in mRNA level relative to serum-starved, unstimulated cells.
3.5
3
2.5
2
1.5
1
0.5
0
Serum – + – –
Control Vehicle – – + –
LPA – – – +
E
Fold increase in mRNA level relative to serum-starved, unstimulated cells.
Serum – + –
Boiled Serum – – +
D
p-ERK 1
p-ERK 2
ERK 1
1 2 3 4 5 6 7 8
Serum – + – – – – – –
vehicle – – + – + – + –
EGF – – – + – – – –
PDGF – – – – – + – –
LPA – – – – – – – +

Figure 4.2: Ser/Thr kinases are involved in the upregulation of CdGAP mRNA. NIH 3T3 cells were serum starved overnight and were then incubated in serum-free medium in the presence or absence of 0.01% DMSO or 1μM Staurosporine for 30 minutes, followed by continued serum starving or stimulation with 20% serum, in the presence or absence of 0.01% DMSO or 1μM Staurosporine for 4 hours. Quantitative analysis of CdGAP mRNA levels was performed as above.


Fold Increase in mRNA level relative to serum-starved, unstimulated cells.
6
5
4
3
2
1
0
Serum − + − + − +
DMSO − − + + − −
Staurosporine − − − − + +

In order to identify the Ser/Thr kinases that may be involved, we inhibited various Ser/Thr kinases and examined the effect on CdGAP mRNA levels. Cells were treated with 10 μM of SB 239063, a P38 MAPK inhibitor, or its control vehicle, 0.04% DMSO for one hour prior to and then during a stimulation with serum. The inhibitor had no effect on the upregulation of CdGAP mRNA (Fig. 4.3A). Cells were also treated with 2 nM rapamycin, an inhibitor of mTOR, or its control vehicle, 0.01% DMSO, and like SB 239063, there was no effect on the up-regulation of CdGAP mRNA (Fig. 4.3B). Cells were then treated with U0126, an inhibitor of MEKK, which blocks the ERK MAP kinase signaling pathways. This inhibitor at a concentration of 40 μM was able to block the phosphorylation of ERK, as shown by Western blotting (Fig. 4.3D). When cells were treated with U0126 or its control vehicle DMSO for one hour prior to and then during a four hour stimulation with serum, U0126 was also unable to block the upregulation of CdGAP mRNA by serum (Fig 4.3C). These results indicate that the MAP kinase, P38 MAPK, and mTOR pathways are not necessary in the signal transduction leading to upregulation of CdGAP mRNA upon serum stimulation.

Another classic pathway regulated by many components of serum is the PI3 kinase pathway. In order to assess the role of PI3 kinase in the upregulation of CdGAP mRNA NIH 3T3 cells were treated with 50 μM LY 294002 or its control vehicle, 0.1% DMSO, for one hour prior to and then during a four hour stimulation with serum (Fig. 4.4A). The compound blocked PI3K activation as evidenced by reduced phosphorylation of AKT (Fig. 4.4B). LY 294002 was able to dramatically reduce the increase of CdGAP mRNA levels upon serum stimulation (Fig 4.4A). In order to further confirm these results, cells were treated with 1 μM wortmannin or its control vehicle, 0.1% DMSO, for

Figure 4.3: P38 MAPK, mTOR and ERK MAP kinase pathways are not necessary for the upregulation of CdGAP mRNA levels. **A.** NIH 3T3 cells were serum-starved overnight and were then incubated in serum-free medium in the presence or absence of 0.04% DMSO or 10 μM SB 239063 for 1 hour, followed by stimulation with 15% serum in the presence or absence of 0.04% DMSO, or 10 μM SB 239063 for 4 hours. Quantitative analysis of CdGAP mRNA levels was performed as above. **B.** NIH 3T3 cells were serum starved overnight and were then incubated in serum-free medium in the presence or absence of 0.01% DMSO, or 2nM rapamycin for 1 hour, followed by stimulation with 15% serum in the presence or absence of 0.01%DMSO or 2nM rapamycin for 4 hours. Quantitative analysis of CdGAP mRNA levels was performed as above. **C.** NIH 3T3 cells were serum-starved overnight and were then incubated in serum-free medium in the presence or absence of 0.2% DMSO, or 40 μM U0126 for 1 hour, followed by continued serum starving or stimulation with 20% serum in the presence or absence of 0.2% DMSO, or 40 μM U0126 for 4 hours. Quantitative analysis of CdGAP mRNA levels was performed as above. **D.** NIH 3T3 cells treated as in **C**, except stimulation with serum was for 10 minutes. Protein lysates were subjected to SDS-PAGE followed by Western blotting against phospho-ERK1/2 (top panel). The membrane was stripped and re-probed with anti-ERK-1 antibody (bottom panel).

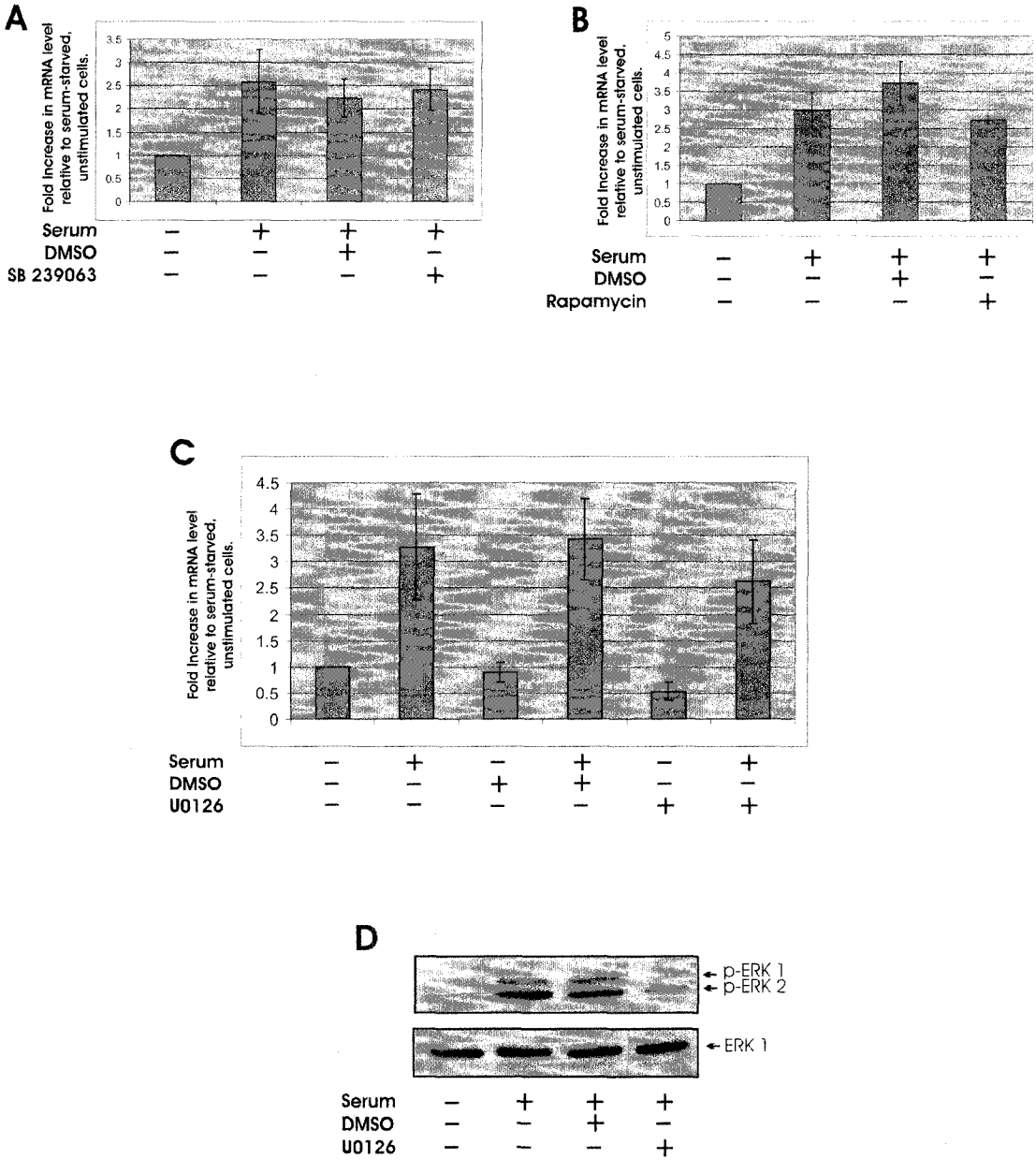
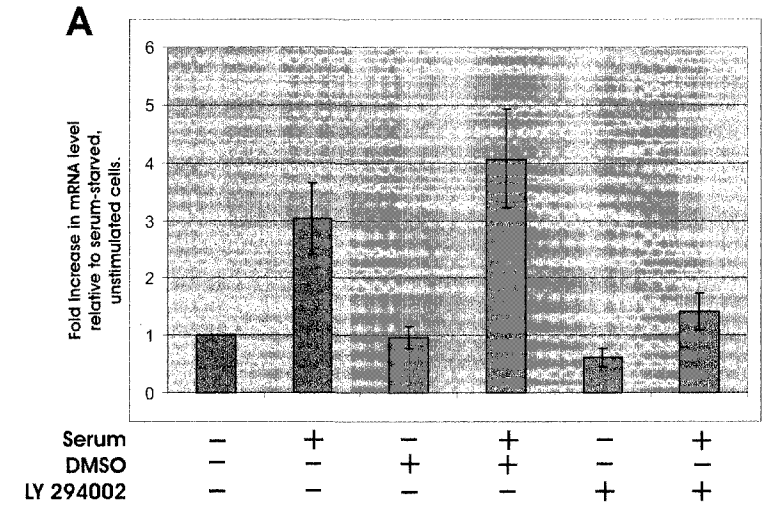
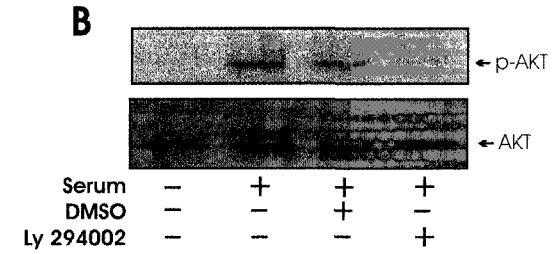
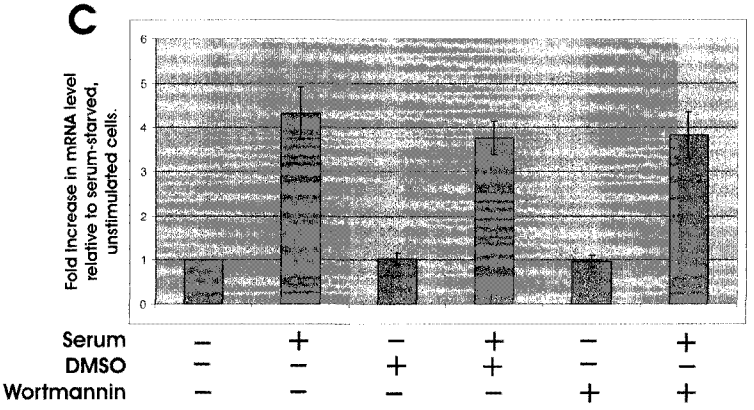
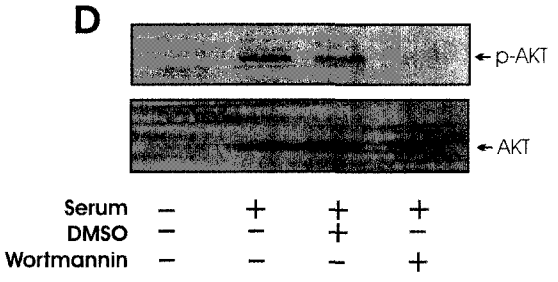

A
Fold Increase in mRNA level relative to serum-starved, unstimulated cells.
3.5
3
2.5
2
1.5
1
0.5
0
Serum – + + +
DMSO – – + –
SB 239063 – – – +
B
Fold Increase in mRNA level relative to serum-starved, unstimulated cells.
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Serum – + + +
DMSO – – + –
Rapamycin – – – +
C
Fold Increase in mRNA level relative to serum-starved, unstimulated cells.
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Serum – + – + – +
DMSO – – + + – –
U0126 – – – – + +
D
p-ERK 1
p-ERK 2
ERK 1
Serum – + + +
DMSO – – + –
U0126 – – – +

Figure 4.4: PI3 Kinase may be involved in the upregulation of CdGAP mRNA. A. NIH 3T3 cells were serum-starved overnight and were then incubated in serum-free medium in the presence or absence of 0.1% DMSO, or 50 μM LY 294002 for 1 hour followed by continued serum starving, or stimulation with 20% serum in the presence or absence of 0.1% DMSO, or 50 μM LY 294002 for 4 hours. Quantitative analysis of CdGAP mRNA levels was performed as above. **B.** NIH 3T3 cells were treated as in **A**, except stimulation with serum was for 20 minutes. Protein lysates were subjected to SDS-PAGE followed by Western blotting against phospho-AKT (top panel). The membrane was stripped and re-probed for total AKT (bottom panel). **C.** NIH 3T3 cells were serum-starved overnight and were then incubated in serum-free medium in the presence or absence of 0.1% DMSO, or 1 μM wortmannin for 1 hour followed by continued serum starving, or stimulation with 20% serum in the presence or absence of 0.1% DMSO, or 1 μM wortmannin for 4 hours. Quantitative analysis of CdGAP mRNA levels was performed as above. **D.** NIH 3T3 cells were treated as in **C**, except stimulation with serum was for 20 minutes. Protein lysates were subjected to SDS-PAGE followed by Western blotting against phospho-AKT (top panel). The membrane was stripped and re-probed for total AKT (bottom panel).


A
Fold increase in mRNA level relative to serum-starved, unstimulated cells.
0
1
2
3
4
5
6
Serum
DMSO
LY 294002


B
p-AKT
AKT
Serum
DMSO
Ly 294002


C
Fold increase in mRNA level relative to serum-starved, unstimulated cells.
0
1
2
3
4
5
6
Serum
DMSO
Wortmannin

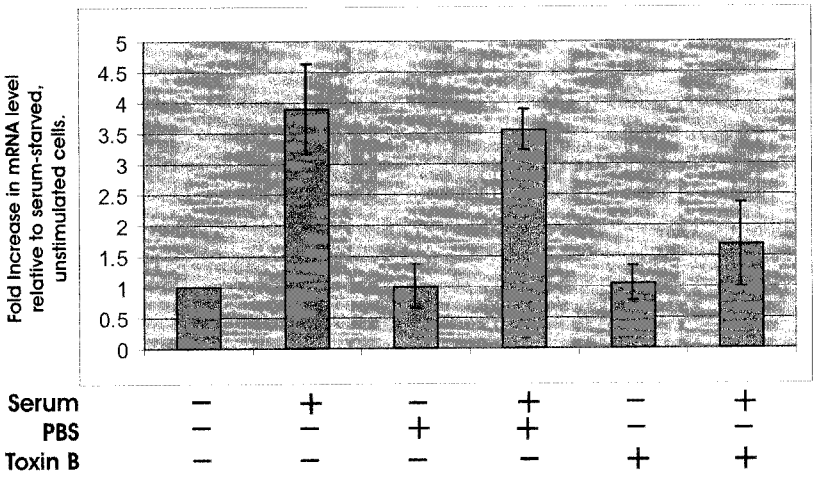

D
p-AKT
AKT
Serum
DMSO
Wortmannin

one hour prior to and then during a four hour stimulation with serum (Fig. 4.4C). As for LY 294002, wortmannin was able to block the phosphorylation of AKT indicating that it was blocking PI3K (Fig. 4.4D). Unlike LY 294002, wortmannin was unable to prevent the increase in CdGAP mRNA levels (Fig. 4.4C). These results indicate that the increase in CdGAP mRNA levels may be due to PI3K activity; however, it is possible that other kinases also blocked by LY 294002 may be involved.

The Rho subfamily of proteins are heavily implicated in signal transduction upon serum stimulation (47, 424, 425). In order to test the involvement of this family of proteins, serum-starved NIH 3T3 cells were treated with 10 ng/mL of Toxin B, an inhibitor of RhoA, Rac1 and Cdc42, for two hours prior to and then during a four hour stimulation with serum. Toxin B was able to inhibit the Rho GTPases as evidenced by cell rounding (data not shown), and was able to dramatically block the increase in CdGAP mRNA levels (Fig. 4.5). PBS, the control vehicle, had no effect on this process (Fig. 4.5). This result indicates that RhoGTPases are involved in the upregulation of CdGAP mRNA by serum.

To further explore the pathways involved in the upregulation of CdGAP mRNA, and to identify the promoter regions involved in this process, we set up a luciferase assay system. We inserted into the pGL-3 basic vector the first 1497 bp upstream of the first coding ATG of CdGAP, as well as the first 1137, 907, 677 and 362 base pairs upstream of the ATG of CdGAP (1497ATG, 1137ATG, 907ATG, 677ATG and 362ATG, respectively). These constructs were able to express luciferase when compared against the empty vector at a basal level (data not shown). NIH 3T3 cells expressing these constructs, and serum-starved overnight, were stimulated with serum for four hours, lysed

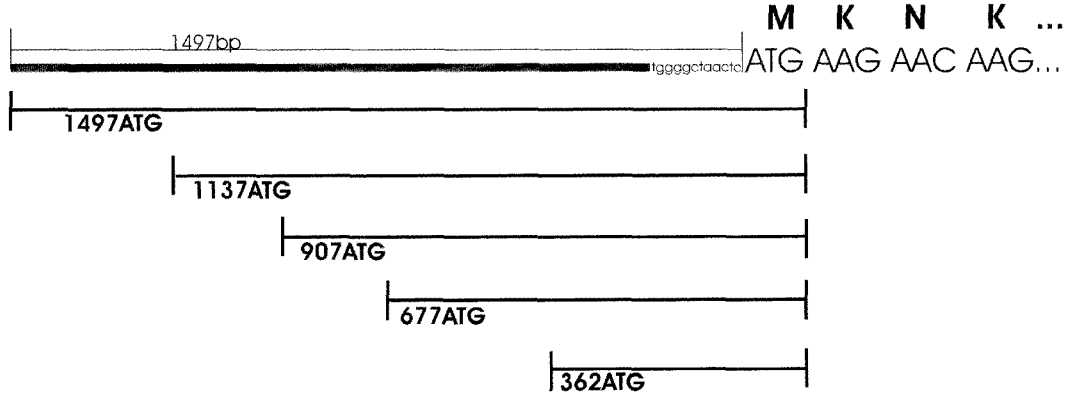
Figure 4.5: RhoGTPases are involved in the upregulation of CdGAP mRNA levels. NIH 3T3 cells were serum-starved overnight and were then incubated in serum-free medium in the presence or absence of 0.04% PBS, or 10 ng/mL Toxin B for 2 hours, which was followed by continued serum-starving or stimulation with 20% serum in the presence or absence of 0.04% PBS or 10 ng/mL Toxin B for 4 hours. Quantitative analysis of CdGAP mRNA levels was performed as described earlier.


Fold Increase in mRNA level relative to serum-starved, unstimulated cells.
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Serum
PBS
Toxin B
− + − + − +
− − + + − −
− − − − + +

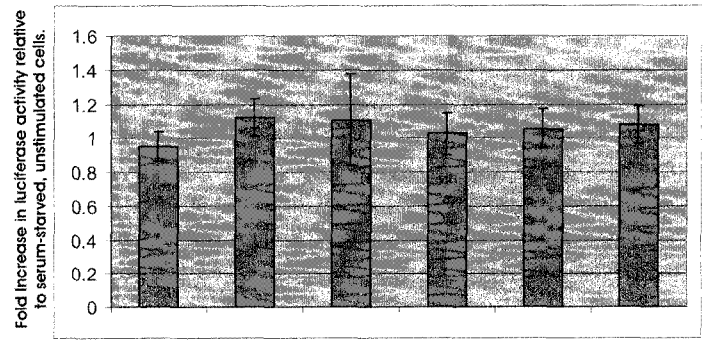
and then the luciferase activity was measured. None of the constructs were able to be regulated by serum (Fig. 4.6B). These results indicate that the isolated 1497 bp upstream of the ATG of CdGAP are not competent on their own to be regulated by serum.

Figure 4.6: The first 1497 bp of the promoter of CdGAP alone are not sufficient to be regulated by serum. A. Schematic showing the fragments of the promoter region of CdGAP that were cloned into the PGL-3 basic vector. **B.** NIH 3T3 cells were transfected with empty PGL3 vector or with 1497ATG, 1162ATG, 907ATG, 677ATG, or 362ATG along with a β-galactosidase expression vector. Cells were serum starved overnight and stimulated with 20% serum for 4 hours. Lysates were probed for luciferase and β-galactosidase activities. Differences in transfection levels were adjusted for using β-galactosidase expression. Data is shown as fold increase over non-stimulated cells, error bars correspond to the standard deviation of three experiments.

A



B



empty vector	+	–	–	–	–	–
1497ATG	–	+	–	–	–	–
1137ATG	–	–	+	–	–	–
907ATG	–	–	–	+	–	–
677ATG	–	–	–	–	+	–
362ATG	–	–	–	–	–	+

DISCUSSION

In previous studies, we have shown that serum can stimulate the transcription of CdGAP mRNA in NIH 3T3 fibroblasts. This brought to light a new potential regulatory mechanism for RhoGAP proteins. In the present study, we have sought to expand upon the mechanisms by which serum can regulate these processes. We first tried to identify potential factors from the serum which could regulate CdGAP. Our experiments show that EGF, PDGF and LPA alone are not sufficient to induce an upregulation of CdGAP mRNA. However, this does not exclude the possibility that they may be involved in the process, as co-stimulatory factors. In order to narrow down the possible factors we incubated the serum at 100 degrees Celsius for 10 minutes to denature the proteins prior to stimulating the cells. We found that this did not affect the potency of the serum. Although this indicates that intact proteins are not necessary for this process, caution must be taken as it is possible that there are proteins within the serum that were not denatured by boiling, and further experimentation, such as treating the serum with proteases will be necessary to rule out the necessity of proteins in this process. If intact proteins within the serum are indeed not necessary to stimulate the upregulation of CdGAP levels, this opens the interesting possibility that some lipids may regulate CdGAP levels.

We then proceeded to look at the signaling cascades involved in this process, and we found that staurosporine, a general Ser/Thr kinase inhibitor was able to block the process quite effectively. We then tried to identify specific Ser/Thr kinases involved in the upregulation of CdGAP. We found that P38 MAPK, mTOR, and ERK were not

necessary for serum-induced induction of CdGAP mRNA levels. The role of PI3 kinases is less clear; LY 294002 was able to effectively block the upregulation of CdGAP mRNA, while wortmannin was not able to do so. There are a few possible explanations of these results. The first, and most likely, is that LY 294002 is acting on other kinases that are involved in this process. It is in fact well known that LY 294002 can act on CKII with equal affinity as for PI3K (426), and, although not as strongly, it can also block phosphorylase kinase, and GSK3beta (426). We have previously shown that GSK-3beta is not necessary in the upregulation of CdGAP mRNA (422), but it remains possible that CKII and/or phosphorylase kinase are involved, and this will have to be examined further. Also, it is possible that LY 294002 more completely blocks PI3K than wortmannin, and that wortmannin although seemingly blocking PI3K activity, may still allow a small, hard to detect residual activity that is enough to propagate signaling leading to upregulation of CdGAP mRNA. It will be necessary to use RNAi of PI3K or its downstream effectors to fully resolve the role of PI3K in these processes.

Rho GTPases are intimately linked with signal transduction and are involved in the signal transduction pathways leading to an upregulation of CdGAP mRNA after serum stimulation. It remains to be determined which members are specifically involved, and it would be ideal to test this using RNAi to selectively knock-out particular GTPases. The fact the Rho GTPases are involved in this process creates an interesting potential for regulation of CdGAP. It is possible that Rac1 or Cdc42 are involved in transducing signals leading to the upregulated expression of their own repressor. It is likewise possible that one of the other GTPases is signaling to increase the levels of CdGAP to inhibit Rac1 and Cdc42 while itself remaining active.

The promoter region of CdGAP has numerous putative binding sites for transcription factors. In order to characterize the promoter region involved in the upregulation of CdGAP, we attempted to setup a luciferase reporter assay system. This is a powerful tool not only to characterize the promoter region of CdGAP, but also to investigate the upstream signaling pathways involved, as one can co-transfect cells with the luciferase reporter along with cDNAs, or siRNA constructs. It allows one to read exclusively cells that have been transfected and is thus an effective tool to reduce background noise when using cells with low transfection efficiency. The constructs that we created, with varying lengths of the CdGAP promoter used to drive luciferase expression were all able to express luciferase; however, they were not regulated by serum. This could be explained by a number of possibilities; the likely ones being that with a small isolated region in a vector, it is constantly exposed to transcription factors, and thus may lose regulation dependant on availability, or that although there is enough promoter to drive a basal expression, the elements regulated by serum are further upstream or downstream of the ATG, and thus, the promoter can drive the expression of luciferase at a basal level, but will not respond to serum stimulation.

In conclusion, the Rho GTPases, and possibly the PI3kinases or other LY 294002 sensitive kinases are involved in the up-regulation of CdGAP by serum. This presents yet another complexity to the regulation of CdGAP as it is the RhoGTPases themselves that are necessary to induce the expression of one of their repressors.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 Major Findings

1. Prior to the studies in this thesis, very little was known about the regulation of CdGAP. In the second chapter we describe a second, longer isoform of CdGAP that contains the entire short form (820 amino acids) of CdGAP, and extends at the C-terminal side to a total of 1425 amino acids. We have characterized the phosphorylation of CdGAP, and we have examined how phosphorylation regulates CdGAP. We have shown that both the short and long isoforms of CdGAP are phosphorylated, and that this phosphorylation is mainly in the PRD (amino acids 516-820). We have also shown that the phosphorylation is mostly of serine residues. We demonstrated that MEK-1 inhibition leads to decreased phosphorylation of CdGAP downstream of PDGF signaling. We identified RSK-1 and ERK1 as kinases for CdGAP, and we further identified the site which ERK1 phosphorylates. We have shown that this site is important in the regulation of the catalytic activity of CdGAP, and phosphorylation of this site diminishes GAP activity. The phosphorylation of CdGAP by RSK and ERK1, and the ability of the latter to regulate GAP activity highlight an important potential role for CdGAP in mediating cross-talk between Ras/MAPK pathways and RhoGTPase signaling.

2. In the third chapter of this thesis we set out to identify binding partners for the PRD of CdGAP and characterize their interaction with CdGAP. We identified GSK-3 as both an interacting partner of CdGAP as well as a kinase for CdGAP. We demonstrated that CdGAP is phosphorylated *in vivo* by GSK-3 at Thr-776, the same site that is phosphorylated by ERK1 and known to regulate CdGAP activity. This is particularly interesting as there are only two other known examples where both ERK1 and GSK-3 regulate the same site, and little is known about the consequences of this

phosphorylation. ERK1 is generally active at times when GSK-3 is inactive, and GSK-3 is generally active at times when ERK1 is not active. This suggests an interesting potential for temporal regulation of the GAP activity of CdGAP. We have also shown that CdGAP mRNA is transcriptionally upregulated by serum stimulation. This is a novel potential mechanism for RhoGAP protein regulation and expands the regulation repertoire of the RhoGAP proteins, and consequently that of the Rho GTPases. Furthermore, we have shown that GSK-3 is involved in the upregulation of CdGAP protein levels after the transcriptional upregulation caused by serum stimulation, however the mechanism for this is still unknown.

3. In the fourth chapter, we further examined the pathway by which serum upregulates CdGAP mRNA levels. We have demonstrated that intact proteins are not necessary in the serum and that Ser/Thr kinases are involved. The PI3 kinases may be involved in the process, but our data is inconclusive and further testing will be necessary to determine if this is in fact the case. We did find that Rho GTPases are involved in this process, and this opens the door to two interesting possibilities. First, Cdc42 and/or Rac1 activity leads to the upregulation of CdGAP levels, leading to a negative feedback repression of their activity. Second, other Rho GTPases stimulate the upregulation of CdGAP levels in order to turn off Rac1 and Cdc42 signaling.

5.2 Phosphorylation of CdGAP

RSK

RSK was able to phosphorylate CdGAP in the PRD, on a site different from ERK1. Although there is only one predicted site for RSK phosphorylation in the PRD, it still remains to be confirmed that Ser-765 is the RSK-1 phosphorylation site using point mutants of this site. It is likely to be the site; however, the possibility always remains that there is another non-classical site. RSK phosphorylates CdGAP on serine residues, which, *in vivo*, are more phosphorylated than threonine, and thus RSK may be a major contributor to serine phosphorylation of CdGAP. RSK phosphorylation of the PRD seems to be weak, and thus, perhaps its contributions to CdGAP serine phosphorylation may also be due to other phosphorylation sites. The central domain of CdGAP has two such sites, Ser-272 and Ser-295. Preliminary evidence indicates that RSK can phosphorylate recombinant CdGAP central domain, and this phosphorylation is on serine residues (unpublished observations). It remains to be determined which CdGAP residues are phosphorylated by RSK, and, further, what the effects of this phosphorylation are on CdGAP catalytic activity or on the interaction of CdGAP with other binding partners. Interestingly, phosphorylation of Ser-272 would create a potential binding site for 14-3-3. 14-3-3 is involved in localization of proteins within the cell. This seems to be accomplished by 14-3-3 binding blocking other localization signals, and when it is no longer bound, these signals are exposed and the protein can go to other locations within the cell, such as import to the nucleus which is prevented when 14-3-3 binds to a protein and blocks access to its NLS (427). 14-3-3 is also involved in cytoplasm-mitochondria

shuttling, and cytoplasm-plasma membrane shuttling in addition to nucleo-cytoplasmic shuttling (428). 14-3-3 is also known to bind to proteins and stabilize active conformations, as in the case of AANAT (which regulates melatonin synthesis), which is phosphorylated and then bound by 14-3-3. 14-3-3 binds its active conformation and holds it in this state (427, 429). Any of these possibilities lead to regulation possibilities for CdGAP, which could have its subcellular location altered by interaction with 14-3-3, or, it could also have its active or inactive form stabilized by interaction with 14-3-3.

ERK

Elimination of Thr-776 was not able to cause a complete loss of phosphorylation of the PRD of ERK1/2, while impairing ERK binding to the PRD did cause a near total loss of phosphorylation. There are 12 other "minimal" consensus sequences for ERK1/2, and thus it is likely that one or more of these is also phosphorylated. It will be necessary to explore these sites and any potential roles they may have in activation of CdGAP. Perhaps mutation of multiple ERK1/2 sites to alanine would lead to a stronger effect on GAP activity. It is also possible that these sites have other roles for CdGAP beyond GAP activity.

GSK-3

In this thesis, we have explored phosphorylation of CdGAP by GSK-3 and determined that GSK-3 could phosphorylate CdGAP at Thr-776 in the PRD. However, our studies do not necessarily exclude the possibility that GSK-3 phosphorylates CdGAP at other residues. As our *in vitro* phosphorylation assay using Thr-776 substituted with

alanine has shown, there is still some residual phosphorylation of CdGAP. Assessing the roles of other phosphorylation sites is quite difficult in the context of the Thr-776, as, GSK-3 normally needs a substrate to be primed with a phosphorylation event +4 amino acids C-terminal to its own site. In the case of the PRD of CdGAP the Thr-776 is a "self-primed" site, with a glutamic acid substituting for a phosphorylated residue. This allows its bacterially expressed product to be a strong substrate for GSK-3 in *in vitro* kinase reactions using recombinant GSK-3. The other potential sites for GSK-3 are all classical sites that require a priming phosphorylation event, and as such should make weak substrate sites using bacterially expressed CdGAP-PRD in an *in vitro* kinase assay with recombinant GSK-3. The weak residual phosphorylation seen after the substitution of Thr-776 to alanine could be indicative of one or more GSK-3 phosphorylation sites that are poor recombinant substrates. It will be interesting to mutate potential GSK-3 sites in a PRD that already contains Thr-776 mutated to alanine, as this will remove efficient phosphorylation of this site, which could cloud the phosphorylation of the PRD by weaker sites that normally require a priming phosphorylation. It will also be interesting to test the central domain of CdGAP for phosphorylation by GSK-3, as it also harbors potential sites for phosphorylation by GSK-3.

The PKC family

Aside from GSK-3, ERK1 and RSK, there are many other kinases that could potentially phosphorylate CdGAP. One particularly interesting candidate is PKCζ. CdGAP contains one potential PKCζ phosphorylation site, S323, in the central domain. Cdc42 activation leads to activation of PKCζ and subsequent inactivation of GSK-3 (367,

368). This could lead to a couple of interesting regulation options for CdGAP. In the first case, assuming that phosphorylation of CdGAP by PKCζ is inhibitory to GAP activity, Cdc42 activity would lead to an increase of PKCζ activity, which could phosphorylate CdGAP and inactivate it, leading to sustained Cdc42 activation. This could be particularly interesting, since GSK-3 can phosphorylate CdGAP leading to decreased activity, and, since PKCζ inactivates GSK-3, it would be relieving GSK-3 induced dampening of CdGAP activity. A balance would have to be reached between PKCζ phosphorylation and the lack of GSK-3 phosphorylation leading to decreased GAP activity. Furthermore, it is also possible that Cdc42 activity leads to ERK1 activity, and, as such, induces phosphorylation of CdGAP at the site regulated by GSK-3. As a result this site would remain phosphorylated in addition to the phosphorylation mediated by PKCζ. This is based on the assumption that phosphorylation of CdGAP by PKCζ leads to decreased GAP activity. It is also equally possible that phosphorylation by PKCζ leads to increased GAP activity, as is seen with FilGAP which has its activity upregulated after phosphorylation by ROCK (283). In this way, Cdc42 could control the duration of its signaling, activating PKCζ, which would then phosphorylate and activate CdGAP which would then inactivate Cdc42. In this mechanism, again, GSK-3 would presumably also be inactivated, and thus would not be able to diminish GAP activity through its phosphorylation, further encouraging an active GAP protein. Of course in this case ERK1 could phosphorylate CdGAP at Thr-776 and thereby act antagonistically to PKCζ phosphorylation, and again a balance would have to be reached. It is also possible that PKCζ phosphorylation of CdGAP occurs in a different location from ERK1 or GSK-3, or that due to temporal restrictions the kinases do not overlap in their function.

Cdc42 activity is also able to activate PLCγ which is an upstream activator of PKCδ (430, 431), and, further, PKCζ may also be involved in activation of PKCδ, giving Cdc42 a second potential mechanism by which to activate PKCδ (432, 433). In the central regions of CdGAP there are five potential PKCδ phosphorylation sites, three serines and two threonines, and there is one potential serine residue in the proline rich domain. Cdc42 (and RhoA) can also activate PKCα (434), and there are five potential phosphorylation sites in the central domain, four serines and two threonines. Thus, through various PKC isoforms, it may be possible for Cdc42, and other RhoGTPases to regulate CdGAP activity. This could be in a number of possible fashions, either by directly affecting catalytic activity of CdGAP, altering the location of active CdGAP, or blocking or encouraging binding of other interacting partners.

With so many potential kinase sites in CdGAP, it will be of great help to find *in vivo* phosphorylation sites in a systematic fashion. Current mass spectrometric tools allow for the identification of *in vivo* phosphorylation sites using immunoprecipitated proteins. This will be invaluable to identify new phosphorylation sites of CdGAP. Having a starting point of a known *in vivo* phosphorylation site, it will then be necessary to identify the kinase(s) that phosphorylates the site, and to characterize the effects of phosphorylation of these sites on CdGAP function.

5.3 ERK and GSK-3 mediated phosphorylation of Thr-776

In this study we have shown that ERK1 and GSK-3 can both phosphorylate Thr-776, which, when phosphorylated, leads to diminished GAP activity. This allows for an

interesting regulation potential, as ERK1 and GSK-3 are generally not active under the same conditions. GSK-3 is normally active in resting cells, and stimulation of cells with agents such as growth factors transiently inactivates GSK-3 (435, 436). On the other hand, ERK1 is normally inactive in resting cells and is transiently activated upon stimulation of cells with agents such as growth factors (437, 438). This sets up the potential for temporal regulation of GAP activity. For example, when cells are stimulated, ERK1 is activated, and GSK-3 would be inactivated, and then, there would be a time, as little as twenty minutes after stimulation depending on the factor used, when ERK1 activity wanes (437, 438), and GSK-3 begins to regain its activity. Presumably, there would be a time when both kinases are inactive and a phosphatase could relieve CdGAP of this phosphorylation, and CdGAP would then be more active, until GSK-3 recovers its activity and phosphorylates CdGAP.

5.4 Potential Interacting Partners

In addition to the six clones corresponding to GSK-3α, nine other clones were isolated from the yeast two-hybrid screen carried out in Chapter 3. These clones were able to grow on selective medium lacking histidine, and were able to express β-galactosidase, indicating a possible interaction with the PRD of CdGAP. It was not possible to sequence six of these clones. The other three clones were identified by DNA sequencing as ZFHX1B, MCM7, and ZBTB4 (Table 5.1). Although GSK-3α came out as nearly half of the screen, enrichment during the propagation of the library cannot be

Table 5.1: CdGAP has multiple potential interacting partners. Table representing the positive clones that were found in addition to GSK-3α in the yeast two-hybrid screen described in chapter 3. β-galactosidase activity is represented on a scale where (+) is weakly positive and (+ + +) is highly positive within 2 hours. " Not determined" refers to clones that were not assayed for β-galactosidase activity. Growth on 3-amino-1,2,4-triazole (3-AT) medium plates is represented on a scale where (+) indicates weak growth and (+ + +) represents strong growth. The identity of the clones was determined by sequencing, "No signal" refers to clones which were not able to be sequenced.

Clone #	β-galactosidase activity	Growth on 3-AT medium	Identity
5A	+	+++	MCM7
8A	not determined	+++	ZFHX1B
134A	++	+++	ZBTB4
56A	+++	+++	no signal
200A	+++	+++	no signal
100A	+++	+++	no signal
174A	++	+++	no signal
3A	+	+++	no signal
29	not determined	+++	no signal

ruled out, and thus, the relative abundance of GSK-3α may not reflect a higher binding affinity for the PRD of CdGAP.

ZFHX1B (also known as SIP1) is a gene involved in multiple human pathologies. Mutations in ZFX1B cause Hirschsprung disease (HSCR), which is characterized by mental retardation, microcephaly and distinct facial features (439). Nonsense mutations of ZFHX1B are also involved in Mowat-Wilson syndrome, which is a multiple congenital anomaly-mental retardation (440). ZFHX1B interacts with R-Smads following receptor-mediated Smad activation (441). ZFHX1B is a member of the δEF1 family of two-handed zinc finger homeodomain proteins and it binds to a bipartite sequence; a CACCT and a CACCTG sequence, and acts as a transcriptional repressor (441).

ZBTB4 is a zinc finger containing protein that binds to methylated DNA and blocks transcription (442).

MCM7 (also known as Cdc47), is a member of the MCM family of proteins, which consists of MCM2, 3, 4, 5, 6 and 7 (443). The MCM proteins are involved in the initiation of DNA replication, and in replication fork migration (443). MCM7 exists either in a complex with MCM2, 3, 4, 5 and 6 or as part of a smaller complex with MCM2, 4 and 6 (443). It is normally localized in the nucleus, and approximately one quarter of the total pool of MCM7 is associated with insoluble nuclear material. This association decreases as the cell progresses through S phase (443, 444). MCM7 is expressed throughout the cell cycle, with a slight increase in expression as cells enter S phase (444). MCM7 is localized to the nucleus, throughout the cell cycle (444).

It is interesting that two of these three potential interacting partners for CdGAP are transcriptional repressors, and the third is involved in the initiation of DNA replication. This indicates a possible role for CdGAP in regulating gene expression or cell proliferation. Of course, it must first be determined whether these interactions are in fact true interactions that occur with full-length proteins in mammalian cells, but assuming they are real interacting partners opens the door to some interesting possibilities. It will be important to determine the role of an interaction of these proteins with CdGAP on their function. CdGAP could either aid their function, leading to the repression of gene expression or to enhanced or permitted DNA synthesis. Or it could prevent their function leading to static or upregulated expression of certain genes, or a block of DNA synthesis. CdGAP may play a role in altering the location of these factors. For example, it may sequester them in the cytoplasm and prevent them from entering the nucleus, thereby preventing the repressors from inhibiting their target, or preventing MCM7 from aiding in the initiation of DNA replication. This sequestration of transcription factors in the cytoplasm is seen with another GAP protein, p190RhoGAP, which binds to the transcription factor TFII-I and keeps it in the cytosol, preventing it from entering into the nucleus (445). It is also possible that CdGAP functions as a shuttle, binding the transcriptional repressors or MCM7 and shuttling them into the nucleus, as is seen with MgcRacGAP which is involved in the shuttling of STAT5A to the nucleus (281). Preliminary evidence from our laboratory indicates that there may be a pool of CdGAP within the nucleus (unpublished observations). Further work will have to be done in order to confirm this, and to see what effect altering the localization of CdGAP would have on the activity or location of the two repressors or MCM7.

It also remains to be determined whether or not these interactions are related to the ability of CdGAP to function as a GAP protein. It is conceivable that CdGAP could be bound to one or more of these proteins, but when it interacts with active Cdc42 or Rac1 the conformation of CdGAP may shift and release the protein. In this fashion, CdGAP could act as a GAP and an effector of Rac1 and/or Cdc42 at the same time. It is also possible for this to occur without CdGAP enhancing GTPase activity, with CdGAP acting just as an effector, as is seen with another GAP, n-chimaerin which can act as an effector for Rac1 and Cdc42 (265). It is also possible that the interaction of CdGAP with active Rac1 or Cdc42 alters the conformation of CdGAP allowing proteins such as MCM7, ZFHX1B or ZBTB4 to bind to CdGAP. In these ways Cdc42 or Rac1 could affect the activity and or localization of these proteins through the CdGAP GAP domain. It is also possible that interaction with these proteins has consequences on GAP activity. Perhaps when bound to CdGAP, GAP activity is enhanced or repressed, and thus, expression or localization of these proteins could be used as a switch to turn on or off GAP activity.

It is interesting to note that none of the aforementioned proteins contain SH3 domains, since the PRD of CdGAP contains multiple consensus SH3 binding motifs. It is possible that interacting partners of the proline-rich sequences of CdGAP are weak interacting partners that were not detected in our screen, and, it is also possible that the PRD of CdGAP needs to be modified by other proteins, such as kinases, in order for it to become a more attractive protein for SH3 domain containing proteins. It is also possible that modifications such as phosphorylation could make CdGAP a less attractive substrate for interacting partners such as SH3 domain containing proteins. This is quite possible,

as, for example, Thr-776 lies within a consensus SH3 binding site. It is possible that the phosphorylation state of this site could affect binding of other proteins to CdGAP. It is also possible that phosphorylation sites further away from these SH3 consensus binding sites could also affect conformation of the PRD, and thus accessibility of sites for interaction with other proteins.

5.5 Intersectin and CdGAP

The original motive for the yeast two-hybrid screen of chapter 3 was to find interacting partners of the PRD to help elucidate the role of the PRD in regulating GAP activity. It was previously shown that intersectin could bind to the central region of CdGAP and regulate CdGAP's activity; however, it could only do so if the PRD was present (325). We have now identified five potential binding partners of the PRD (GSK-3, ERK1, ZBTB4, ZFHX1B and MCM7), and we have shown that two of these, GSK-3 and ERK1, interact with the PRD. RSK-1 also interacts with CdGAP, and can phosphorylate the PRD, although the region of CdGAP to which it binds is still unknown. Phosphorylation of CdGAP in the PRD by GSK-3 and by ERK1 leads to decreased GAP activity. It will be interesting to find out whether the phosphorylation of this site affects the ability of intersectin to regulate CdGAP. It is possible that the phosphorylation could help or hinder this. In the former case, it is possible that this phosphorylation is necessary to stabilize a conformation of CdGAP that interacts with intersectin in such a fashion that it is able to regulate GAP activity. In the latter case, it is possible that this phosphorylation could destabilize the interaction of CdGAP and intersectin preventing

intersectin from regulating GAP activity. Since ERK, GSK-3 and RSK all bind to CdGAP it is also possible that their binding can block the binding of intersectin to CdGAP and thus block its ability to regulate GAP activity. It is likewise possible that the other potential CdGAP interacting partners could interfere with or help the interaction of CdGAP with intersectin, and help or interfere with the regulation of CdGAP catalytic activity by intersectin.

It is also interesting to note that the long isoform of intersectin contains a Cdc42 GEF domain, and can activate Cdc42 (446). Intersectin could therefore possibly activate Cdc42, while also binding to and inhibiting CdGAP, which can turn off Cdc42. This would enable intersectin to activate Cdc42 and keep it active. It is also possible that intersectin may function to bind and inactivate CdGAP, and keep it inactive in the vicinity of active Cdc42, and at the right time, release CdGAP, and thus release inhibition of CdGAP, allowing CdGAP to turn off Cdc42. It will be interesting to further explore the interaction of intersectin and CdGAP, and the roles of CdGAP post-translational modifications, or of other CdGAP binding partners in this interaction.

5.6 CdGAP Protein Upregulation

The question of just how GSK-3 leads to an upregulation of CdGAP protein levels remains to be answered. There are two potential mechanisms by which GSK-3 could achieve this. The first is through promotion of CdGAP mRNA translation. Although this is possible, it should be noted that in general GSK-3 activity has negative effects on translation (447). The second possibility is that GSK-3 activity leads to

stabilization of CdGAP protein. There are many potential mechanisms by which this could be achieved, such as by creating binding sites for proteins which bind to CdGAP and prevent its degradation. Another potential mechanism is phosphorylation leading to a conformational change rendering CdGAP resistant to degradation. In the latter case, it is possible that proline isomerization is involved. Proline isomerization is implicated in protein stability generally by altering the conformation of the protein and allowing or preventing it from interacting with other proteins such as ubiquitin ligases (448-451). The prolyl isomerase Pin1 recognizes pSer/pThr-Pro motifs (451), such as the one created by GSK-3 when Thr-776 is phosphorylated. It is possible that GSK-3 phosphorylates the newly translated CdGAP, and that Pin1 then interacts with and changes the conformation of CdGAP, rendering it more resistant to degradation. A potential change of conformation brought on by prolyl isomerases may also have roles beyond protein stability. For example, it may be a player in the in the regulation of GAP activity, by means of causing or relieving intramolecular inhibition, or encouraging or preventing protein-protein interactions that could enhance or disrupt catalytic GAP activity.

5.7 Conclusions

In this thesis we characterized the regulation of CdGAP. We identified new interacting partners for CdGAP, characterized the phosphorylation of CdGAP in general and characterized in detail the phosphorylation by ERK1 and GSK-3 of Thr-776, which is involved in regulation of GAP activity. We have also shown that serum stimulation upregulates CdGAP mRNA, and that the RhoGTPases are involved in this process. With the latter, we have identified a novel potential mechanism of RhoGAP regulation.

Regulation of a RhoGAP protein extends beyond the RhoGAP itself: regulation of RhoGAP activity becomes regulation of the RhoGTPases that are regulated by the RhoGAP. In our case, the studies we have undertaken to better understand CdGAP give us greater insight into the regulation of Cdc42 and Rac1. In this context, we now know that GSK-3 and ERK1 could regulate Cdc42 and Rac1 activity through CdGAP. CdGAP can serve as a link mediating cross-talk between Ras signaling and Cdc42/Rac1 signaling through ERK and CdGAP. PI3K and or WNT pathways could modulate Cdc42/Rac1 through GSK-3 and CdGAP, and of course, CdGAP can serve as a convergence point of ERK and GSK-3 signaling, suggesting potential temporal regulation of CdGAP. We also know that serum stimulation could lead to later inhibition of Cdc42 and Rac1 by upregulating CdGAP mRNA and subsequently CdGAP protein levels.

Understanding the regulators of the GTPases is extremely important in order to be able to design effective strategies to cope with such a devastating illness as cancer. The fundamental problem is that the GTPases are involved in both normal cell processes as well as pathological states, and as a result, targeting a GTPase directly may have adverse

effects in addition to the desired effects. Ideally, we would be able to turn a GTPase on or off in a specific context or cell type while leaving the GTPase to function normally in other contexts or cell types. The positive and negative regulators of these GTPases are attractive therapeutic options, as they could be used to turn on or turn off GTPases in specific contexts. For example, if a tumor cell has a specific GAP that is not found in other cell types and this GAP becomes activated, it could then turn off the GTPase in the tumor cell, and not in other cells. It is also possible for the RhoGAP proteins to serve as markers. RhoGAP proteins are deleted in various cancers and their absence could serve as markers for the severity of a cancer and help to design appropriate therapeutic strategies.

As we learn more about the molecular regulation of CdGAP, it becomes apparent that we need to also learn more about the clinical contexts of CdGAP. The GTPases that are regulated by CdGAP are intertwined in numerous cell signaling and response pathways and their aberrant signaling can lead to numerous problems. Most notably Cdc42 and Rac1 are implicated in cancer at many different stages, from proliferation leading to tumorigenesis, through to metastasis. CdGAP is a negative regulator of Cdc42 and Rac1 and is regulated by signaling pathways that are well known for their roles in cell survival and proliferation: GSK-3 and ERK1. As such it is likely that CdGAP may have a role or even multiple roles in tumorigenesis and cancer.

In conclusion, through this work we have gained a better understanding of how CdGAP is regulated, and thus, we have gained new insights into how a cell can control Cdc42/Rac1 signaling.

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