Phosphoregulation of CdGAP and DCC, proteins involved in actin dynamics

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CONTRIBUTIONS OF AUTHORS

This thesis is a manuscript-based structure. According to the faculty regulations, manuscripts co-authored by others must be accompanied with an explicit statement as to who contributed to such work and to what extent. Copyright waivers form the co-authors and the publishers appear in the appendix.

Chapter 2

My contribution to the manuscript entitled "Extracellular signal-regulated kinase 1 interacts with and phosphorylates CdGAP at an important regulatory site" involved performing all the experiments except for the Western blot of mouse tissues in Figure 1B, which was performed by Ibtissem Triki. The CdGAP deletion mutants were produced by Dr. Sarah Jenna and Eric Danek who also made the His-tagged CdGAP-PRD-T776A protein mutant. Both Dr. Nathalie Lamarche-Vane and I contributed to the writing of the manuscript.

Chapter 3

My contribution to the manuscript entitled "The human ortholog of CdGAP is a phosphoprotein and a GTPase-activating protein for Cdc42 and Rac1 but not RhoA" involved making Figure 1 and performing the experiments in Figure 5. The experiments in Figure 2 and 3 were performed by Ibtissem Triki and the experiments in Figure 4 were conducted by Dr. Nathalie Lamarche-Vane. Both Dr. Nathalie Lamarche-Vane and I contributed to the writing of the manuscript.

Chapter 4

My contribution to the manuscript entitled "Phosphorylation of DCC by Fyn mediates netrin-1 signaling in growth cone guidance" involved initiating the project and performing the experiments in Figure 1C, D, E, F and performing the experiments in Figure 2A. The subsequent experiments were performed by Dr. Mayya Meriane except for Figure 3 that was performed by Christine Webber. The GST-tagged intracellular domain of DCC wild type and tyrosine mutants were produced by Ibtissem Triki. Dr. Nathalie Lamarche-Vane, Dr. Mayya Meriane and, myself contributed to the writing of the manuscript.

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Chapter 4

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ABSTRACT

The Rho GTPases are members of the Ras superfamily of small monomeric GTP binding proteins that regulate multiple cellular processes affecting both cell proliferation and cytoskeletal dynamics. They are positively and negatively regulated by the guanine nucleotide exchange factors (GEFs) and the GTPase-activating proteins (GAPs), respectively. In principle, extracellular signals such as growth factors and other agents bind to plasma membrane receptors and modulate Rho GTPase function through intracellular mechanisms that affect GEF or GAP activities. There is now compelling evidence that both receptors and cytoplasmic proteins implicated in Rho GTPase signaling are modulated by post-translational modifications such as phosphorylation.

CdGAP (Cdc42 GTPase-activating protein) is a negative regulator of Rac1 and Cdc42 and the netrin-1 receptor DCC (deleted in colorectal cancer) is an upstream activator of these GTPases during axon guidance. To date, very little is known about the biochemical regulation of these two proteins. Given that CdGAP protein migrates higher than its expected molecular weight and contains a number of consensus phosphorylation sites for different kinases, and that the DCC ortholog in *C. elegans* UNC-40 is tyrosine phosphorylated, it is likely that both proteins are regulated through mechanisms involving phosphorylation.

In the first part of this thesis, we have demonstrated that CdGAP phosphorylation *in vivo* is stimulated by external cues that activate the Ras-MEK-ERK pathway. We also found that CdGAP is phosphorylated *in vivo* on serine, threonine but not tyrosine residues. Phosphorylation of Thr776 by ERK and that this phosphorylation is important in regulating CdGAP's activity towards Rac1.

In the second part, we found that the human ortholog of CdGAP is also active both *in vitro* and *in vivo* on Cdc42 and Rac1 but not RhoA. Furthermore, we found that the human protein is also highly phosphorylated *in vivo* on serine and threonine residues but not tyrosine.

In the last part, we have combined classical biochemical approaches and the dissection of rat primary commissural neurons and found that DCC is phosphorylated *in vivo* upon netrin-1 stimulation on serine, threonine and tyrosine residues. We have also

found that the phosphorylation and function of DCC in axon outgrowth and guidance is mainly regulated by the Src family tyrosine kinases.

RÉSUMÉ

Les petites GTPases Rho sont des protéines de signalisation intracellulaire de la famille Ras. Activées par des signaux extracellulaires très variés, elles induisent en aval des réponses multiples impliquant notamment l'organisation du cytosquelette d'actine, l'activation de facteurs de transcription et la progression du cycle cellulaire. Leur activité régulatrice est fondée sur leur capacité à cycler entre une forme inactive liée au GDP et une forme active liée au GTP, cette dernière transmettant un signal d'action aux protéines dites 'effectrices' située en aval de la cascade de signalisation. Les Rho GTPases sont activés par les GEFs (guanine nucleotide exchange factors) et inactivés par les GAPs (GTPase-activating proteins). En principe, les signaux extracellulaires régulent les Rho GTPases en se liant à des récepteurs membranaires, ce qui engendre une chaîne d'événements qui provoquent la modification de l'activité des GEFs ou des GAPs. De nombreuses recherches ont démontré que les récepteurs et les régulateurs impliqués dans les voies de signalisation des Rho GTPases sont régulés par des modifications post-traductionnelles tel que la phosphorylation. CdGAP (Cdc42 GTPase-activating protein) est une protéine cytoplasmique qui inhibent l'activité de Rac1 et de Cdc42 et le récepteur de la nétrine-1, DCC (deleted in colorectal cancer) est un activateur de ces mêmes GTPases. Actuellement, les mécanismes de régulation biochimique de CdGAP et DCC sont inconnus. Étant donné que CdGAP contient de nombreux sites consensus pour différentes kinases et que l'homologue de DCC chez *C.elegans* UNC-40 est phosphorylé sur des résidus tyrosines, il est probable que ces deux protéines soit régulées par des mécanismes implicant la phosphorylation.

Dans la première partie de ma thèse, nous avons démontré que la phosphorylation de CdGAP *in vivo* est modifiée par des facteurs extracellulaires qui activent la voie de signalisation Ras-MEK-ERK. Nous avons également démontré que CdGAP est phosphorylé *in vivo* sur des résidus de sérine, thréonine mais non tyrosine. La phosphorylation de CdGAP sur Thr776 par ERK semble important pour la régulation de l'activité de CdGAP envers Rac1.

Dans la deuxième partie de ma thèse, nous avons découvert que l'orthologue humaine de CdGAP est également active *in vivo* et *in vitro* envers Rac1 et Cdc42 mais

pas envers RhoA. De plus, la forme humaine est aussi phosphorylée *in vivo* sur des résidus de sérine et thréonine mais non tyrosine.

Finalement, en combinant plusieurs méthodes de biochimie classique avec la dissection de neurones commissuraux, nous avons déterminé que DCC est phosphorylée *in vivo* suite à une stimulation par la nétrine-1. Nous avons également démontré que les tyrosines kinases de la famille Src sont sont responsables pour la phosphorylation et la fonction de DCC en ce qui a trait à la guidance axonale.

LIST OF ABBREVIATIONS

Abl	Abelson tyrosine kinase
Abr	Active-Bcr-related
ARF	ADP-ribosylation factor
Arp2/3	Actin-related protein 2 and 3 complexes
ATP	Adenosine triphosphate
BAR	BinAmphiphysinRvs domain
Bcr	Breakpoint cluster region
3BP-1	SH3 binding protein-1
BPGAP1	BNIP-2 and Cdc42GAP Homology (BCH) domain-containing, Proline-rich and Cdc42GAP-like protein subtype-1
BSA	Bovine serum albumin
C1	Cysteine-rich phorbol ester binding
C2	Calcium-dependent lipid binding
CaM	Calmodulin
CAMGAP1	CIN85 associated multi-domain containing RhoGAP1
cAMP	Cyclic adenosine monophosphate
CC	Coiled-coil
Cdc42	Cell division cycle 42
CdGAP	Cdc42 GTPase-activating protein
Cdk	Cyclin-dependent kinase
CeGAP	*C.elegans* GAP
cGMP	Cyclic guanosine monophosphate
CRIB	Cdc42/Rac interactive binding
CRMP-2	Collapsin response mediator protein-2
Dbl	Diffuse B cell Lymphoma
DCC	Deleted in colorectal cancer
DEP	Domain found in Dishevelled, Egl-10, and Pleckstrin
DH	Dbl homology
DLC	Deleted in liver cancer
Dock	Dreadlocks
Dok	Downstream of tyrosine kinase
DRacGAP	Drosophila RacGAP
Dscam	Down syndrome cell adhesion molecule
E13	Embryonic day 13
ECM	Extracellular matrix molecules
EGF	Epidermal growth factor
EH	Eps15 homology
Eph	Erythropoietin-producing hepatocellular receptor

Ephexin	Eph-interacting exchange factor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FCH	Fes/CIP4
FGF	Fibroblast growth factor
FF	Domain with two conserved phenylalanine (F) residues
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GMIP	Gem-interacting protein
GPI	Glycosylphosphatidylinositol
Graf	GAP for Rho associated with focal adhesion kinase
GRIT	Rho/Rac/Cdc42 small GTPases
GST	Glutathione S-transferase
GTP	Guanine triphosphate
IQ	Calmodulin binding motif
HEK	Human embryonic kidney
HGF	Hepatocyte growth factor
Ig	Immunoglobulin
Ig-CAM	Immunoglobulin family cell adhesion molecules
JNK/SAPK	c-jun N-terminal kinase/stress-activated protein kinase
LARG	Leukemia-associated RhoGEF
LIM	Zinc-binding domain present in Lin-11, Isl-1, and Mec-3
LIMK	LIM kinase
LOH	Loss of heterozygosity
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MEK	MAP/ERK kinase
MBS	Myosin-binding subunits
MgcRacGAP	Male Germ Cell RacGAP
MLC	Myosin light chain
MRCK	Myotonic dystrophy kinase-related Cdc42-binding kinase
Myr	Myosin from rat
MyTH4	Myosin tail homology
Nadrin	Neuron-associated developmentally regulated protein
NCAM	Neuron cell adhesion molecule
NGF	Nerve growth factor
OCRL-1	Oculocerebrorenal syndrome of Lowe-1

PAK	P21 activated kinase
PARG1	PTPL1-associated RhoGAP 1
PBS	Phosphate-buffered saline
PC12	Pheochromocytoma cell line
PDGF	Platelet-derived growth factor
PDZ	Domain present in PSD-95, Dlg, and ZO-1/2
PH	Pleckstrin homology
PI3-K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5 biphosphate
PIP3	Phosphatidylinositol 3,4,5 triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PSGAP	PH and SH3 domain containing rhoGAP protein
PTB	Phosphotyrosine-binding
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PX	Phox-homology
RA	Ras-associating domain
Rac	Ras-related C3 botulinum toxin substrate
RalBP1	Ral-binding protein 1
Ral BR	Ral GTPase binding region
Ras	Rat Sarcoma
RARhoGAP	Ra and RhoGAP domain containing protein
RBD	Rho-binding domain
RGC	Retinal ganglion cell
Rho	Ras homologous
Rich	RhoGAP interacting with CIP4 homologues
RIP1	Ral-interacting protein 1
RLIP76	Ral-interacting protein of 76 kDa
RnGAP	Rotund GAP
Robo	Roundabout
ROCK	Rho kinase
RPTP	Receptor protein tyrosine phosphatase
RT	Reverse transcriptase
RTK	Receptor tyrosine kinase
SAM	Sterile alpha motif
Scar	Suppressor of cAMP receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sec14	Sec14-like
S/T kinase	Serine and Threonine kinase

SH2	Src-homology 2
SH3	Src-homology 3
Shh	Sonic hedgehog
SLAP	SLP-76 associated protein
Sos	Son of sevenless
SRE	Serum response element
srGAP	Slit-Robo GAP
START	Star-related lipid transport domain
TCGAP	TC10/Cdc42 GTPase activating protein
tGAP1	Testicular GAP domain-containing protein 1
TGF	Transforming growth factor
Tiam1	T-lymphoma invasion and metastasis-1 gene
TOR	Target of rapamycin
TRITC	Tetramythylrhodamine isothiocyanate
UNC	Uncoordinated
VASP	Vasodilator-stimulated phosphoprotein
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP family Verprolin-homologous protein
WH1	WASP-homology domain 1
WIP	WASP interacting protein
WW	Domain with two conserved tryptophan (W) residues
XrGAP	Xenopus Rho-GTPase activating protein

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 GENERAL INTRODUCTION

A century ago, Phoebus Levene found that the protein Vitellin contained phosphate (Levene, 1906), and later with Fritz Lipmann reported that the phosphate was linked to serine residues (Lipmann F.A., 1932). Between 1940 and 1980, a number of studies on various aspects of hormone action and metabolism established serine/threonine phosphorylation as a key mechanism for rapidly modulating protein function through phosphorylation (Bishop and Larner, 1969; Cori, 1943; Fischer and Krebs, 1955; Krebs and Fischer, 1956; Soderling et al., 1970; Walsh et al., 1968). In 1979, Tony Hunter and colleagues identified phosphotyrosine as the product of a protein kinase activity of a viral oncoprotein, necessary for cellular transformation (Eckhart et al., 1979).

Today, it is recognized that reversible phosphorylation of proteins regulates nearly every aspect of the cell. Phosphorylation and dephosphorylation, catalysed by protein kinases and protein phosphatases, can modify the function of a protein in a number of ways. These include, modulation of its biological activity, stabilizing the protein or targeting it for degradation, facilitating or inhibiting movement between subcellular compartments, or affecting protein-protein interactions. The simplicity, flexibility and reversibility of phosphorylation, coupled with the ready availability of ATP, explain why it is the most general regulatory device adopted by eukaryotic cells. In fact, more than a third of cellular proteins appear to be phosphorylated (Ahn and Resing, 2001).

A wide range of cellular processes including gene expression, cytoskeletal architecture, cell cycle and cell adhesion, to name only a few, are regulated through mechanisms involving phosphorylation. The Rho GTPases are key regulators of actin dynamics, and many components of Rho signaling pathways have been shown to be regulated through phosphorylation. However, the role of phosphorylation in regulating some of these molecules such as CdGAP and DCC remains to be unraveled.

1.1 RAS GTPASES

1.1.1. Ras oncogenes

Surprisingly, our knowledge of tumor formation has its roots not in humans, but in the chicken. In 1908, Ellerman and Bang reported that an avian leukosis could be transmitted by a filterable agent, a virus, which caused lymphomas in chickens (Ellerman, 1908). Evidence from several laboratories in the 1970s demonstrated that the Kirsten and Harvey murine sarcoma virus as well as the Rous avian sarcoma virus had an additional gene (the *src* gene), which was not required for viral growth, but was necessary for oncogenic transformation (Ash et al., 1976; Collett and Erikson, 1978; Rasheed et al., 1978; Scolnick, 1979; Young et al., 1979). When the viral DNA sequences of the *src* gene were isolated and characterized they were found to have high similarity to genes that already existed in animals. It was proposed that these viral oncogenes had actually been captured from a normal cellular proto-oncogene. By incorporating these genes and mutating them, the virus increased host cell proliferation, and consequently, its own replicative potential. This hypothesis led to the prediction that mutated forms of these same genes would likely be found in naturally occurring tumor cells as well. In fact, the cellular oncogenes of the Harvey (Ha) and Kirsten (Ki) sarcoma virus transforming gene v-Ras, were identified in humans, and mutations in the protein sequences at residues Gly12, Gly13 or Gln61 were found in 30 % of human carcinomas (Barbacid, 1987; Quilliam et al., 1994). The mutated forms were then shown to stimulate proliferation and transformation of cultured cells.

1.1.2. Ras, a molecular switch

The three human *ras* genes encode four highly related proteins, H-Ras, N-Ras, K-Ras4A and K-Ras4B that are associated with the inner face of the plasma membrane where they relay signaling initiated by diverse extracellular stimuli such as growth factors, cytokines, hormones and neurotransmitters to numerous intracellular signal transduction pathways that influence cell morphology, differentiation and survival (Campbell et al., 1998). Like most GTPases their biological activities are controlled by a regulated GDP/GTP cycle. This cycle is tightly regulated by Guanine nucleotide exchange factors (GEFs), like RasGRF/mCDC25 and SOS1/2 that promote the exchange

of GDP for GTP and Ras GTPase activating proteins (GAPs) such as p120RasGAP and NF1-GAP/neurofibromin that accelerate the intrinsic GTPase activity, thus inhibiting Ras (Downward, 2003). The best-characterized Ras-mediated signal transduction pathway involves the activation of mitogen-stimulated receptor tyrosine kinases (RTKs) (Repasky et al., 2004). The mitogen-stimulated RTK undergoes autophosphorylation of specific tyrosine residues in its cytoplasmic domain, which creates binding sites for the Src homology 2 (SH2) and/or phosphotyrosyl binding (PTB) domains of the Shc and/or Grb2 adaptor proteins. Shc binding to activated RTK induces its autophosphorylation which creates recognition sites for the SH2 domain of Grb2. Given that Grb2 and SOS are stably associated, the translocation of Grb2 to the plasma membrane induces Ras activation (Downward, 2003). The transition of Ras between the GDP- and GTP-bound states is accompanied by changes in protein conformation that greatly influence the affinity of Ras binding to effector molecules that stimulate diverse cytoplasmic signaling activities.

1.1.3. Ras-Raf-MEK-ERK signaling cascade

The most widely studied effectors for Ras signaling are the Raf serine/threonine kinases (c-Raf-1, A-Raf, B-Raf). Ras promotes Raf association with the plasma membrane, where other events such as phosphorylation by Src, PKC and PAK facilitate Raf activation (Chong et al., 2003). Subsequently, Raf phosphorylates and activates its only known substrate the MEK1 and MEK2 enzymes. These are dual specificity kinases, which phosphorylate and activate ERK1 and ERK2 mitogen-activated protein kinases (MAPKs). ERK1 and ERK2 are proteins of 43 and 41 KDa that are approximately 85 % identical in the entire protein sequence and belong to a growing family of MAPKs (ERK1 to ERK8) (Bogoyevitch and Court, 2004). Both are ubiquitously expressed, although their relative abundance in tissues is variable (Pearson et al., 2001). Activated MAPKs either interact with membrane-bound or cytoplasmic proteins or translocate to the nucleus and activate transcription factors, which regulate gene expression. Moreover, activated MAPKs also phosphorylate and activate a downstream substrate the RSK serine/threonine kinase which interacts with overlapping substrates as well as specific substrates (Blenis, 1993). In mammals, there are four RSK isoforms, termed RSK1 to -4. Comparative

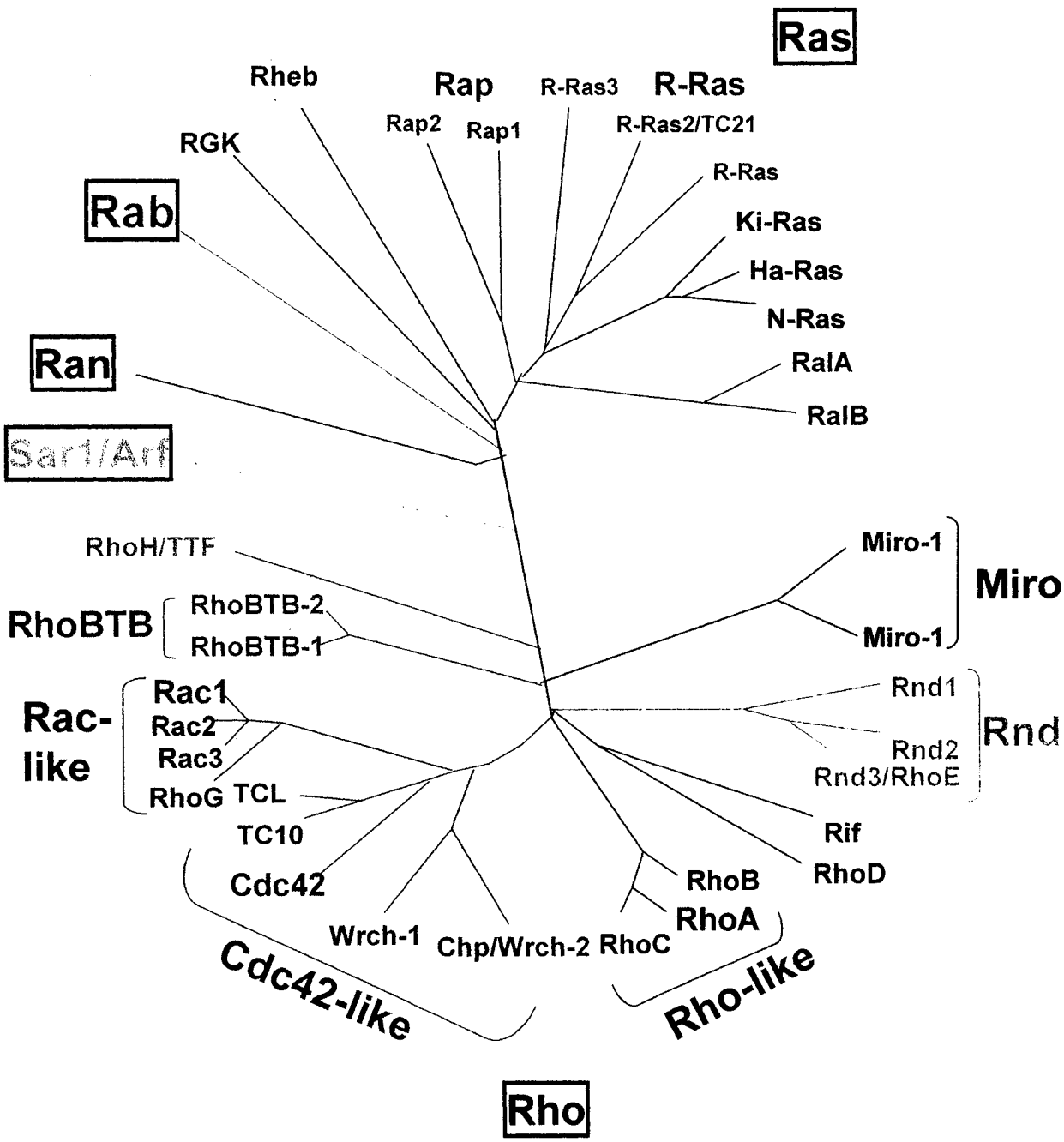
analyses of RSK isoforms propose that these proteins may have distinct roles for specifying ERK signals. Biochemical studies of RSK1-3 show that these enzymes have distinct binding specificities for ERK and associate with ERK for different lengths of time (Roux et al., 2003; Zhao et al., 1996). Moreover, RSK1 has limited interaction with identified targets of RSK2 (Clark et al., 2001), and all four RSK genes are differently expressed during late embryonic stages and in adult tissues (Alcorta et al., 1989; Kohn et al., 2003; Yntema et al., 1999). The biochemical differences among the family members suggest that there may be functional differences among the RSK proteins at a cellular level.

1.1.4. Ras superfamily of small GTPases

Soon after the discovery of Ras, other small monomeric GTP-binding proteins with molecular masses of 20-40 KDa with relative homology to Ras were identified and together constitute the Ras superfamily. Today, more than 100 small G-proteins have been identified in eukaryotes ranging from yeast to human (Wennerberg et al., 2005). In mammals, approximately 60 members have been identified and based on sequence homology and biological function, they have been grouped into five subfamilies including Ras, Rho, Rab, Sar1/Arf, and Ran (Fig. 1.1). The Ras and Rho family members are mainly involved in regulating gene expression, proliferation, differentiation, and morphology (Etienne-Manneville and Hall, 2002). The Rab and Sar1/Arf families are regulators of intracellular vesicular transport and the trafficking of proteins between different organelles of the endocytic and secretory pathways (Zerial and McBride, 2001). The Ran protein regulates nucleocytoplasmic transport during the G1, S and G2 phase of the cell cycle and microtubule organization during the M phase (Weis, 2003).

Figure 1.1. Phylogenic tree of the Ras superfamily.

The Ras superfamily of small GTPases is comprised of more than 60 members in mammals, and based on sequence homology and function, they have been grouped into five subfamilies including Ras, Rho, Rab, Sar1/Arf, and Ran. The Rho family can further be divided into six major branches: RhoA-related, Rac-related, Cdc42-related, Rnd proteins, RhoBTB proteins and Miro proteins. RhoD, Rif and TTF/RhoH do not obviously fall into any of these subfamilies. This figure was generated by modification of those found in the literature (Takai et al., 2001 and Wennerberg and Der, 2004).


Ras
Rheb
Rap
R-Ras3
R-Ras
Rap2
Rap1
R-Ras2/TC21
RGK
R-Ras
Rab
Ki-Ras
Ha-Ras
N-Ras
Ran
RalA
RalB
Sar1/Arf
RhoH/TTF
Miro-1
Miro
RhoBTB
RhoBTB-2
RhoBTB-1
Miro-1
Rac1
Rnd1
Rac-
like
Rac2
Rac3
Rnd
Rnd2
RhoG
TCL
Rnd3/RhoE
TC10
Rif
Cdc42
RhoB
RhoD
RhoA
Wrch-1
Chp/Wrch-2
RhoC
Cdc42-like
Rho-like
Rho

1.2. RHO GTPASES: REGULATION AND FUNCTION

1.2.1. Role of Rho GTPases in cellular transformation

Key steps in invasion and metastasis include alterations in cell adhesion, cell-matrix and cell-cell interactions, and the acquisition of an increased migratory phenotype. These cellular properties are regulated, in part, by Rho family GTPases and their control of actin organization (Van Aelst, 1997). The aberrant activities of Rho GTPases have been implicated in contributing to a metastatic and invasive phenotype. The oncogenic properties of Ras have been shown to be critically dependent on Rho GTPase function (Sahai and Marshall, 2002). The inhibition of Ras transformation by dominant–negative mutants of specific Rho family proteins (RhoA, RhoB, RhoG, Rac1, Cdc42 and TC10), coupled with the ability of activated Rho family members to cooperate with Raf to cause synergistic transformation, has implicated these proteins as key mediators of Ras transformation. Given that Rho family proteins are regulators of actin cytoskeletal organization, gene expression and cell-cycle progression, their importance in Ras function is likely to be significant (Shields et al., 2000). The observation that activated Rho family proteins alone can cause tumorigenic transformation, and promote invasion and metastasis, suggests that they can facilitate the ability of Ras to cause these aberrant cellular activities. Consequently, Ras regulation of Rho GTPase function may contribute to tumor cell invasion and metastasis.

1.2.2. The Rho family

Members of the Rho family were identified on the basis of their homology to the Ras proteins. Within their GTPase domain, they share approximately 30 % amino acid identity with the Ras proteins and 40-95 % identity to each other (Wennerberg and Der, 2004). Like Ras proteins, Rho and Rho-like proteins signal by cycling between an active GTP and inactive GDP-bound forms. However, unlike Ras oncogenes, few activating mutations have been found in Rho GTPases in cancer, and aberrant regulation of expression and/or GTP/GDP-bound ratios appear to be critical for their role in cellular transformation (Sahai and Marshall, 2002). Moreover, the putative effector domain of the Rho proteins is significantly different from that of Ras and therefore interacts with distinct cellular targets. The Rho proteins are ubiquitously expressed across species from

yeast to human and so far, 21 human genes encoding at least 23 proteins have been described. Based on primary amino acid sequence identity, structural motifs and biological function, the Rho family is divided into six subfamilies that display similar, but not identical, properties (Fig. 1.1). These are: the RhoA-related subfamily (RhoA, RhoB and RhoC); the Rac1-related subfamily (Rac1a, Rac1b, Rac2, Rac3 and RhoG); the Cdc42-related subfamily (Cdc42Hs, G25K, TC10, TCL, Chp/Wrch-2 and Wrch-1); the Rnd subfamily (Rnd1, Rnd2, and RhoE/Rnd3); the RhoBTB subfamily; and the Miro subfamily.

1.2.3. Rho GTPases and the actin cytoskeleton

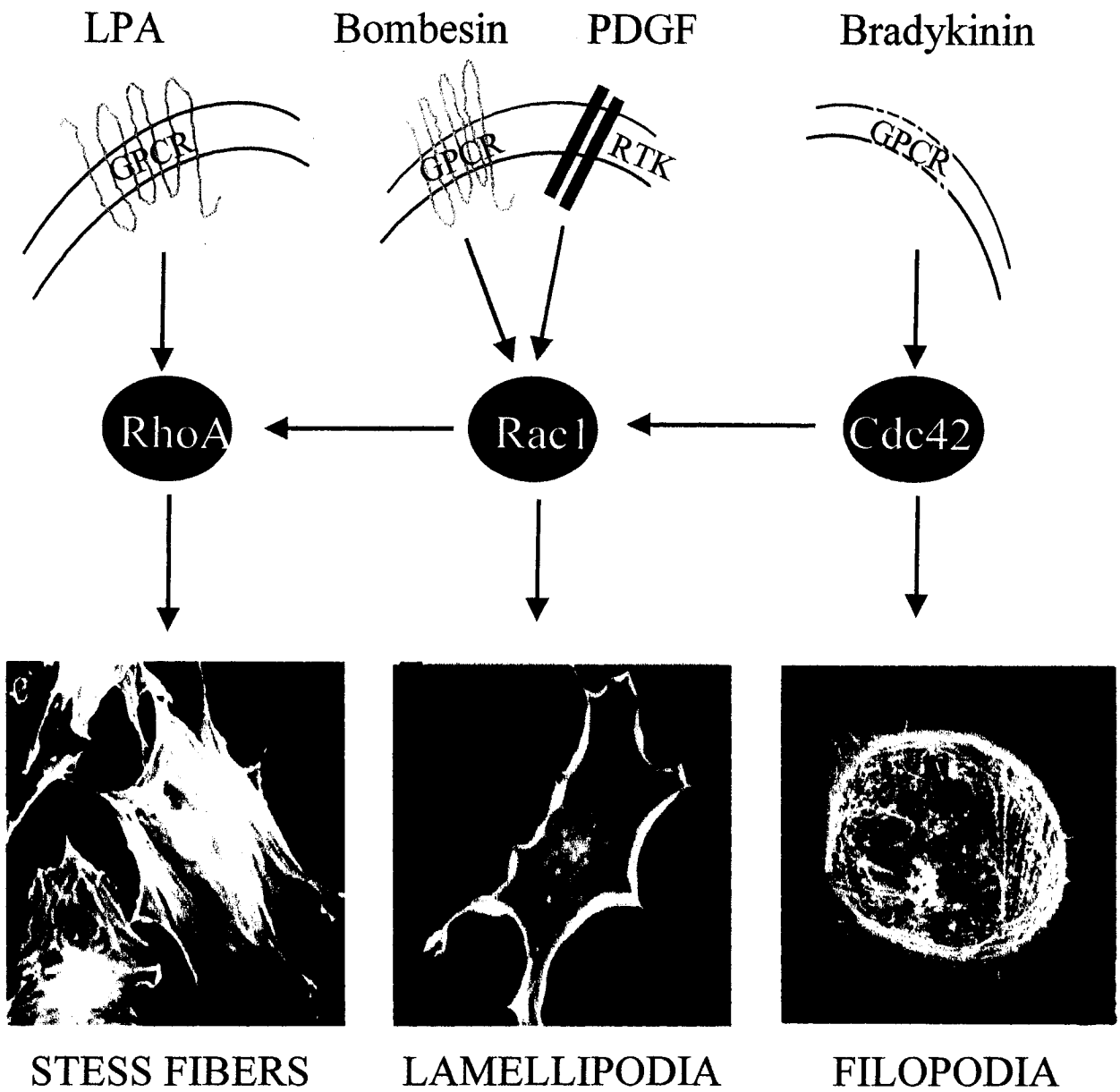
For several years, cell biologists had noticed that cells in culture displayed distinct actin structures such as stress fibers and membrane ruffles and treatment of cells with growth factors and other agents led to changes in these structures. However, it was not until the early 1990s that the Rho family members RhoA (Ras homologous) and Rac-1 (Ras-related C3 botulinum toxin substrate) took center stage and were shown to be key regulators of actin cytoskeleton dynamics (Ridley and Hall, 1992b; Ridley et al., 1992).

More specifically, it was reported that activation of RhoA by Lysophosphatidic acid (LPA) promotes actin-myosin contractility, and thereby, the formation of stress fibers and focal adhesion, regulating cell shape, attachment and motility (Ridley and Hall, 1992b)(Fig.1.2). LPA and several other external cues like sphingosine-1-phosphate (S1P), bombesin, endothelin, and thrombin activate Rho mainly through G protein-coupled receptors (GPCRs) (Goetzl and An, 1998; Seasholtz et al., 1999). These receptors activate RhoA primarily through two Rho specific GEF proteins, p155RhoGEF and PDZ-RhoGEF (Hart et al., 1998; Kozasa et al., 1998; Mao et al., 1998).

In response to growth factors like PDGF, EGF and insulin which activate receptor tyrosine kinases (RTKs), Rac1 promotes the assembly of lamellipodia, which are curtain-like extensions that consist of thin protrusive actin sheets at the leading edge of migrating cells (Ridley et al., 1992)(Fig1.2). Rac1 activation by growth factors is thought to be induced in part by PI3K which generates phosphoinositides that are required for the activation of RhoGEFs such as Tiam-1 and Vav (Han et al., 1998; Michiels et al., 1997). Later, it was reported that activation of Cdc42 (Cell division cycle) by bradykinin induced

Figure 1.2. Rho GTPases and the actin cytoskeleton

A number of extracellular signals regulate actin dynamics mainly by interacting with plasma membrane receptors like receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs). Intracellular signaling components downstream of these receptors activate RhoA, Rac1 and Cdc42 which induce changes in actin cytoskeleton structures such as stress fibers, lamellipodia, and filopodia, respectively. In Swiss 3T3 fibroblasts, Cdc42, Rac1, and RhoA have been placed in a hierarchical cascade wherein Cdc42 activates Rac1, which in turn activates RhoA. This figure has been generated by modification of that from Hall, 1998.


LPA
Bombesin
PDGF
Bradykinin
GPCR
GPCR
RTK
GPCR
RhoA
Rac1
Cdc42
STESS FIBERS
LAMELLIPODIA
FILOPODIA

formation of filopodia, which are thin finger-like cytoplasmic protusions of actin bundles at the periphery of the cell (Kozma et al., 1995; Nobes and Hall, 1995) (Fig.1.2).

These findings established Rho proteins as important actin cytoskeleton regulators and also as molecular switches that control signaling pathways downstream of growth factors and other cues that act on receptors at the cell surface. The Rho proteins have been implicated in almost every fundamental biological processes. Some of these are dependent on their effects on actin cytoskeleton remodeling such as cytokinesis (Prokopenko et al., 2000), phagocytosis (Caron and Hall, 1998), pinocytosis (Ridley et al., 1992) cell migration (Nobes and Hall, 1999), axon guidance (Govek et al., 2005) and endocytosis (Symons and Rusk, 2003), while others like gene expression, differentiation and membrane trafficking are not (Etienne-Manneville and Hall, 2002).

1.2.4. Cycling of Rho GTPases

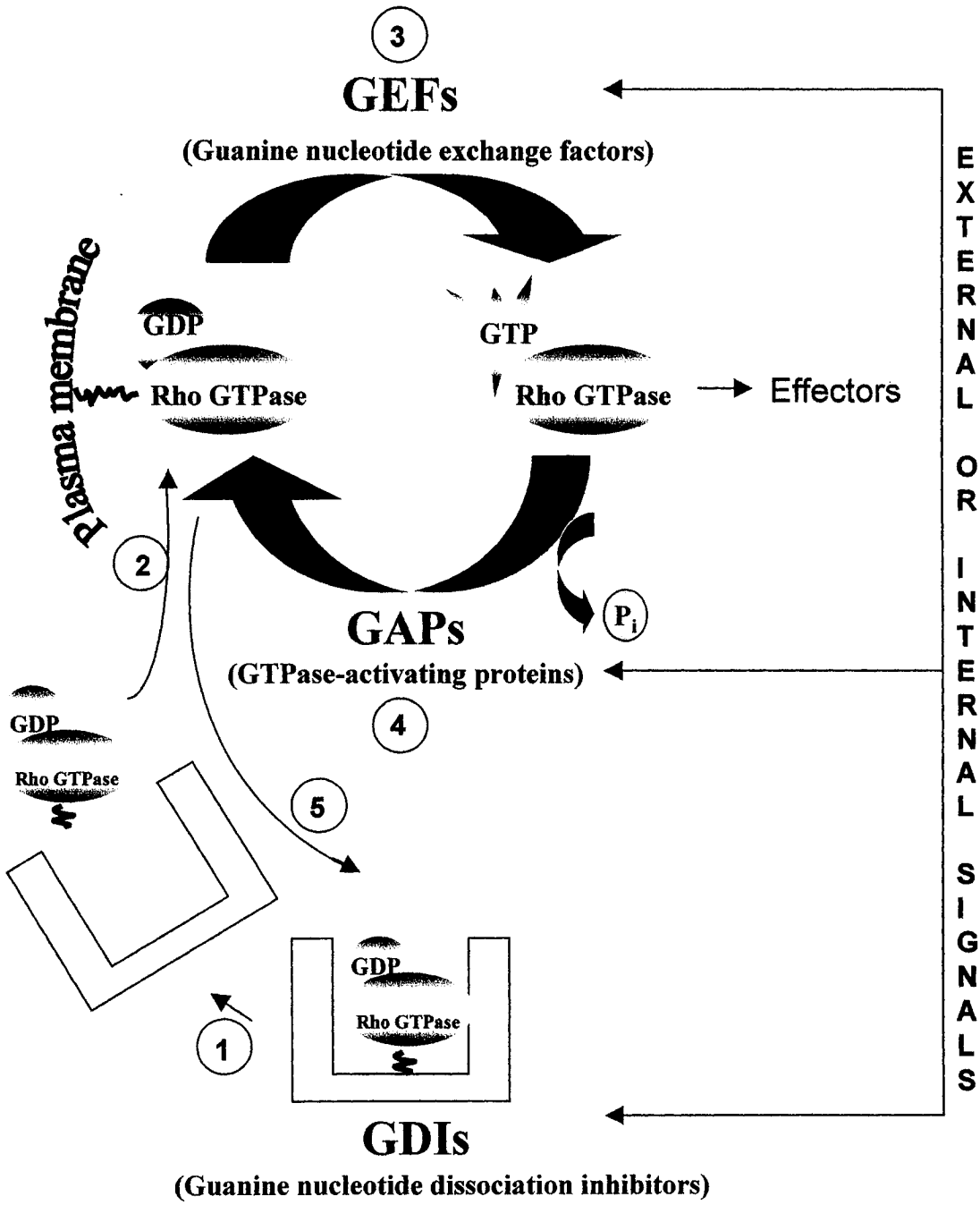
Like other small GTPases, the Rho proteins are turned on (GTP-bound) and off (GDP-bound) by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). A third family of proteins the guanine nucleotide dissociation Inhibitors (GDIs) stabilize the inactive GDP-bound Rho proteins in the cytoplasm until signal input from extracellular factors induces the release of Rho proteins from the complex for subsequent activation at the plasma membrane (Fig.1.3). RhoGDIs maintain Rho proteins as soluble cytosolic proteins by forming high-affinity complexes that mask the C-terminal geranylgeranyl membrane-targeting moiety within a hydrophobic pocket formed by the immunoglobulin-like domain of RhoGDI (Gosser et al., 1997; Keep et al., 1997; Hoffman et al., 2000). When Rho proteins are released from GDIs, they insert into the plasma membrane through their isoprenylated C-terminus. They are then activated by membrane-associated GEFs, and can interact with effector proteins, which induce a wide spectrum of biological effects. Reassociation with GDI, possibly initiated by GTP hydrolysis, is thought to induce recycling of the GTPase to the cytosol.

1.2.5. The RhoGEF proteins

In principle, GTPase activation in response to external or internal cellular signals can occur through the modulation of the activity of any of the three types of Rho family

Figure 1.3. Cycling of Rho GTPases

Small GTPases exist in either an inactive, GDP-bound form or an active, GTP-bound form. This GDP/GTP cycle is tightly regulated by three families of proteins, including the GEFs, GAPs, and GDIs. In the absence of intracellular or extracellular signals, the Rho GTPases are maintained in the cytosol by the GDIs which mask their lipid modifications. External or internal cues promote their release from the inhibitory complex (1) which enables them to associate with the plasma membrane (2) where they are activated by GEFs (3) and can signal to effector proteins. Then, GAP proteins inactivate the GTPases by accelerating the intrinsic GTPase activity, leading to the GDP-bound form (4). Once again, the GDI molecules stabilize the inactive GDP-bound form in the cytoplasm, waiting for further instructions (5).


3
GEFs
(Guanine nucleotide exchange factors)
GDP
Rho GTPase
GTP
Rho GTPase
Effectors
Plasma membrane
2
GAPs
(GTPase-activating proteins)
P_i
4
GDP
Rho GTPase
5
GDP
Rho GTPase
1
GDIs
(Guanine nucleotide dissociation inhibitors)
EXTERNAL OR INTERNAL SIGNALS

regulatory proteins (Fig 1.3). However, RhoGEF regulation has attracted considerable more attention, mainly because they were initially identified as potent oncogenes capable of transforming cells into which they were introduced. The Dbl (**D**iffuse **B** cell **L**ymphoma) oncogene is the founder of the RhoGEF family and was originally shown to induce focus formation in NIH 3T3 fibroblasts (Eva and Aaronson, 1985). Dbl was subsequently shown to function as a GEF for Cdc42Hs and RhoA (Hart et al., 1994). In addition, deletion analysis of the Dbl protein revealed that the DH (Dbl homology) domain was required for Rho GTPase activation and also necessary to induce cellular transformation. Since the discovery of Dbl two decades ago, more than 40 proteins containing DH domains have been identified in different species from yeast to human (Whitehead, 2003)(Table 1.1). The human genome is predicted to encode approximately 69 Rho GEF proteins (Rossman et al., 2005). Interestingly, all the DH-containing proteins share a pleckstrin homology (PH), which is also present in a number of signaling molecules. The PH domains bind to phosphoinositides and have been proposed to localize GEF proteins to plasma membranes, and to regulate their activity through allosteric mechanisms. Outside the DH–PH domains, Dbl-family proteins show significant divergence and typically contain other protein domains that underlie the unique cellular functions of the different family members. All RhoGEF proteins contain a single DH domain followed by one or two PH domains, exept for the protein Trio which harbors two functional GEF domains. The first GEF domain (GEFD1) has specific exchange activity towards RhoG and Rac1, while the second GEF domain (GEFD2) is active on RhoA (Estrach et al., 2002).

An important, unresolved issue is how cell surface receptors modulate RhoGEF activity in response to extracellular factors. Recent studies have shed some light on some possible mechanisms for GEF regulation. First, the fact that GEF proteins contain a PH domain suggests a potential role for phosphoinositides in GEF regulation. In fact, phosphatidylinositol 3,4,5-triphosphate (PIP3) was shown to bind to the PH domain of Vav, leading to enhance GEF activity towards Rac1. In contrast, binding of phosphatidylinositol 4,5-bisphosphate (PIP2) to the PH domain of Vav causes inhibition of GEF activity, suggesting that the PH domain of Vav regulates its activity through binding to PI3K products (Han et al., 1998). Furthermore, it was reported that the binding

Table 1.1 Specific GEF activity and properties of mammalian RhoGEFs

More than 40 RhoGEF proteins have been identified in mammals. Some GEF proteins display a broad specificity while others are specific to a single GTPase. Also, they display overlapping and specific effects on a variety of cellular processes. NR: not reported. This table has been adapted from Whitehead et al., 2003.

Name	Specifity	Comments
Dbl	Cdc42, RhoA	Oncogene
Lbc	RhoA	Oncogene
Lfc	RhoA	Oncogene
Lsc/p115-RhoGEF	RhoA	Oncogene; RGS domain GAPs on Gα13
Dbs/Ost	Cdc42, RhoA	Oncogene
Tiam1	Rac1	Invasive phenotype in T lymphoma cells
Vav1	Rac1, Cdc42	Oncogene
Vav2	Rac1, Cdc42, RhoA	Oncogene
Vav3	Rac1, RhoA, RhoG	May regulate GTP-hydrolases
FGD1	Cdc42	Disrupted in faciogenital dysplasia
Trio	RhoG, RhoA,	Encodes two DH/PH domain modules
Abr	Cdc42, RhoA, Rac1	Similar to Bcr but lacks kinase domain
Bcr	Cdc42, RhoA, Rac1	Contains a GAP domain
Ect-2	Cdc42, RhoA, Rac1	Oncogene; may regulate cytokinesis
Tim	NR	Oncogene
NET1	RhoA	Oncogene
Sos	Rac1	Contains a Ras GEF domain
RasGRF1	Rac1	Contains a Ras GEF domain
RasGRF2	Rac1	Contains a Ras GEF domain
ARHGEF3	NR	Widely expressed
ARHGEF4	NR	Expression restricted to brain
Brx	NR	Modulates estrogen receptor
CDEP	NR	Expressed in chondrocytes
Collybist in/hPEM-2	Cdc42	Induces clustering of gephryin
Ephexin	RhoA, Cdc42	Regulates growth cone dynamics
Frabin	Cdc42	Binds actin filaments
GEF337	RhoA	Promotes actin stress fiber formation
GEF-H1	Rac1, RhoA,	Binds microtubules
intersectin	NR	Component of the endocytic machinery
KIAA0380	RhoA	Contains PDZ and RGS domains
LARG	RhoA	Regulates neurite outgrowth
Ngef	NR	Oncogene
p114-RhoGEF	RhoA	Widely expressed
p116-RIP	RhoA	Promotes neurite outgrowth
p190-RhoGEF	RhoA	Binds microtubules
PDZ-RhoGEF	NR	Binds Gα subunits through RGS domain
Pix/Cool/ARFGEF6	Rac1	Mutated in X-linked mental retardation
Kalirin	Rac1	Regulates dendritic morphogenesis
Duet	NR	Contains serine/threonine kinase activity
STEF	Rac1	Closely related to Drosophila Sif

of the PH domain of Dbl to PIP2 and PIP3 results in the inhibition of its GEF activity towards Cdc42 (Russo et al., 2001). Thus, cell surface receptor-mediated activation of lipid kinases and subsequent generation of phosphoinositides seems to be an efficient mechanism by which extracellular factors modulate GEF activity. Another interesting regulatory mechanism for GEF proteins involves phosphorylation by receptor kinases or receptor-activated kinases. For instance, the neurotrophin-3 receptor TrkC phosphorylates and stimulates the catalytic activity of Dbs, the major Cdc42-GEF expressed in primary Schwann cells, leading to JNK activation and induction of neuronal cell migration (Yamauchi et al., 2005). Moreover, binding of ephrin-A ligand to the EphA4 receptor induces tyrosine phosphorylation of ephexin-1 by the Src tyrosine kinase family and switches ephexin-1 preference from all three GTPases to that of only RhoA, leading to growth cone collapse (Sahin et al., 2005). Likewise, the GEF activities of Vav, Dbl, LARG, and FRG are also modulated by mechanisms involving phosphorylation (Kato et al., 2000; Miyamoto et al., 2003; Schuebel et al., 1998; Suzuki et al., 2003). To date, tyrosine phosphorylation seems to be the only phospho-regulatory mechanism in GEF regulation but future studies will surely highlight the importance of serine and threonine phosphorylation.

1.2.6. The RhoGAP proteins

1.2.6.1.The RhoGAP family

The GAP proteins were initially viewed as signal terminators with a secondary role in comparison to the GEF proteins, which were known to activate Rho GTPases in response to growth factors and other agents. However, this misconception rapidly disappeared as growing evidence suggested that GAP proteins were also regulated by external cues and played pivotal roles in a number of Rho-mediated signaling pathways.

The first RhoGAP protein was discovered 16 years ago (Garrett et al., 1989), and since then, more than 70 members have been characterized in eukaryotes ranging from yeast to human, and together they form a rapidly growing family (Fig.1.4). The human genome is predicted to encode between 59 and 70 proteins containing a RhoGAP domain and to date more than half of them have already been characterized (Bernards, 2003; Peck et al., 2002; Venter, 2001) (Table 1.2). In the fruit fly *Drosophila melanogaster*

Fig 1.4 Phylogenic tree of the RhoGAP family from yeast to human

An unrooted tree based on sequence homology of the conserved RhoGAP domain. The RhoGAP domains of 72 characterized GAP proteins were aligned with the Clustal-W program and the phylogenic tree was generated with the Phylodraw program.

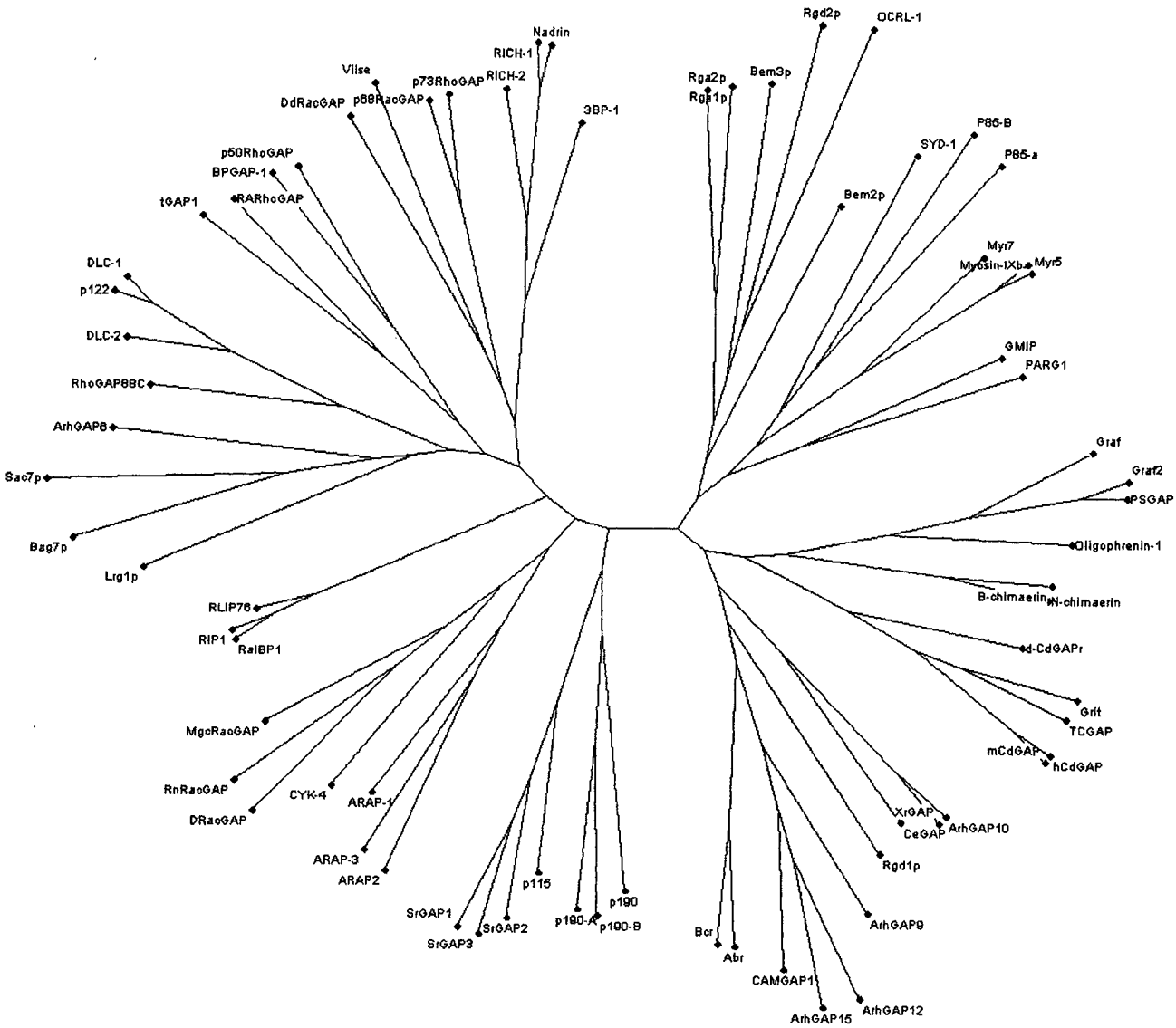

Nadrin
RICH-1
Vilse
RICH-2
p73RhoGAP
DdRacGAP
p68RacGAP
3BP-1
p50RhoGAP
BPGAP-1
tGAP1
RARhoGAP
DLC-1
p122
DLC-2
RhoGAP68C
ArhGAP6
Sac7p
Bag7p
Lrg1p
RLIP76
RIP1
RalBP1
MgcRacGAP
RnRacGAP
DRacGAP
CYK-4
ARAP-1
ARAP-3
ARAP2
SrGAP1
SrGAP2
SrGAP3
p115
p190
p190-A
p190-B
Rgd2p
OCRL-1
Rga2p
Rga1p
Bem3p
P85-B
SYD-1
P85-a
Bem2p
Myr7
Myosin-IXb
Myr5
GMIP
PARG1
Graf
Graf2
PSGAP
Oligophrenin-1
B-chimaerin
N-chimaerin
d-CdGAPr
Grit
TCGAP
mCdGAP
hCdGAP
XrGAP
CeGAP
ArhGAP10
Rgd1p
ArhGAP9
Bcr
Abr
CAMGAP1
ArhGAP15
ArhGAP12

Table 1.2 Properties of RhoGAP proteins from yeast to human

More than 70 RhoGAP proteins have been identified in different species ranging form yeast to human. Some GAP proteins display a broad specificity while others are specific to a single Rho GTPase. For some GAP proteins, the *in vitro* and *in vivo* GTPase preference seems to be different. Bold: preferred substrate. ND: not determined

Name	***Organism***	***In vitro* specificity**	***In vivo specificity***
p50RhoGAP	*H.sapiens*	**Cdc42**, Rac1, RhoA	RhoA
BPGAP1	*H.sapiens*	**Cdc42**, RhoA	RhoA
Bcr	*H.sapiens*	Cdc42, **Rac1,** Rac2	Rac1
Abr	*H.sapiens*	Cdc42, Rac1,Rac2	ND
mCdGAP	*M.musculus*	Cdc42, Rac1	Cdc42, Rac1
TCGAP	*M.musculus*	**Cdc42, Rac1**, RhoA	No activity
GRIT	*H.sapiens*	**Cdc42**, Rac1, **RhoA**	**Cdc42**, Rac1, **RhoA**
hCdGAP	*H.sapiens*	Cdc42, Rac1	Cdc42, Rac1
d-CdGAPr	*D.melanogaster*	ND	ND
ARAP1	*H.sapiens*	Cdc42, Rac1, **RhoA**	RhoA
ARAP2	*H.sapiens*	ND	ND
ARAP3	*H.sapiens*	Cdc42, Rac1, **RhoA**	RhoA
ARAP3	*M.musculus*	RhoA	RhoA
SrGAP1	*M.musculus*	ND	Cdc42, RhoA
SrGAP2	*M.musculus*	ND	ND
SrGAP3	*H.sapiens*	**Rac1**, Cdc42	ND
p115	*H.sapiens*	ND	RhoA
p85-α	*H.sapiens*	None	None
p85-β	*H.sapiens*	ND	ND
RIP1	*M.musculus*	**Cdc42**, Rac1	ND
RalBP1	*R.norvegus*	**Cdc42**, Rac1	ND
RLIP76	*H.sapiens*	**Cdc42**, Rac1	ND
DLC-1	*H.sapiens*	ND	ND
DLC-2	*H.sapiens*	RhoA, Cdc42	RhoA
p122RhoGAP	*R.norvegus*	RhoA	RhoA
RhoGAP80C	*D.melanogaster*	ND	Rho1, Rac1. Rac2
α1-chimaerin	*H.sapiens*	Rac1	Rac 1
β1-chimaerin	*R.norvegus*	Rac1	ND
Rich-1	*H.sapiens*	Cdc42, Rac1	Cdc42, Rac1
Rich-2	*H.sapiens*	Cdc42, Rac2	ND
Nadrin	*R.norvegus*	Cdc42, Rac1, RhoA	ND
3BP-1	*M.musculus*	Cdc42, Rac1	Rac1
Oligophrenin-1	*H.sapiens*	Cdc42, Rac1, RhoA	RhoA, Cdc42
Graf	*G.gallus*	Cdc42, RhoA	**RhoA**
Graf-2	*H.sapiens*	Cdc42, RhoA	ND
PSGAP	*M.musculus*	**RhoA**, Cdc42	RhoA, **Cdc42**
GMIP	*H.sapiens*	RhoA	RhoA
PARG1	*H.sapiens*	Cdc42, Rac1, **RhoA**	RhoA
ArhGAP9	*H.sapiens*	**Cdc42, Rac1**, RhoA	ND
ArhGAP12	*H.sapiens*	ND	ND
Arhgap15	*H.sapiens*	Rac1	Rac1
CAMGAP1	*R.norvegus*	Cdc42, Rac1	ND
Myosin-IXb	*H.sapiens*	RhoA	ND

Myr5	*R.norvegus*	Cdc42, **RhoA**, Rac1	RhoA
Myr7	*R.norvegus*	RhoA	RhoA
ArhGAP10	*H.sapiens*	**Cdc42**, RhoA, Rac1	ND
XrGAP	*X.laevis*	ND	ND
CeGAP	*C.elegans*	Rac1, Cdc42, RhoA, Ras, Rab3A	ND
MgcRacGAP	*H.sapiens*	Rac1, Cdc42, RhoA	Cdc42, RhoA
RnGAP	*D.melanogaster*	Rac1, Cdc42	Rac1, Cdc42
DRacGAP	*D.melanogaster*	ND	Rac1, Cdc42
CYK-4	*C.elegans*	**Rac1, Cdc42**, RhoA	ND
RARhoGAP	*R.norvegus*	RhoA	RhoA
tGAP1	*R.norvegus*	None	ND
p73RhoGAP	*H.sapiens*	ND	RhoA
p68RacGAP	*M.musculus*	Rac-1	Rac-1
ArhGAP6	*H.sapiens*	RhoA	RhoA
OCRL-1	*H.sapiens*	Rac-1	Rac-1
Vilse	*D.melanogaster*	**Rac1**, Cdc42	Rac1, Cdc42
SYD-1	*C.elegans*	None	ND
p190-A	*R.norvegus*	Rac1, Cdc42, **RhoA**	RhoA
p190-B	*H.sapiens*	Rac1, Cdc42, **RhoA**	ND
p190	*D.melanogaster*	ND	RhoA
Sac7p	*S.cerevisiae*	Rho1p	Rho1p
Bag7p	*S.cerevisiae*	Rho1p	Rho1p
Rga1p	*S.cerevisiae*	Cdc42	Cdc42p
Rga2p	*S.cerevisiae*	Cdc42	Cdc42p
Bem3p	*S.cerevisiae*	Cdc42p	Cdc42p
Lrg1p	*S.cerevisiae*	Cdc42p, Rho2p	ND
Bem2p	*S.cerevisiae*	Rho1p	Rho1p
Rgd1p	*S.cerevisiae*	Rho3p, Rho4p	Rho3p
Rgd2p	*S.cerevisiae*	Cdc42p, Rho5p	ND
DdRacGAP1	*D.discoideum*	DdRac1A, DdRacC, Rac1, RhoA	ND

approximately 20 different genes are predicted to encode for RhoGAP proteins and six of them have already been identified (Bernards, 2003; Billuart et al., 2001) (Table 1.2). In the yeast *Saccharomyces cerevisiae* eight out of the ten predicted RhoGAP proteins have been identified and characterized (Jenna and Lamarche-Vane, 2003; Venter, 2001) (Table 1.2). Two RhoGAP proteins have been identified in the worm *Caenorhabditis elegans* where approximately 20 are predicted (Jenna and Lamarche-Vane, 2003; **Venter**, 2001) (Table 1.2).

1.2.6.2. Too many GAPs

Approximately six Rho GTPases have been identified in worm, yeast and fly and 21 in mammals (Wherlock and Mellor, 2002). Interestingly, in all cases the RhoGAP proteins outnumber the Rho GTPases they regulate by 2- to 3-fold. The obvious question is why are there so many GAPs. This question emerged in the first review on RhoGAPs more than a decade ago (Lamarche and Hall, 1994), and at the time only a dozen RhoGAP proteins were identified and answers could only be speculative. However, studies over the last decade have enabled us to shed some light on this question and have provided at least four possible explanations.

First, unlike the Rho GTPases which are ubiquitously expressed, some GAP proteins are predominantly found in a particular tissue and seem to have tissue-specific functions. For instance, the vascular endothelium cell restricted p73RhoGAP protein is involved in regulating Rho GTPases during angiogenesis and the brain specific GAP protein Grit is involved in neuritogenesis (Nakamura et al., 2002; Su et al., 2004). However, since most GAPs are also ubiquitous, this explanation is unlikely to be the sole cause for the overabundance of RhoGAP proteins.

Second, some GAP proteins specifically act on a single GTPase such as p112RhoGAP, GMIP, ArhGAP6 and mouse ARAP3 which act specifically on RhoA, while chimearin, ArhGAP15 and OCRL-1 are Rac1 specific (Table 1.2). Given that most GAP proteins display activity towards multiple Rho proteins at least *in vitro*, this also provides only partial explanation.

Third, 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. This assumption is best supported from studies in yeast and fly. In yeast, Rga1p, Rga2p and Bem3p are Cdc42p specific and Bag7p, Sac7p and Bem2p target only Rho1p. Interestingly, each one seems to play a role in modulating specific aspects of Cdc42p and Rho1p function (Schmidt et al., 2002; Smith et al., 2002). A recent genome-wide analysis of RhoGAP functions in the fly was assessed by RNAi and revealed that out of the 17 *Drosophila* RhoGAPs analyzed some caused lethality while others gave distinct morphological defects in particular tissues (Billuart et al., 2001). Moreover, several mammalian RhoGAP proteins have been shown to be implicated in specific Rho GTPase-mediated biological functions including cytokinesis, differentiation, cell migration and neuronal morphogenesis (Table 1.3).

Fourth, the GAP domain may simply serve as a recognition module and RhoGAP proteins could act as effectors or scaffold proteins mediating cross-talk between Rho GTPases and other signaling pathways. For example, full length α1-chimaerin lacking GAP activity but retaining binding ability to GTPases, seems to cooperate with Rac1 and Cdc42 to promote formation of lamellipodia and filopodia (Kozma, 1996). Moreover, TCGAP has been shown to bind to Rho GTPases through its GAP domain and is involved in insulin-mediated glucose transport signaling, even though it does not display *in vivo* GAP activity (Chiang et al., 2003). Although the p85 subunit of PI3K does not contain a functional GAP domain, it binds to the Rho proteins and acts both upstream and downstream. Very few if any RhoGAP proteins only harbor a GAP domain, most are large proteins that contain additional functional modules (Fig.1.5). These multidomain proteins are thought to integrate signals from a number of signaling pathways. For instance, Bcr contains both a RhoGAP and RhoGEF domain which are both active *in vivo* on Rac1 and Cdc42, respectively (Korus et al., 2002; Ridley et al., 1993). Therefore, Bcr may simultaneously inactivate Rac1 while activating Cdc42. Similarly, DracGAP1 also contains both domains and each one interacts with a specific subset of GTPases and deletion of either domain causes specific phenotypes (Knetsch et al., 2001; Ludbrook et al., 1997). CdGAP which contains an ERK docking site was shown to be phosphorylated and regulated by ERK, suggesting cross-talk between mitogenic and Rho signaling pathways (Tcherkezian et al., 2005). Graf may also mediate cross-talk between mitogen signaling pathways and Rho signaling since it has been shown to be phosphorylated by

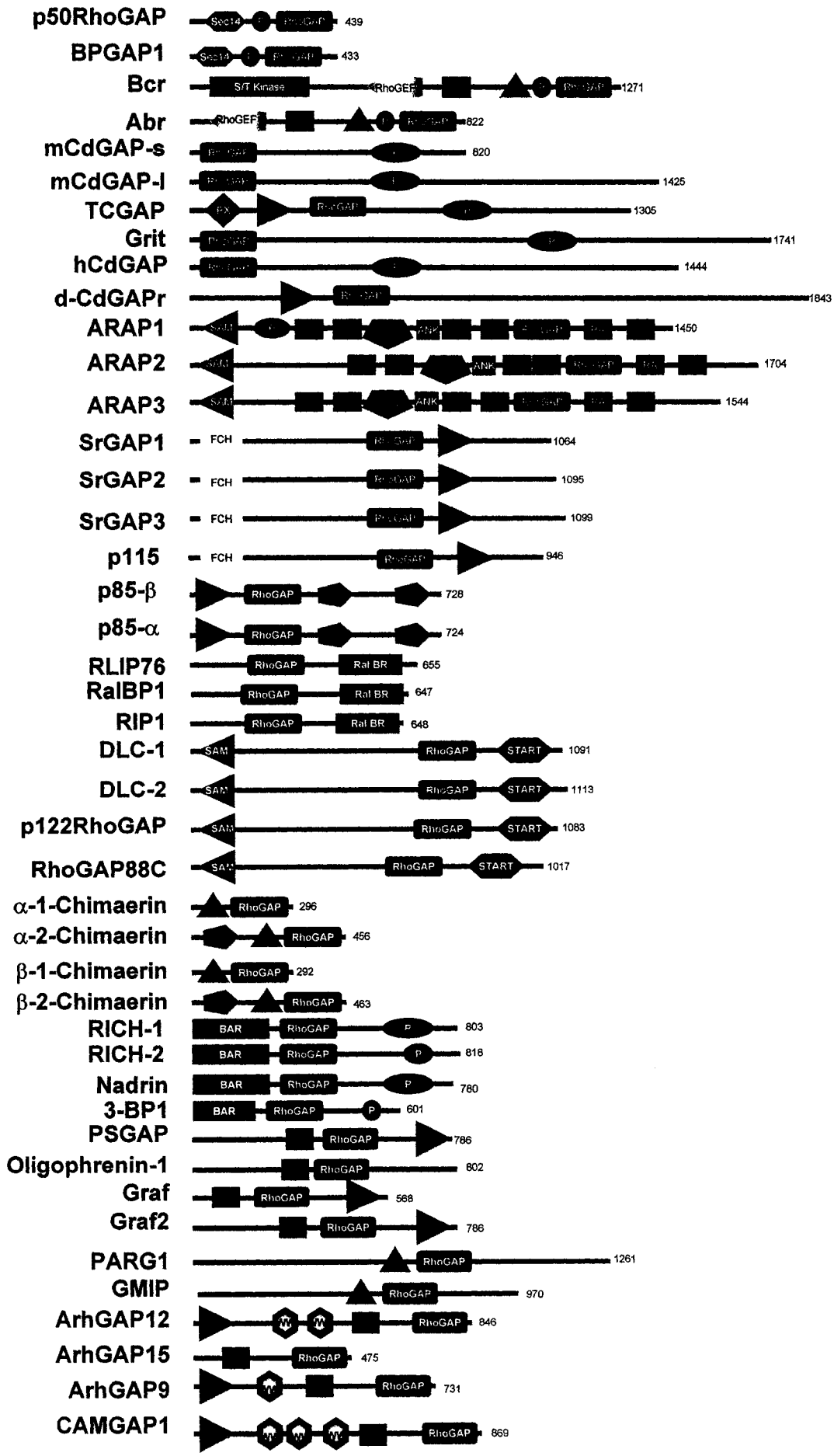
Table 1.3. RhoGAP regulation, biological function and disease

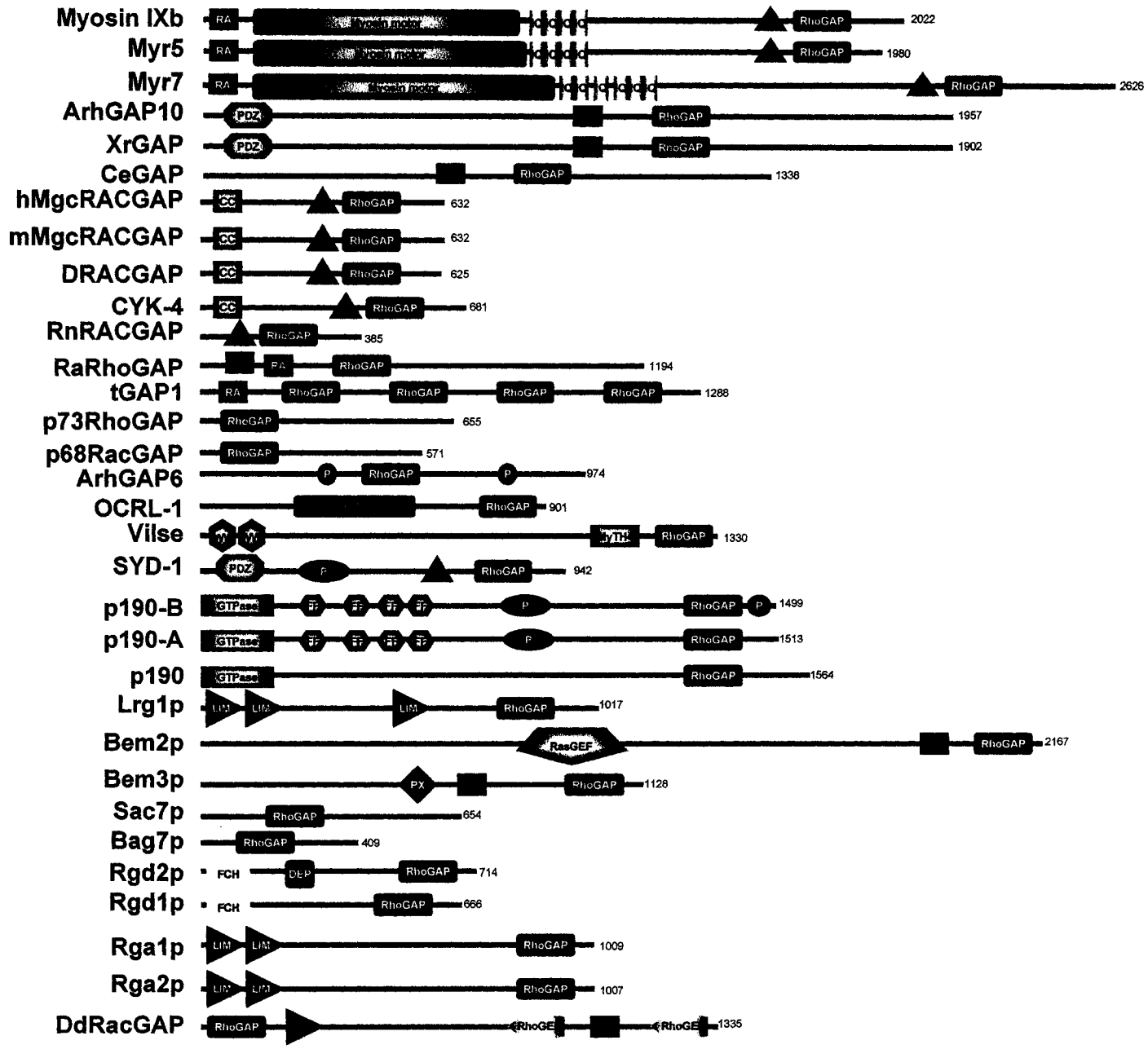
RhoGAP proteins are regulated by a number of mechanisms including phosphorylation, protein-protein interaction, lipids and protein degradation. They are also implicated in a variety of biological activities such as exocytosis, endocytosis, cytokinesis, cell differentiation, migration, neuronal morphogenesis, angiogenesis and tumor suppression. Disruptions in genes encoding RhoGAP proteins are suspected to be involved in a diverse array of diseases such as Bardet-Biedl Syndrome (BBS), microphthalmia with linear skin defects syndrome (MLS), X-linked mental retardation and cancer.

Regulation mechanism	Name
Phosphorylation	p190-A, CdGAP, MgcRacGAP, RICS
Protein-protein interaction	CdGAP, ARAP3, MgcRacGAP
Lipids	α1-chimaerin
Protein degradation	p190-A
Biological function	
Exocytosis	Nadrin, TCGAP
Endocytosis	RalBP1, RLIP76, CAMGAP1
Cytokinesis	MgcRacGAP, CYK-4, p190-A
Cell differentiation	MgcRacGAP, p190-B
Cell migration	ARAP1, ARAP3, BPGAP1, p190-A
Neuronal morphogenesis	SrGAP1, Grit, α2-chimaerin, p190,
	p190-A, RARhoGAP
	Nadrin, Vilse, SYD-1
Angiogenesis	P73RhoGAP, p68RhoGAP
Tumor suppression	DLC1 and DLC-2, p190-A
Diseases	
MLS	ArhGAP6
Cancer (leukemia, liver cancer)	GRAF, Bcr, DLC-1, DLC-2
X-linked mental retardation	SrGAP3, Oligophrenin-1, OCRL-1
Bardet-Biedl Syndrome (BBS)	Myosin-IXa

Figure 1.5 The multidomain features of RhoGAPs

Most RhoGAP proteins harbour a number of functional domains and are thought to mediate cross-talk between Rho GTPases and other signaling pathways. Abbreviations: C1, cysteine-rich phorbol ester binding; C2, calcium-dependent lipid binding; CC, coiled-coil; RhoGEF, Guanine nucleotide exchange factor for Rho-like GTPase; EH, Eps15 homology; FCH, Fes/CIP4 homology; IQ, calmodulin binding motif; P, proline-rich; PH, pleckstrin homology; RA, Ras-associating domain; RhoGAP, Rho GTPase activating protein; SH3, Src homology 3; Sec14, sec14-like; SH2, Src homology 2; PX, Phox homology; START, Star-related lipid transport domain; S/T kinase, serine and threonine kinase; BAR, BinAmphiphysinRvs domain; Ral BR, Ral GTPase binding region; Lim, Zinc-binding domain present in Lin-11, Isl-1, Mec-3; DEP, domain found in Dishevelled, Egl-10, and Pleckstrin; RasGEF, Guanine nucleotide exchange factor for Ras-like GTPase; FF, domain with 2 conserved phenylalanine (F) residues; WW, domain with 2 conserved tryptophan (W) residues; MyTH4, myosin tail homology 4; PDZ; domain present in PSD-95, Dlg, and ZO-1/2; SAM, Sterile alpha motif; ArfGAP; Arf GTPase activating protein.


p50RhoGAP
439
BPGAP1
433
Bcr
S/T Kinase
RhoGEF
1271
Abr
RhoGEF
822
mCdGAP-s
820
mCdGAP-l
1425
TCGAP
1305
Grit
1741
hCdGAP
1444
d-CdGAPr
1843
ARAP1
1450
ARAP2
1704
ARAP3
1544
SrGAP1
FCH
1064
SrGAP2
FCH
1095
SrGAP3
FCH
1099
p115
FCH
946
p85-β
RhoGAP
728
p85-α
RhoGAP
724
RLIP76
RhoGAP
Ral BR
655
RalBP1
RhoGAP
Ral BR
647
RIP1
RhoGAP
Ral BR
648
DLC-1
SAM
RhoGAP
START
1091
DLC-2
SAM
RhoGAP
START
1113
p122RhoGAP
SAM
RhoGAP
START
1083
RhoGAP88C
RhoGAP
START
1017
α-1-Chimaerin
RhoGAP
296
α-2-Chimaerin
RhoGAP
456
β-1-Chimaerin
RhoGAP
292
β-2-Chimaerin
RhoGAP
463
RICH-1
BAR
RhoGAP
803
RICH-2
BAR
RhoGAP
818
Nadrin
BAR
RhoGAP
780
3-BP1
BAR
RhoGAP
601
PSGAP
RhoGAP
786
Oligophrenin-1
RhoGAP
802
Graf
RhoGAP
568
Graf2
RhoGAP
786
PARG1
RhoGAP
1261
GMIP
RhoGAP
970
ArhGAP12
RhoGAP
846
ArhGAP15
RhoGAP
475
ArhGAP9
RhoGAP
731
CAMGAP1
RhoGAP
869


Myosin IXb
RA
RhoGAP
2022
Myr5
RA
RhoGAP
1980
Myr7
RA
RhoGAP
2626
ArhGAP10
PDZ
RhoGAP
1957
XrGAP
PDZ
RhoGAP
1902
CeGAP
RhoGAP
1338
hMgcRACGAP
CC
RhoGAP
632
mMgcRACGAP
CC
RhoGAP
632
DRACGAP
CC
RhoGAP
625
CYK-4
CC
RhoGAP
681
RnRACGAP
RhoGAP
385
RaRhoGAP
PA
RhoGAP
1194
tGAP1
RA
RhoGAP
RhoGAP
RhoGAP
RhoGAP
1288
p73RhoGAP
RhoGAP
655
p68RacGAP
RhoGAP
571
ArhGAP6
P
RhoGAP
P
974
OCRL-1
RhoGAP
901
Vilse
SYTH
RhoGAP
1330
SYD-1
PDZ
P
RhoGAP
942
p190-B
GTPase
RhoGAP
P
1499
p190-A
GTPase
P
RhoGAP
1513
p190
GTPase
RhoGAP
1564
Lrg1p
LIM
LIM
LIM
RhoGAP
1017
Bem2p
RasGEF
RhoGAP
2167
Bem3p
PX
RhoGAP
1128
Sac7p
RhoGAP
654
Bag7p
RhoGAP
409
Rgd2p
FCH
DEP
RhoGAP
714
Rgd1p
FCH
RhoGAP
666
Rga1p
LIM
LIM
RhoGAP
1009
Rga2p
LIM
LIM
RhoGAP
1007
DdRacGAP
RhoGAP
RhoGE
RhoGE
1335

the ERK MAPK pathway (Taylor et al., 1998). Also, p155 interacts with MEKK1 and reduces MEKK1 signaling to the AP-1 transcription factor, offering cross-talk between MEKK1 and RhoGTPase signaling (Christerson et al., 2002). The Gmip and PARG1 GAP proteins interact with Gem and Rap2 and may act as effector molecules for Ras-related proteins to regulate Rho-induced actin remodeling and provide additional cross-talk mechanism between these two families (Aresta et al., 2002; Myagmar et al., 2005). Moreover, RalBP1 seems to be an effector of Ral in mediating changes in the actin cytoskeleton. The ARAP family members mediate PI3K-dependent cross-talk between Ras, Rho, and Arf family of small GTPases. Furthermore, ARHGAP10 seems to mediate cross-talk between ARF1 and Cdc42 signaling pathways.

1.2.6.3. Rho GAP regulation

The incredibly large number of RhoGAP proteins strongly suggests a tight regulation of their activities at specific sites within the cell (Table 1.3). Indeed, accumulating evidence indicates that RhoGAP activities are regulated by a wide variety of mechanisms, including lipid binding, protein-protein interaction, phosphorylation, and proteolytic degradation (Ahmed et al., 1993; Jenna et al., 2002; Roof et al., 1998; Su et al., 2003). For example, tyrosine phosphorylation of p190RhoGAP by Src is necessary for its association with p120RasGAP and activation of its rhoGAP activity *in vivo* (Hu and Settleman, 1997; Roof et al., 1998). 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 (Okabe et al., 2003). Interestingly, MgcRacGAP, known to be involved in cytokinesis and a GAP for Rac1 and Cdc42, is functionally converted to a GAP for RhoA after serine phosphorylation by Aurora B kinase both *in vitro* and *in vivo* during cytokinesis (Minoshima et al., 2003). Moreover, phosphorylation of CdGAP by the ERK MAPK inhibits its GAP activity towards Rac1 both *in vitro* and *in vivo* (Tcherkezian et al., 2005). In addition to phosphorylation, CdGAP's activity has also been shown to be regulated through protein-protein interaction by the endocytic protein intersectin (Jenna et al., 2002). Another example is the regulation of ARAP3 and RARhoGAP rhoGAP activities through direct interaction with the Ras-related protein Rap1 (Krugmann et al., 2004; Yamada et al., 2005). Moreover, the protein

regulator of cytokinesis (PRC1) binds to the GAP domain of MgcRacGAP and down-regulates its GAP activity both *in vitro* and *in vivo* during metaphase (Ban et al., 2004). The expression of p190-A is cell cycle regulated through ubiquitin-mediated degradation (Su et al., 2003). The *in vitro* and *in vivo* GAP activities of α1-Chimaerin towards Rac1 are both positively and negatively regulated through binding of phorbol esters to its C1 domain (Ahmed et al., 1993; Caloca et al., 2001) .

1.2.6.4. Involvement of RhoGAPs in human deseases

Finally, alterations in genes coding for RhoGAP proteins are involved in a number of human genetic disorders, notably X-linked mental retardation, oculocerebrorenal syndrome of Lowe (OCRL), leukemias, liver cancer, Bardet-Biedl syndrome (BBS) and, microphthalmia with linear skin defects syndrome (MLS) (Attree et al., 1992; Billuart et al., 1998; Borkhardt et al., 2000; Gorman et al., 1999; Schaefer et al., 1997; Yuan et al., 1998).

1.2.6.5. The RhoGAP subfamilies

BPGAP1 and p50RhoGAP

The discovery of a Ras GTPase activating protein (p120RasGAP) from cytosolic extracts of bovine brain prompted the search for a GAP protein for other small GTPases (Gibbs et al., 1988). p50RhoGAP also known as Cdc42GAP was isolated from human spleen tissue and from platelets, and is the founder of the RhoGAP family of proteins (Barfod et al., 1993; Garrett et al., 1989; Lancaster et al., 1994). This ubiquitous protein contains a C-terminal RhoGAP domain and an N-terminal putative lipid binding domain (Sec14) that may be required for its ability to bind to phosphoinositides and possibly for membrane localization (Krugmann et al., 2002). In addition, it contains a proline-rich region upstream from the GAP domain that binds to the SH3 domain of p85-α and c-Src *in vitro*, but the biological relevance of these interactions is unknown. p50RhoGAP is active *in vitro* on RhoA, Rac1 and Cdc42 with significant preference for the latter (Barfod et al., 1993; Hart et al., 1991; Lancaster et al., 1994; Ridley et al., 1993). When expressed in Swiss 3T3 cells, p50RhoGAP inhibits RhoA-induced stress fibers but not Rac1-induced lamellipodia while Cdc42 downregulation *in vivo* has not yet been demonstrated

(Ridley et al., 1993). The crystal structure of p50RhoGAP alone or in complex with Cdc42 was elucidated and provided valuable knowledge on how GAP proteins bind and enhance GTPase activity (Barrett et al., 1997; Rittinger et al., 1997). BPGAP1 was recently discovered by searching the human genome for proteins with high homology to the GAP domain of p50RhoGAP. Similar to p50RhoGAP, BPGAP1 contains an N-terminal Sec14/BCH domain followed by a proline-rich region and a C-terminal RhoGAP domain which is active *in vitro* towards RhoA and Cdc42 but not Rac1 (Shang et al., 2003). BPGAP1 co-immunoprecipitates with both Cdc42 and RhoA, but only the latter seems to be downregulated inside the cells. BPGAP1 coordinately regulates cell morphology and migration via the interplay of its Sec14/BCH, RhoGAP and proline-rich domains (Shang et al., 2003). Interaction of the SH3 domain of cortactin with the proline-rich sequence of BPGAP1 facilitates cortactin translocation to the cell periphery and enhances cell migration (Lua and Low, 2004). Recently, the SH3 domain of the endocytic protein EEN/endophilin II was reported to bind to the proline-rich sequence of BPGAP1, and together mediate EGF receptor endocytosis and MAPK pathway activation (Lua and Low, 2005).

Bcr and Abr

Bcr (Breakpoint Cluster Region) was first discovered as part of the Bcr-Abl fusion oncogene in human leukemias positive for the Philadelphia chromosome (Heisterkamp et al., 1985). Later, Bcr was identified as a RhoGAP protein based on sequence homology to a peptide derived from p50RhoGAP (Diekmann et al., 1991). Bcr is a 160 KDa phosphoprotein mainly expressed in brain and hematopoietic cells (Diekmann et al., 1991; Maru and Witte, 1991). It has a complex structure with six domains including an N-terminal kinase domain followed by a Dbl-homology (DH) domain, a pleckstrin-homology (PH) domain, a protein kinase C conserved region 2 (C2), a proline-rich region and a C-terminal RhoGAP domain. Abr (Active Bcr-Related) is a 92 KDa protein mainly present in brain and was originally identified by its close relatedness to Bcr (Heisterkamp et al., 1993; Tan et al., 1993). Abr is structurally similar to Bcr but lacks the N-terminal kinase domain. The DH domain of both proteins displays GEF activity *in vitro* towards Cdc42, RhoA, Rac1 and Rac2 but not Rap1 or Ha-Ras (Chuang et al., 1995) and BCR

displays GEF activity *in vivo* towards Cdc42 but not Rac1 or RhoA (Korus et al., 2002). The GAP domains of both proteins are active *in vitro* towards Rac1, Rac2 and Cdc42 but not RhoA (Diekmann et al., 1991; Heisterkamp et al., 1993; Ridley et al., 1993; Tan et al., 1993). The GAP domain of Bcr is able to inhibit Rac1-induced membrane ruffling but not RhoA-mediated stress fiber formation in Swiss 3T3 fibroblasts (Ridley et al., 1993). Interestingly, the GAP and GEF domains of both proteins bind to the GTPases in a non-competitive manner, suggesting that these two domains may interact simultaneously with two GTPases and may coordinately regulate Rho GTPases (Chuang et al., 1995). In Bcr-null mice, neutrophils display a significant increase in reactive oxygen species (ROS) production upon activation as well as increased Rac2 membrane translocation compared to control mice (Voncken et al., 1995), suggesting a role for Bcr in regulating Rac-mediated superoxide production. In addition, phosphorylation of Bcr by the non receptor tyrosine kinase FES regulates the association of Bcr with multiple signaling molecules, and also its kinase activity (Li and Smithgall, 1996; Maru et al., 1995; Peters and Smithgall, 1999).

CdGAP, Grit, and TCGAP

Mouse CdGAP (for Cdc42 GTPase-activating protein) was initially identified in a yeast two-hybrid screen by using the Y40C effector mutant of Cdc42 as a bait. CdGAP is a serine- and proline-rich protein showing GAP activity against Cdc42 and Rac1 but not Rho A, both *in vitro* and *in vivo* (Lamarche-Vane and Hall, 1998). In addition to its N-terminal GAP domain, CdGAP contains a C-terminal proline-rich domain harboring five consensus Src homology 3 (SH3)-binding sites whose functions are still unclear. 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 (Jenna et al., 2002). Recently, it was reported that CdGAP's activity is also regulated through threonine phosphorylation by ERK (Tcherkezian et al., 2005). Moreover, a longer isoform of CdGAP (CdGAP-l) has been identified in mice, which seems to be structurally and functionally similar to short CdGAP (CdGAP-s) (Tcherkezian et al., 2005). Proteins with high homology to CdGAP have been characterized in human, mouse and fly. Mouse TCGAP (for TC10/cdc42

GTPase activating protein) shares 67 % sequence homology to CdGAP within the RhoGAP domain and similar to CdGAP it contains an N-terminal RhoGAP domain, a C-terminal proline-rich sequence, but also contains an N-terminal phox homology (PX) and Src homology 3 (SH3) domains. The GAP domain of TCGAP is active *in vitro* on Cdc42 and Rac1 and less on Rho (Chiang et al., 2003). TCGAP does not seem to display GAP activity inside the cells. The PX domain of TCGAP has been shown to interact with plasma membrane phosphoinositides and is necessary but not sufficient for the translocation of TCGAP to the plasma membrane (Chiang et al., 2003). TCGAP has been reported to be involved in insulin-mediated glucose transport signaling (Chiang et al., 2003). In human, there are two proteins that are highly homologous to mCdGAP. One encodes a previously characterized protein named Grit (also known as p200RhoGAP, RICS, p250GAP and GC-GAP) (Nakamura et al., 2002; Moon et al., 2003; Zhao et al., 2003; Okabe et al., 2003; Taniguchi et al., 2003) which shares 68 % homology to mCdGAP within the RhoGAP domain. Grit also contains an N-terminal GAP domain and five potential SH3 binding motifs. Both in *vitro* and *in vivo* studies show that the GAP domain of Grit preferentially stimulates GTP hydrolysis of RhoA and Cdc42 over that of Rac1 (Nakamura et al., 2002). Moreover, Grit has been reported to regulate neurite extension through binding with the TrkA receptor and the adaptor molecules N-Shc and CrkL/Crk (Nakamura et al., 2002). Moreover, the *in vitro* GAP activity of RICS is inhibited by phosphorylation from Ca^{2+}/calmodulin-dependent protein kinase II (Okabe et al., 2003). The human ortholog of mouse CdGAP is a phosphoprotein of 1,444 amino acids that shares 97 % homology to mCdGAP within the rhoGAP domain and 84 % homology within the entire protein sequence. Human CdGAP (hCdGAP) is structurally and functionally similar to the mouse protein (Tcherkezian et al, 2005). The drosophila ortholog, dCdGAP-r is a 1843 amino acid protein that contains an N-terminal SH3 domain followed by a RhoGAP domain which has not yet been shown to have a GAP activity (Sagnier et al., 2000).

ARAP1, ARAP2 and ARAP3

This family comprises five ubiquitous members including human ARAP-1, -2, and-3, and mouse ARAP-3. These multidomain proteins contain a sterile α motif (SAM) domain,

five PH domains, an ArfGAP domain, ankyrin repeats, a RhoGAP domain and a Ras-associating (RA) domain (I et al., 2004; Krugmann et al., 2002; Miura et al., 2002). Both ARAP1 and ARAP3 (human and mouse) display functional ArfGAP domains, which are regulated by PIP3 binding. ARAP1 is active *in vitro* mainly on Arf1 and Arf5 whereas human and mouse ARAP3 prefer Arf6 and Arf5, respectively (I et al., 2004; Krugmann et al., 2002; Miura et al., 2002). Human ARAP1 and ARAP3 display phosphoinositide-independent GAP activity *in vitro* towards RhoA and significantly less on Rac1 and Cdc42 whereas mouse ARAP3 exclusively stimulates GTP hydrolysis of RhoA (I et al., 2004; Krugmann et al., 2002; Miura et al., 2002). However, *in vivo* only RhoA seems to be a target of all three ARAP proteins. Both human ARAP1 and mouse ARAP3 inhibit cell spreading and mouse ARAP3 also inhibits cell migration (I et al., 2004; Miura et al., 2002). Mouse ARAP3 is phosphorylated both *in vitro and in vivo* by the Src tyrosine kinase family, which seems to partially inhibit the ability of ARAP3 to induce the formation of elongated membrane projections, suggesting a negative regulation of its GAP activity through tyrosine phosphorylation (I et al., 2004). ARAP1 associates with the Golgi apparatus and mediates changes in the distribution of Golgi proteins (Miura et al., 2002). Furthermore, interaction of human ARAP3 with the Ras-related protein Rap1b through its RA domain leads to increase GAP activity both *in vitro and in vivo* (Krugmann et al., 2004). Taken together, it seems that ARAP family members mediate PI3K-dependent cross-talk between Ras, Rho, and Arf family of small GTPases.

SrGAP1, SrGAP2, SrGAP3 and p115

The three SrGAP proteins were identified in a yeast two-hybrid screen searching for proteins that interacted with the C-terminal region of the neuronal Robo1 receptor and p115 protein was identified by searching for genes encoded in the long arm of human chromosome X (Endris et al., 2002; Tribioli et al., 1996; Wong et al., 2001). All four members are structurally similar and contain an N-terminal Fes/CIP4 homology (FCH) domain, a central RhoGAP domain followed by an SH3 domain. Mouse SrGAP1 is mainly expressed in lung and to a lower extent in brain and kidney whereas human SrGAP3 is predominantly expressed in brain, and mouse SrGAP2 is ubiquitous (Endris et al., 2002; Wong et al., 2001). Human p115 is predominantly expressed in spleen thymus

and leukocytes and in cell lines of B lymphoid, T lymphoid, myeloid or Hodgkin derivation (Tribioli et al., 1996). The p115 protein binds to MEKK1 *in vitro* and *in vivo*, which reduces MEKK1-induced signaling to the transcription factor AP-1 (Christerson et al., 2002). Hence, p115 provides a link between MEKK signaling and cytoskeletal dynamics. SrGAP1 is active *in vivo* on Cdc42 and RhoA and SrGAP3 is mainly active *in vitro* on Rac1 and to a lesser extent Cdc42 (Endris et al., 2002; Wong et al., 2001). Expression of recombinant p115 in cells causes loss of stress fibers, suggesting that it may downregulate RhoA (Tribioli et al., 1996). It has been proposed that SrGAP1 binds to the Robo receptor through its SH3 domain and a proline-rich sequence in the intracellular tail of the receptor. This interaction is increased in the presence of the Slit ligand and leads to an increased association of SrGAP1 with Cdc42, thus leading to Cdc42 downregulation and disruption of local actin structures (Wong et al., 2001). Recently, it was reported that SrGAP3 gene is located on chromosome 3p25 which is deleted in patients suffering form severe mental retardation, suggesting that SrGAP3 may have an important role in the development of neuronal structures that are important for normal cognitive function (Endris et al., 2002).

p85-α and p85-β

The ubiquitously expressed human p85-α and p85-β proteins are the regulatory subunits for the 110 KDa catalytical subunit of phosphatidylinositol-3 kinase (PI3K) which regulates a number of signaling pathways through generation of potent phosphoinositides. Both proteins are structurally analogous and contain an N-terminal SH3 domain followed by a RhoGAP domain and two SH2 domains that mediate interaction with protein tyrosine kinases (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991). Although, the RhoGAP domain of p85-α is homologous to that of Cdc42GAP and binds specifically to Cdc42 and Rac1 in a GTP-dependent manner, and contains the essential arginine residue, it does not seem to display GAP activity towards GTPases (Zheng et al., 1994a; Zheng et al., 1994b). This led to the assessment that a RhoGAP protein that harbors the critical arginine residue will not necessarily display GAP activity. However, interaction of Rac1 but not RhoA with p85-α increases PI-3 kinase activity *in vitro* (Bokoch et al., 1996a). In addition, it has been demonstrated that

PI3K-mediated phosphoinositides activate RhoGEFs like Tiam-1 which in turn promote GTPase activation. Therefore, PI3K seems to have a dual role as an upstream activator and a downstream effector of Rho GTPases.

RalBP1, RLIP76 and RIP1

These three ubiquitous proteins were identified during the same period by three different laboratories searching for binding partners to the Ral small GTPase. The human RLIP76, the mouse RIP1 and the rat RalBP1 proteins are structurally and functionally similar. They are constituted of a RhoGAP domain followed by a C-terminal Ral binding region which interacts with Ral in a GTP-dependent manner. They also contain two ATP binding regions and present functional ATPase activity (Awasthi et al., 2002). All three proteins display GAP activity *in vitro* towards Cdc42 and to a lesser extent on Rac1 but not RhoA (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995). RIP1 binding to Ral requires the Ral binding sequence but not the RhoGAP domain and activated Ral does not affect RIP1 GAP activity (Park and Weinberg, 1995), suggesting that these two domains may function independently and may integrate signal from both Ral and Rho signaling simultaneously. Several lines of evidence suggest that RLIP76 and RalBP1 proteins may play a pivotal role in Ral-mediated protein trafficking. In fact, RLIP76 and RalBP1 interact with a number of proteins involved or suspected to be involved in endocytosis such as Reps1, the AP2 adaptor and POB1 (Ikeda et al., 1998; Jullien-Flores et al., 2000; Yamaguchi et al., 1997). Given that Ral GTPases are involved in EGF receptor endocytosis (Ikeda et al., 1998) and that EGF activates the Rho GTPases, RalBP1 could provide a connection between mitogen signaling (Ras-Ral), endocytosis (Ral-RalBP1) and cytoskeleton dynamics (RalBP1-Cdc42). In addition to its role in signal transduction, RLIP76 can catalyze ATP-dependent transport of glutathione conjugates and xenobiotics, and may contribute to the multidrug resistance of cancer cells (Awasthi et al., 2000).

DLC-1, DLC-2, p122RhoGAP and RhoGAP80C

The human DLC-1 and DLC-2 genes are deleted or downregulated in a significant number of hepatocellular carcinomas (HCCs) and have been shown to function as tumor

suppressors (Ching et al., 2003; Yuan et al., 1998). The rat p122RhoGAP was identified as an interacting partner for phospholipase C-delta1 (PLC-δ1) (Homma and Emori, 1995). Recently, Drosophila RhoGAP88C was identified in a search for genes that regulate Drosophila morphogenesis (Denholm et al., 2005). All four proteins are expressed in a number of tissues and are structurally related. They contain an N-terminal SAM domain that has been thought to function as a protein interaction module through homo- and hetero-oligomerization with other SAM domains, a RhoGAP domain and a C-terminal STAR-related lipid transfer domain (START). Both DLC-2 and p122RhoGAP have been reported to have functional RhoGAP domains whereas the GAP activity of DLC-1 has not been studied. Studies indicate that both DLC-2 and p122RhoGAP have GAP activity towards RhoA in *vitro* and *in vivo* (Ching et al., 2003; Homma and Emori, 1995; Sekimata et al., 1999). The *in vitro* GAP activity of RhoGAP88C has not been demonstrated yet but like other members of this family, it seems to be active *in vivo* on RhoA (Denholm et al., 2005). Moreover, p122RhoGAP associates with PLC-δ1 and promotes its phosphatidylinositol 4,5-biphosphate (PIP2) hydrolyzing activity both *in vitro and in vivo* (Homma and Emori, 1995; Sekimata et al., 1999). Since many actin-associated cytoskeletal proteins like gelsolin and profilin are regulated by PIP2, p122RhoGAP may act as an effector for Rho GTPases in inducing cytoskeletal dynamics. Furthermore, p122RhoGAP seems to be localized in caveolae and is involved in the intracellular distribution of caveolin (Yamaga et al., 2004).

α1-Chimaerin, α2-Chimaerin, β1-Chimaerin and β2-Chimaerin

The four members of this subfamily are small proteins (34 to 46 KDa) that are alternative splice variants from the α-chimaerin and β-chimaerin genes. All four contain an N-terminal Cysteine-rich (C1) domain and a C-terminal RhoGAP domain. In addition, α2 and β-Chimaerin variants harbor an N-terminal Src homology 2 (SH2) domain. Moreover, α1-Chimaerin is exclusively expressed in brain and α2-Chimaerin is present in brain and testis (Hall et al., 1990; Hall et al., 1993), whereas β1-Chimaerin seems to be exclusively present in testis and in germ cells during acrosomal assembly (Leung et al., 1993), and β2-Chimaerin is predominantly present in brain (Leung et al., 1994). Both α-chimaerin and β-chimaerin variants have functional RhoGAP domains that specifically

stimulate the GTPase activity of Rac1 *in vitro* (Diekmann et al., 1991; Leung et al., 1993). The GAP domain of α1-Chimaerin has been reported to inhibit Rac-mediated lamellipodia formation in Swiss 3T3 fibroblasts (Kozma, 1996). Surprisingly, full length α1-Chimaerin lacking GAP activity but retaining binding to the GTPases, seems to cooperate with Rac1 and Cdc42 to promote formation of lamellipodia and filopodia (Kozma, 1996), suggesting a double function for α1-Chimaerin as a Rho GTPase-activating protein and a Rho GTPase effector. The *in vitro* GAP activity of α1-Chimaerin towards Rac1 is positively and negatively regulated through binding of phorbol esters to its C1 domain (Ahmed et al., 1993) and treatment of cells with phorbol esters seems to induce changes in the subcellular localization of chimaerins (Caloca et al., 2001). Moreover, the brain specific α2-Chimearin was shown to induce neurite outgrowth in N1E-115 neuroblastoma cells and is involved in Semaphorin-3A induced growth cone collapse (Brown et al., 2004; Hall et al., 2001).

RICH-1, RICH-2, Nadrin and 3BP-1

Members of this family comprise the human RICH-1 and RICH-2, the rat Nadrin and the mouse 3BP-1 proteins. All four contain an N-terminal BIN/Amphiphysin/Rvsp (BAR) domain followed by a RhoGAP domain and a C-terminal proline rich-region (Cicchetti et al., 1995; Harada et al., 2000; Richnau and Aspenstrom, 2001). A marked difference between the members of this family is that 3BP-1 contains a single proline-rich sequence whereas Nadrin and RICH harbor four. The GAP domains of all four proteins are functionally similar and stimulate the intrinsic GTPase activity of Cdc42, and Rac1 *in vitro* and Nadrin also displays some activity towards RhoA (Cicchetti et al., 1995; Harada et al., 2000; Richnau and Aspenstrom, 2001). In addition, RICH-1 and 3BP-1 inhibit Rac-mediated lamellipodia formation in Swiss 3T3 fibroblasts. The expression of RICH-1 seems to be ubiquitous whereas RICH-2 and Nadrin are mainly found in brain and 3BP-1 is predominantly expressed in spleen. Nadrin was reported to promote the fusion of synaptic vesicles with the plasma membrane during exocytosis through its RhoGAP domain, possibly by inducing changes in the underlying cortical actin filament network (Harada et al., 2000). At least four variants of Nadrin have been identified with molecular weights ranging from 102 to 126 KDa. The 116 KDa variant significantly inhibits NGF-

dependent neurite outgrowth in a GAP activity-dependent manner (Furuta et al., 2002). The second proline-rich sequence of RICH-1 binds to the SH3 domain of Cdc42-interacting protein 4 (CIP4) and the first proline-rich sequence is required for binding to the SH3 domains of the endocytic proteins endophilin and amphiphysin but the biological relevance of these interactions have not been established (Richnau and Aspenstrom, 2001; Richnau et al., 2004). Also, the BAR domain of RICH-1 binds to phospholipids and seems to be involved in membrane deformation events (Richnau et al., 2004).

Graf, Graf-2, PSGAP, Oligophrenin-1

GRAF and related members have been characterized in human, mouse and chicken. Human Graf-2 and Oligophrenin-1, mouse PSGAP and chicken Graf are structurally and functionally similar. They contain a PH domain followed by a RhoGAP domain and all except Oligophrenin-1 have a C-terminal SH3 domain. All members are expressed in most tissues and Oligophrenin-1 is predominantly expressed in brain. The GAP domain of all four proteins stimulates GTP hydrolysis of Cdc42 and RhoA *in vitro* and Oligophrenin-1 also displays some activity towards Rac-1 (Billuart et al., 1998; Hildebrand, 1996; Ren et al., 2001; Shibata et al., 2001). However, *in vivo* Graf prefers RhoA and PSGAP is active on Cdc42 whereas the GAP domain of Oligophrenin-1 seems to be active on all three GTPases (Fauchereau et al., 2003; Ren et al., 2001; Taylor et al., 1999). Moreover, full length Oligophrenin-1 is not active and its GAP activity seems to be regulated by intra-molecular interactions (Fauchereau et al., 2003). Graf binds to the C-terminal domain of FAK in an SH3 domain-dependent manner and may function to mediate cross-talk between FAK and the Rho GTPase during integrin signaling (Hildebrand et al, 1996). Graf may also mediate cross-talk between mitogen signaling pathways and Rho signaling since it has been shown to be phosphorylated by the ERK MAPK pathway (Taylor et al., 1998). Human GRAF has been reported to have a possible role in development and progression of hematopoietic disorders (Borkhardt et al., 2000). Mutations resulting in Oligophrenin-1 loss of function are related to non-specific X-linked mental retardation (MRX) (Billuart et al., 1998). Recently, it was reported that Oligophrenin-1 associates with Homer, a protein involved in dendritic spine morphogenesis and synaptic transmission and knock-down of Oligophrenin-1 by RNAi

revealved that it is implicated in dendritic spine morphogenesis (Govek et al., 2004). PSGAP interacts with PYK2 trough its SH3 domain and a proline-rich region in PYK2. Also, PYK2 negatively regulates PSGAP activity *in vivo* possibly through mechanisms involving phosphorylation (Ren et al., 2001). However, a direct correlation between PSGAP phosphorylation by PYK2 and regulation of its GAP activity remains to be established.

Gmip and PARG1

Both the human Gmip (Gem-interacting protein) and its close homologue human PARG1 (PTPL1-associated RhoGAP1) were identified by a yeast two hybrid screen in a search of proteins interacting with the Ras-related Gem protein and the PTPL1 tyrosine phosphatase, respectively (Aresta et al., 2002; Saras et al., 1997). Both proteins are ubiquitously expressed and contain a cysteine-rich domain related to a phorbol ester/diacylglycerol binding domain followed by a RhoGAP domain. The RhoGAP domain of PARG1 and Gmip are active on RhoA both in *vitro and in vivo* (Aresta et al., 2002; Myagmar et al., 2005; Saras et al., 1997). PARG1 was recently shown to interact with the Ras-related protein Rap2 and PARG1 inhibition of Rho-mediated stress fibers seems to be suppressed by Rap2 (Myagmar et al., 2005). Hence, Gmip and PARG1 may act as effector molecules for Ras-related proteins to regulate Rho induced actin remodeling and provide an additional cross-talk mechanism between these two families.

ArhGAP9, ArhGAP12, ArhGAP15 and CAMGAP1

Human ArhGAP9, ArhGAP12 and ArhGAP15, and rat CAMGAP1 are composed of a PH domain and a C-terminal RhoGAP domain. All except ArhGAP15 also contain an N-terminal SH3 domain followed by one to three WW domains, which are known to mediate protein–protein interactions by binding to proline-rich region. ArhGAP12 is ubiquitously expressed in a wide range of normal tissues and tumor cell lines (Zhang et al., 2002). ArhGAP15 and CAMGAP1 are also ubiquitously expressed whereas ArhGAP9 is mainly present in spleen and thymus and in cell lines from B-cell leukemias, T-cell leukemias and myeloid or Hodgkin lymphomas. The ArhGAP15 protein stimulates the GTPase activity of Rac1, but not Cdc42 and RhoA *in vitro* (Seoh et al., 2003). In

contrast, ArhGAP9 is active *in vitro* towards Cdc42 and Rac1 but to a lesser extent towards RhoA (Furukawa et al., 2001) and CAMGAP1 is also active *in vitro* towards Cdc42 and Rac1 but not RhoA (Sakakibara et al., 2004). CAMGAP1 was recently identified from a yeast two hybrid screen as an interacting partner to Cbl-interacting protein of 85 kDa (CIN85), a recently discovered protein that interacts with the Cbl proto-oncogene. Recent studies have shown that CIN85 is involved in the regulation of clathrin-mediated endocytosis of several plasma membrane receptors (Petrelli et al., 2002; Soubeyran et al., 2002). It was shown that a deletion mutant containing the three WW domains of CAMGAP1 was sufficient to inhibit internalization of transferrin receptors (Sakakibara et al., 2004), suggesting that CAMGAP1 may be involved in clathrin-mediated endocytosis.

Myosin-IXb, Myr 5 and Myr 7

Members of the unconventional Myosin-IX subfamily are the largest known RhoGAP containing proteins. They are multidomain proteins containing an N-terminal Ras-associating domain (RA) followed by a myosin large ATPase domain, four to six short calmodulin binding motifs (IQ), a cysteine-rich domain (C1), and a C-terminal RhoGAP domain. Rat Myr 5 is expressed in several tissues whereas its human ortholog Myosin-IXb is mainly expressed in spleen and peripheral blood leukocytes (Reinhard et al., 1995; Wirth et al., 1996). Rat Myr 7 is significantly homologous to human Myosin-IXb and to Rat Myr 5 and is predominantly expressed in the developing and adult brain (Chieregatti et al., 1998). All three proteins seem to be highly active *in vitro* towards RhoA but not Cdc42 or Rac1 (Chieregatti et al., 1998; Post et al., 1998; Reinhard et al., 1995). In addition Myr 5 has been shown to inhibit stress fibers *in vivo* (Muller et al., 1997). The role of this RhoGAP family in regulating Rho-mediated signal transduction pathways remains to be unraveled. Recently, Myosin-IXa which also contains a RhoGAP domain has been identified as a candidate gene involved in the Bardet-Biedl syndrome (BBS), an heterogenous autosomal recessive disorder partially characterized by mental retardation, obesity, short stature and hypogenitalism (Gorman et al., 1999).

ArhGAP10, XrGAP and CeGAP

CeGAP was identified over a decade ago in *C.elegans*, and has recently found a place in a novel subfamily composed of human ArhGAP10 and a putative Xenopus Rho GTPase-activating protein (XrGAP) gene predicted to encode a protein which shares high degree of homology to ArhGAP10 (Basseres et al., 2002; Chen and Lim, 1994; Kim et al., 2003). All three are large proteins that contain a PH domain and a RhoGAP domain. Both ArhGAP10 and XrGAP also harbor an N-terminal PDZ domain which is found in many proteins and are involved in mediating protein–protein interactions. ArhGAP10 is present in many tissues and seems to be up-regulated during cell differentiation (Basseres et al., 2002). Arhgap10 is a potent GAP for Cdc42 but is much less effective on RhoA and Rac1 *in vitro*. Recently, it was reported that ArhGAP10 interacts with ARF1 and localizes to the Golgi complex in a GTP-ARF1-dependent manner. It appears that ArhGAP10 can regulate the Arp2/3 complex and F-actin dynamics, suggesting cross-talk between ARF and Rho signaling (Dubois et al., 2005). CeGAP is active *in vitro* towards human and C.*elegans* Rac1, Cdc42 and RhoA and unlike many other RhoGAPs, CeGAP displays activity towards C.*elegans* and human Ras proteins (Chen and Lim, 1994). The biological relevance of this cross reactivity has not been determined yet.

MgcRacGAP, RnRacGAP, DRacGAP and CYK-4

Members of this group comprise the human and mouse MgcRacGAP, the drosophila DRacGAP and RnGAP, and the *C.elegans* CYK-4 proteins. These proteins are structurally and functionally similar. They contain an N-terminal coiled-coil region, a cysteine-rich sequence (C1) followed by a RhoGAP domain. MgcRacGAP is active *in vitro* towards Cdc42 and Rac1 but displays very little GAP activity towards RhoA (Toure et al., 1998). However, phosphorylation of MgcRacGAP by the AuroraB kinase within the RhoGAP domain downregulates its GAP activity towards Cdc42 and Rac1 while significantly increasing its preference for RhoA (Minoshima et al., 2003). Moreover, during cytokinesis, AuroraB phosphorylates MgcRacGAP which significantly increases its activity towards RhoA which seems to be an important step for proper cell division (Hirose et al., 2001; Minoshima et al., 2003). During metaphase, PRC1 (protein-regulating cytokinesis 1) binds to the GAP domain of MgcRacGAP and down-regulates

its GAP activity and thereby contributes to Cdc42 activation and proper formation of the spindle (Ban et al., 2004). Furthermore, phosphorylation of MgcRacGAP by Aurora B kinase prevents its binding to PRC1 and the inhibition of its GAP activity (Ban et al., 2004). MgcRacGAP has also been shown to play a key role in controlling growth and differentiation of hematopoietic cells, independently of its GAP domain (Kawashima et al., 2000; Kitamura et al., 2001). CYK-4 stimulates GTP hydrolysis of Cdc42 and Rac1 with very little effect on RhoA *in vitro,* and has also been implicated in central spindle formation and cytokinesis (Jantsch-Plunger et al., 2000). RnRacGAP displays *in vitro* activity towards DRac1 and DCdc42 but not DRho1 and interferes with eye development and embryonic dorsal closure in the fly, two processes controlled by the Rho GTPases DRac1 and DCdc42 (Raymond et al., 2001). DRacGAP seems to behave as a negative regulator of DRac1 and DCdc42 *in vivo*. During drosophila development DRac1 enhances EGFR/Ras-dependent activation of the MAP Kinase pathway and DRacGAP expression seems to be negatively regulated through a feedback mechanism by the EGFR/Ras pathway (Sotillos and Campuzano, 2000).

RARhoGAP and tGAP1

These two recently identified rat proteins are expressed in male germ cells and RARhoGAP (also known as RA-RhoGAP) is also found in brain and liver (Yamada et al., 2005; Curry et al., 2004; Modarressi et al., 2004). RARhoGAP contains an N-terminal PH domain followed by a Ras-association (RA) domain and a RhoGAP domain which is active both *in vitro* and *in vivo* towards RhoA but not Rac1 or Cdc42 (Yamada et al., 2005). Moreover, the GTP-bound active form of Rap1 binds to the RA domain of RARhoGAP and enhances its GAP activity *in vitro*. RARhoGAP seems to be involved in Rap1-induced neurite outgrowth, mainly through downregulation of RhoA (Yamada et al., 2005), providing a link between Rap1 and Rho signaling during neurite outgrowth. tGAP1 also contains an N-terminal RA domain but lacks the PH domain. Remarkably, it contains four RhoGAP domains and the fourth one shares 35 % sequence identity with RaRhoGAP. tGAP1 is the only protein that contains more than one GAP domain and ironically none of them displays a functional activity towards Rho GTPases. However, all four Rho GAP domains are devoid of the conserved arginine residue important for

GTPase stimulation. Given that tGAP1 is both nuclear and cytoplasmic, it has been proposed that it might use its RhoGAP domains to sequester Rho GTPases inside the nucleus (Modarressi et al., 2004). In addition, tGAP1 was shown to induce apoptosis of somatic cells and it has been proposed that it may have an important role during spermatogenesis since a significant number of male germ cells that result from mitosis and meiosis die by an apoptotic process (Modarressi et al., 2004).

p73RhoGAP and p68RacGAP

These recently characterized members are the first RhoGAP containing proteins to be vascular-specific (Aitsebaomo et al., 2004; Su et al., 2004). Human p73RhoGAP and mouse p68RacGAP share approximately 40 % sequence homology within the entire protein sequence and around 75 % sequence identity in their RhoGAP domains. Overexpression of p73RhoGAP decreases the levels of active RhoA but does not have an effect on Rac1 and Cdc42 activation. Moreover, introducing p73RhoGAP mutated in the critical arginine residue inside cells causes an increase in RhoA activity and also promotes stress-fiber formation, suggesting that RhoA is a target of p73RhoGAP *in vivo* (Su et al., 2004). RhoA but not Rac1 and Cdc42, is a target for p68RacGAP both *in vitro* and *in vivo* (Aitsebaomo et al., 2004). Both proteins have been shown to inhibit endothelial cell capillary tube formation *in vitro* and p73RhoGAP also inhibits angiogenesis *in vivo*. Given that angiogenesis plays an important role in tumor growth and metastasis, these proteins could eventually become interesting therapeutic targets for specifically modulating Rho GTPase activity in this process.

ArhGAP6

ArhGAP6 gene has been localized to the X chromosome in a critical region deleted in patients suffering from microphthalmia with linear skin defect (MLS) (Schaefer et al., 1997). However, whether alteration of the ArhGAP6 gene is responsible of MLS remains to be established. In fact ArhGAP6 deficient mice do not show features of MLS syndrome (Prakash et al., 2000). ArhGAP6 is expressed in a number of human tissues and contains a RhoGAP domain and several putative SH3 binding motifs. The

RhoGAP domain is active both *in vivo* and in *vitro* towards RhoA but not Cdc42 or Rac1 (Prakash et al., 2000).

OCRL-1

The oculocerebrorenal syndrome of Lowe (OCRL) is a rare human X-linked developmental disorder characterized by a pleitropic phenotype including eye and renal abnormalities and severe mental retardation (Attree et al., 1992). The OCRL-1 protein is homologous to inositol polyphosphate 5-phosphatase type II (INPP5B) from human platelets. OCRL-1 is ubiquitously expressed and contains a central inositol phosphate domain followed by a C-terminal RhoGAP domain. OCRL-1 is primarily localized to the trans-Golgi network (TGN) and has been shown to be a catalytically active phosphoinositide 5-phosphatase both in *vitro and in vivo* (Faucherre et al., 2003; Zhang et al., 1995). OCRL-1 does not belong to a particular subfamily because its RhoGAP domain shows a relatively low degree of identity with other RhoGAPs. OCRL-1 weakly stimulates Rac1 GTPase activity *in vitro* (Faucherre et al., 2003). Unlike most RhoGAP members it does not present the conserved arginine residue and it binds efficiently to the GDP bound form of Rac1, this unexpected property may contribute to a low turn-over rate of the substrate, resulting in a reduced overall GAP activity observed *in vitro*. Nevertheless, it seems that OCRL-1 is a potent inhibitor of Rac-mediated membrane ruffles in Swiss 3T3 cells (Faucherre et al., 2003) and is active as a PIP2 5-phosphatase in Rac-induced membrane ruffles (Faucherre et al., 2003).

Vilse

Given that SrGAPs are not found in flies and worms, these proteins cannot provide a link between Robo signaling and Rho GTPases during axon guidance in these invertebrates. It was recently reported that another RhoGAP protein, Vilse (also known as CrGAP) which is found both in vertebrates and invertebrates provides a direct connection between Robo signaling and Rho GTPases (Hu et al., 2005; Lundstrom et al., 2004). The Vilse protein contains two WW motifs at its N-terminal which are necessary and sufficient to mediate binding to the intracellular tail of Robo, and a C-terminal myosin tail homology 4 (MyTH4) and RhoGAP domains. Vilse is active both *in vitro* and *in vivo*

towards Rac1 and Cdc42 but not RhoA (Lundstrom et al., 2004). The biological role of Vilse/CrGAP in Robo signaling seems to be different in midline neurons versus in trachea (Hu et al., 2005; Lundstrom et al., 2004). Overexpression of Vilse in the trachea of *robo* mutants improves the phenotypes of *robo (-/-)*, indicating that Vilse acts downstream of Robo to mediate midline repulsion. In contrast, introduction of exogenous CrGAP/Vilse in midline axons increases the *robo* phenotype. These differences suggest the need for further studies in order to elucidate how precisely Robo activation regulates Vilse/CrGAP function in neurons.

SYD-1

The *C.elegans* SYD-1 protein contains an N-terminal PDZ domain followed by a proline-rich sequence that harbors six putative SH3 binding motifs, a C2 domain and a C-terminal RhoGAP domain. Recently, SYD-1 was reported to have an important role in defining axonal identity during initial polarity determination. In fact, loss-of-function mutations in the gene that encodes SYD-1 caused axonal components to be localized to dendritic processes (Hallam et al., 2002). SYD-1 does not harbor the conserved arginine residue in the RhoGAP domain and lacks both binding and GAP activity *in vitro* towards *C.elegans* Rho, Rac and Cdc42 homologs. However, the GAP domain seems to be important since removing it causes defects in neurite outgrowth (Hallam et al., 2002).

p190-A and p190-B

The p190-RhoGAP subfamily is composed of two closely related proteins, p190-A (originally named p190) and p190-B. Proteins in this family have been found in human, mouse, rat and drosophila (Billuart et al., 2001; Burbelo et al., 1998; Chakravarty et al., 2000; Settleman et al., 1992b; Tikoo et al., 2000). They contain an N-terminal GTPase domain followed by four consecutive FF domains, a C-terminal RhoGAP domain and several SH3 binding sites. Both rat p190-A and human p190-B undergo GAP activity *in vitro* towards Rac1, Cdc42 and RhoA with a preference for the latter (Burbelo et al., 1995; Ridley et al., 1993; Settleman et al., 1992a). However, when expressed in Swiss 3T3 fibroblasts, p190-A displays GAP activity exclusively towards RhoA (Haskell et al., 2001; Ridley et al., 1993). p190-A was initially discovered as a p120 RasGAP-associated

protein in growth-factor stimulated and tyrosine-kinase transformed cells and was predominantly phosphorylated on tyrosine and to a lesser extent on serine residues (Ellis et al., 1990; Moran et al., 1991; Settleman et al., 1992b). Phosphorylation on tyrosine residues by kinases such as Src, have been shown to play a major regulatory role on p190 GAP and GTP-binding activities (Roof et al., 2000; Roof et al., 1998). P190-A and p120RasGAP interact in a phosphotyrosine-dependent manner which regulates p190-A activity towards RhoA (McGlade et al., 1993), suggesting possible cross-talk between Ras and Rho signaling. Recently, p190-A was shown to interact with and sequester the serum-responsive transcriptional regulator TFII-I in the cytoplasm. Upon PDGF-induced phosphorylation of one of the FF domains of p190-A, TFII is released and translocates to the nucleus where it can activate transcription of serum-inducible genes (Jiang et al., 2005). Recent studies established a role for p190-A in regulating cytokinesis and it was shown that expression of p190-A is cell cycle regulated through ubiquitin-mediated degradation (Su et al., 2003). Members of the p190RhoGAP family have been implicated in a number of biological processes such as tumor suppression (Tikoo et al., 2000; Wang et al., 1997), cell migration, cancer cell invasion (Arthur and Burridge, 2001; Nakahara et al., 1998; Zrihan-Licht et al., 2000); and neuronal morphogenesis (Billuart et al., 2001; Brouns et al., 2000; Brouns et al., 2001). Recently it was shown that p190-B deficient mice display reduced cell size, impaired insulin signaling, decreased adipogenesis and myogenesis and decreased incidence of mammary ductal morphogenesis (Chakravarty et al., 2000; Sordella et al., 2003).

S. cerevisiae RhoGAPs

In *Saccharomyces cerevisiae*, eight RhoGAP proteins have been characterized: Bem2p, Bem3p, Rga1p, Rga2p, Rgd1p, Rgd2p, Sac7p, Bag7p and Lrg1p. Sac7p and Bag7p are closely related and are active both *in vitro* and *in vivo* towards Rho1p (Schmidt et al., 1997; Schmidt et al., 2002). Introduction of exogenous Bag7p or Sac7p suppresses the cold sensitivity of a Sac7p mutation and the lethality of Rho1p hyperactivation in response to cell wall injury, whereas Sac7 but not Bag7p regulates the Rho1p-regulated PKC1-MPK1 pathway (Schmidt et al., 2002). Hence, Bag7p and Sac7p regulate similar but also different Rho1p-mediated cellular functions. Rga1p and Rga2p are also highly

homologous and present two N-terminal LIM domains which bind zinc ions and are thought to mediate protein-protein interactions, and a C-terminal RhoGAP domain. Rga1p (also known as Ddm1p) was shown to be a negative regulator of the pheromone response pathway and is thought to be implicated in bud site selection (Stevenson et al., 1995). Recently, biochemical and genetic studies established Rga1 and Rga2 as GAP proteins for Cdc42 (Smith et al., 2002). Bem3p appears to play roles in the regulation of bud site formation (Zheng et al., 1994b). It contains a Phox-homology (PX), a PH and a C-terminal RhoGAP domain. Bem3p is also a specific Cdc42 GTPase-activating protein (Smith et al., 2002; Zheng et al., 1994b; Zheng et al., 1993). Although Rga1, Rga2 and Bem3 are Cdc42 specific, each one seems to play a role in modulating specific aspects of Cdc42 function. For instance, deletion of Rga1p, but not Rga2p or Bem3p causes hyperinvasive growth overproduction and loss of Rga1p and Rga2p but not Bem3p affects the interaction of Cdc42p with Ste20, a p21-activated kinase (PAK) kinase required for haploid invasive growth. Morphological cellular defects are observed in Bem3p deletion strains which are not present in Rga1p or Rga2p deletion strains (Smith et al., 2002). Like Rga1 and Rga2, Lrg1p contains LIM domains and a C-terminal RhoGAP domain. Lrgp1 interacts with and stimulates GTP hydrolysis of Cdc42p and Rho2p (Roumanie et al., 2001). In addition, Lrg1 has been shown to be expressed during sporulation and is thought to play a role during mating (Muller et al., 1994). Bem2p has been reported to have a GAP-dependent role in promoting cell polarity and a GAP-independent role in responding to defects in cell polarity (Bender and Pringle, 1991; Chant et al., 1991; Marquitz et al., 2002). Bem2p was shown to be a GAP for Rho1p both *in vitro and in vivo* (Marquitz et al., 2002; Peterson et al., 1994; Schmidt et al., 1997). Rgd1 and Rgd2 contain an N-terminal FCH domain and a C-terminal RhoGAP domain and Rgd2 also harbors a DEP domain which is specific for G protein signaling, and it is thought to play a role similar to coiled coil domains in dimerization. Rgd1p is active *in vitro* on Rho3p and Rho4p but seems to be specific for Rho3p *in vivo* (Doignon et al., 1999). In contrast, Rgd2 is active on Cdc42p and Rho5p (Roumanie et al., 2001).

DdRacGAP1

DdRacGAP1 is the first RhoGAP protein identified in the slime mold *Dictyostelium discoideum*. This multidomain protein contains an N-terminal RhoGAP domain, an SH3 domain, two RhoGEF domains and a PH domain, suggesting that it may integrate signal from a variety of signaling pathways. The RhoGAP domain was shown to be active in *vitro* towards both *Dictyostelium* and human Rho family GTPases but not human Ras (Ludbrook et al., 1997). Disruption of DdRacGAP1 gene promotes formation of membrane ruffling similar to overexpression of a constitutively active DdRAC1 mutant, suggesting that DdRacGAP1 displays a Rac1-specific GAP activity *in vivo* (Chung et al., 2000). The GAP domain is also active towards the *Dictyostelium* homolog of Rab4, RabD and DdRacGAP1 activity seems to be important for RabD-dependent regulation of the contractile vacuole, suggesting that the RabD-specific activity may have some biological relevance *in vivo* (Knetsch et al., 2001). Moreover, the GEF domain displays activity towards *Dictyostelium* Rac1A but not RacC, RacE, RasG and RabD (Knetsch et al., 2001). Furthermore, DdRacGAP1 has been reported to regulate F-actin dynamics during chemotaxis (Chung et al., 2000).

1.2.7. Rho GTPase effectors

Given the involvement of Rho GTPases in a wide spectrum of biological processes, it is not surprising that they interact with an impressive number of downstream effectors. To date, over 30 potential Rho GTPase effector proteins have been identified (Bishop and Hall, 2000). Once bound to GTP, the Rho proteins undergo conformational changes, which enable them to interact with their effector proteins (Fig. 1.3). Generally, they activate their effectors by disrupting intramolecular autoinhibitory interactions which then exposes functional domains within the effector protein. For instance, the Rac/Cdc42 effector PAK harbors an intramolecular regulatory domain that inhibits its kinase activity. Upon GTPase binding, the inhibitory sequence is displaced, allowing the kinase domain to phosphorylate substrates (Bagrodia et al., 1999; Tu and Wigler, 1999). WASP and N-WASP are two closely related Cdc42 effector proteins which also seem to be regulated by intramolecular interactions, and GTP-bound Cdc42 can activate them by releasing these interactions (Machesky and Insall, 1998; Rohatgi et al., 1999).

Rho GTPases were initially shown to be involved in actin cytoskeleton remodeling. Therefore, significant effort was put into identifying downstream effectors involved in actin dynamics. The serine/threonine kinases, ROKα/Rho Kinase and its close relative, p160ROCK (also known as ROCKII or ROKβ) were the first identified RhoA effectors to be implicated in actin dynamics. Once activated by RhoA, these enzymes phosphorylate the myosin-binding subunit (MBS) of myosin light chain (MLC) phosphatase as well as myosin light chain (MLC), which both regulate the activity of myosin II and subsequently the formation of stress fibers (Amano et al., 1996a; Kawano et al., 1999; Kimura et al., 1996). These kinases can also stabilize actin filaments by phosphorylating and activating LIM kinase which in turn phosphorylates and inactivates cofilin, a protein involved in actin filament disassembly (Bamburg, 1999; Maekawa et al., 1999; Sumi et al., 1999). In addition, they also phosphorylate adducin, a protein associated with the cortical actin network beneath the plama membrane. Phosphorylation of adducin causes it to bind more strongly to F-actin which contributes in reinforcing the cortical actin network (Kimura et al., 1998). However, the kinase domain of ROKs is insufficient to induce stress fibers and requires the contribution of an additional effector of RhoA known as p140mDia (Nakano et al., 1999; Narumiya et al., 1997; Watanabe et al., 1999).

p140mDia is the mammalian ortholog of Bni1 and Bnr1 from *S. cerevisiae* and also diaphanous from *D. melanogaster* (Castrillon and Wasserman, 1994; Imamura et al., 1997), which have been characterized as Rho effectors in actin reorganization. mDia1 and mDia2 are two isoforms of p140mDia that are members of the formin-homology (FH) family of proteins and contain a RhoA binding domain and two FH domains (Watanabe et al., 1997). The FH1 sequence harbors multiple proline-rich sequences that bind to the G-actin-binding protein profilin and cause actin polymerization and F-actin organization into stress fibers (Watanabe et al., 1997).

In addition to ROK and p140mDia, a number of Rho effector proteins have been identified. These include PKN (protein kinase N) and its homolog PRK2 (PKN related kinase 2) which contain a serine/threonine kinase domain that is highly related to that of protein kinase C (PKC) (Amano et al., 1996b; Watanabe et al., 1996). It was reported that a kinase-defective form of PRK2 disrupts actin stress fibers, suggesting a role for PRK2

in actin remodeling (Vincent et al., 1992). Another RhoA target, Citron contains a protein kinase domain that is related to that of ROK. Citron kinase localizes to the cleavage furrow and midline of culture cells and seems to be involved in cytokinesis (Madaule et al., 1998). In addition, RhoA associates with type-I phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) to control the production of phosphatidylinositol 4,5-bisphosphate (PIP2) which is a potent regulator of a number of actin-associated proteins (Ren and Schwartz, 1998). Rhophilin and Rhothekin share homology with PKN in their Rho binding domain and are also targets of RhoA (Reid et al., 1996; Watanabe et al., 1996) but their role in Rho-mediated cytoskeletal rearrangements remains to be established.

So far, several potential effectors of Rac and Cdc42 have been identified and some are common targets for both. Among them is the p21-activated kinase (PAK) family that comprise at least six mammalian isoforms that share a highly conserved C-terminal kinase domain and an N-terminal Cdc42/Rac-interactive binding (CRIB) domain (Burbelo, 1995), also known as the GTPase-binding domain (GBD) (Symons, 1996). PAKs can be categorised into two subgroups based on sequence homology (Jaffer and Chernoff, 2002). The Group I PAKs (PAK1, -2 and -3) share approximately 75 % sequence homology throughout the protein sequence (Manser et al., 1995; Teo et al., 1995), whereas the Group II PAKs (PAK4, -5 and -6) are highly related in the kinase domains but share weak homology in the rest of the protein sequence (Dan et al., 2002; Yang et al., 2001). PAK1, -2 and -3 bind to both Cdc42 and Rac, and this interaction is believed to induce a conformational change, which relieves auto-inhibition and stimulates kinase activity above a low basal level (Sells et al., 1997; Zhao et al., 1998). In contrast, PAK4 and PAK6 interact preferentially with Cdc42, but their kinase activity is not stimulated by this association (Abo et al., 1998; Lee et al., 2002). PAK1, -2 and -3 contain an SH3-binding site N-terminal to the CRIB domain, which is responsible for binding to the adapter protein Nck (Bokoch et al., 1996b). In addition, there is an SH3-binding site between the CRIB domain and the C-terminal kinase domain that mediates association with PIX, a putative exchange factor for Rac (Manser et al., 1998). In contrast, PAK4 has no SH3-binding sites N-terminal to the GBD domain and does not bind Nck or PIX, but has seven putative SH3-binding sites between the GBD and kinase domains (Abo et al., 1998). Both

Group I and Group II PAKs can induce actin rearrangement in cells (Daniels and Bokoch, 1999; Jaffer and Chernoff, 2002) although they may not specifically mediate cytoskeletal effects that are triggered by Cdc42 and Rac (Joneson, 1996; Lamarche, 1996; Sells et al., 1997). A variety of substrates for PAKs have been identified that could affect the actin cytoskeleton. Rac1, like Rho, induces phosphorylation of LIMK, and PAK1 has been shown to phosphorylate LIMK (Edwards et al., 1999). Also, an inactive form of LIMK has been shown to inhibit both Cdc42 and Rac1-induced actin changes (Yang et al., 1998), suggesting that cofilin phosphorylation may be a general requirement in Rho GTPase pathways. Moreover, PAK has been shown to phosphorylate and inactivate MLC kinase, decreasing MLC phosphorylation and reducing actomyosin assembly (Chew et al., 1998; Sanders et al., 1999; Zeng et al., 2000).

IQGAP is also an effector of both Rac1 and Cdc42 and is suspected to be involved in actin dynamics (Brill et al., 1996; Hart et al., 1996; Kuroda et al., 1996; McCallum et al., 1996). IQGAP has been detected in a complex with F-actin and Cdc42, which is enhanced by epidermal growth factor (EGF) and disrupted by dominant negative Cdc42 (Erickson et al., 1997). IQGAP is able to oligomerize and to cross-link F-actin *in vitro*, an activity enhanced by GTP-bound active Cdc42 (Fukata et al., 1997). It has been suggested that IQGAP oligomers may form upon binding to GTPases after dissociation of calmodulin, and this facilitates cross-linking of F-actin. In addition, IQGAP also plays a role in the regulation of E-cadherin-mediated cell-cell adhesion (Kuroda et al., 1998). IQGAP was found to localize and interact with E-cadherin and β-catenin both *in vivo and in vitro*. Overexpression of IQGAP in mouse fibroblasts expressing E-cadherin resulted in a decrease in E-cadherin-mediated cell-cell adhesion (Kuroda et al., 1998)

The Cdc42 effector WASP is expressed in haematopoietic cells, and was originally isolated as the gene mutated in Wiskott-Aldrich Syndrome, a severe X-linked immunodeficiency disease associated with thrombocytopenia, eczema and recurrent infections (Derry et al., 1994). Five WASP family members exist in mammals: WASP, N-WASP, and the three WASP family verprolin homologous (WAVE) proteins. WASP family proteins are characterized by the presence of three independent domains at the carboxy terminus: the verprolin homology (V), central (C), and acidic (A) regions.

Together, these domains form what is known as the VCA module that is necessary and sufficient to activate Arp2/3-dependent actin polymerization *in vitro*. The V and A regions bind actin monomers and Arp2/3, respectively. The C region also binds Arp2/3 and induces crucial changes in its tertiary and quaternary structure, thereby regulating its ability to induce actin polymerization (Panchal et al., 2003). In spite of these similarities, the five mammalian WASP family members can be divided into two categories based on primary sequence homology and biological function: the WASP-like proteins, including hematopoietic WASP and ubiquitous N-WASP, and the WAVE proteins. WASP and N-WASP contain a central GTPase binding domain (GBD/CRIB) and an NH_2-terminal WASP homology domain 1 (WH1), also called Ena-VASP homology domain 1 (EVH1) (Callebaut et al., 1998). By contrast, WAVE proteins lack these domains but harbor a highly specific WAVE homology domain (WHD), also known as a SCAR homology domain (SHD). This structural difference translates to significant variation in the activity and regulation of WASP family proteins. The primary difference in the activity of the WASP and the WAVE proteins can be explained by their specificity for small GTPases. WASP and N-WASP are direct, specific effectors of Cdc42 and are thought to mediate most of the cytoskeletal effects of active Cdc42. By contrast, WAVE proteins play a major role in Rac-induced actin dynamics. WASP and N-WASP bind directly to GTP-bound Cdc42, through their GBD, whereas WAVE proteins do not contain a GBD and bind to Rac indirectly (Caron, 2003; Pollard and Borisy, 2003).

Mammalian MRCKα and β (myotonic dystrophy kinase-related Cdc42-binding kinase) and the *Drosophila* homologue Genguis Khan (Gek) are Cdc42-specific effector proteins (Leung et al., 1998; Luo et al., 1997). MRCKs contain a PH domain and a ROK-like kinase domain which phosphorylates myosin light chain (MLC) on Ser19 which appears to affect actomyosin contractility. A kinase defective mutant of MRCKα inhibits Cdc42-induced filopodia, and overexpression of MRCKα synergizes with Cdc42 to induce large filopodia in cells (Leung et al., 1998).

Two Rac-binding proteins, partner of Rac1 (POR1) and 140 KDa specifically Rac1-associated protein (p140Sra-1), have been involved in Rac-mediated lamellipodia formation but their precise role in this process is not clear (Kobayashi et al., 1998; Van Aelst et al., 1996). A truncated mutant of POR1 was shown to interfere with membrane

ruffling activity (Van Aelst et al., 1996). It was reported that p140Sra-1 localizes to lamellipodia induced by activated Rac and could be co-sedimented with filamentous actin, suggesting a direct interaction between p140Sra-1 and actin at the periphery of the cells (Kobayashi et al., 1998).

1.2.8 Cross-talk between Rho and and Ras families

Over a decade ago, it was reported that in Swiss 3T3 fibroblasts, oncogenic Ras induced actin remodeling mainly by activating Rac-1 (Ridley and Hall, 1992a). Since then, considerable effort has been invested in attempting to elucidate the molecular mechanisms enabling Ras activation of Rac. It was reported that induction of membrane ruffles by several growth factors was blocked by inhibitors of the Ras effector PI 3-Kinase (Nobes et al., 1995). The products of this lipid kinase include PIP3 and PIP2, which are necessary for the activation of a number of Rac specific GEF proteins. In addition, Ras can activate Rac in a PI 3-Kinase independent manner. The Rac specific GEF Tiam-1 contains a Ras binding domain (RBD), similar to the one in Raf. GTP-bound active Ras binds to this domain and stimulates Tiam-1 GEF activity (Lambert et al., 2002). Another protein with the potential to connect Ras and Rho signaling pathways is p120RasGAP which forms a complex in cells with p190RhoGAP. It has been suggested that p120RasGAP can potentiate the ability of p190RhoGAP to act as a downregulator of Rho (McGlade et al., 1993). Furthermore, it was reported that oncogenic Ras activates RhoA through the Raf/Mek/Erk pathway by decreasing the cytosolic activity and changing the subcellular localization of p190RhoGAP (Chen et al., 2003). In addition, it was shown that ERK1/2 can phosphorylate and enhance myosin light chain kinase (MLCK) activity which then phosphorylates myosin light chains (MLC) and induces changes in actin structures (Klemke et al., 1997). Moreover, ERK 1/2 phosphorylates the Rac effector Wave, and treatment of cells with pharmacological inhibitors against the MEK enzyme reduced the intensity of PDGF-induced membrane ruffling (Miki et al., 1999). The Cdc42/Rac effector PAK has emerged as a key molecule that can link Rac and Ras signaling by converging on the MEK-ERK pathway. It has been well established that expression of constitutively active Rac or Cdc42 activates the Jun N-terminal kinases (JNKs) and p38 MAPKs but not the ERKs (Coso et al., 1995; Minden et al., 1995).

However, dominant-negative Rac can block Ras-mediated ERK activation (Frost, 1996). It was also reported that Rac and Cdc42 can synergize with Raf to promote activation of the ERKs mainly through PAK1 phosphorylation of MEK1 and PAK3 phosphorylation of Raf-1 (Frost et al., 1997; King et al., 1998). PAK1 promotes the phosphorylation of T292 and S298 of MEK1 *in vivo* (Coles et al., 2002; Frost et al., 1997) and mutation of these sites to non phosphorylable residues inhibits the association of MEK1 with Raf-1 (Frost et al., 1997). PAK3 can phosphorylate Raf-1 on S338, enhancing Raf-1 activation (King et al., 1998). Also, it appears that Rac signaling regulates the association of MEK1 and ERK2 as well as the activation of ERK2 during cellular adhesion in a PAK-dependent manner (Eblen et al., 2002).

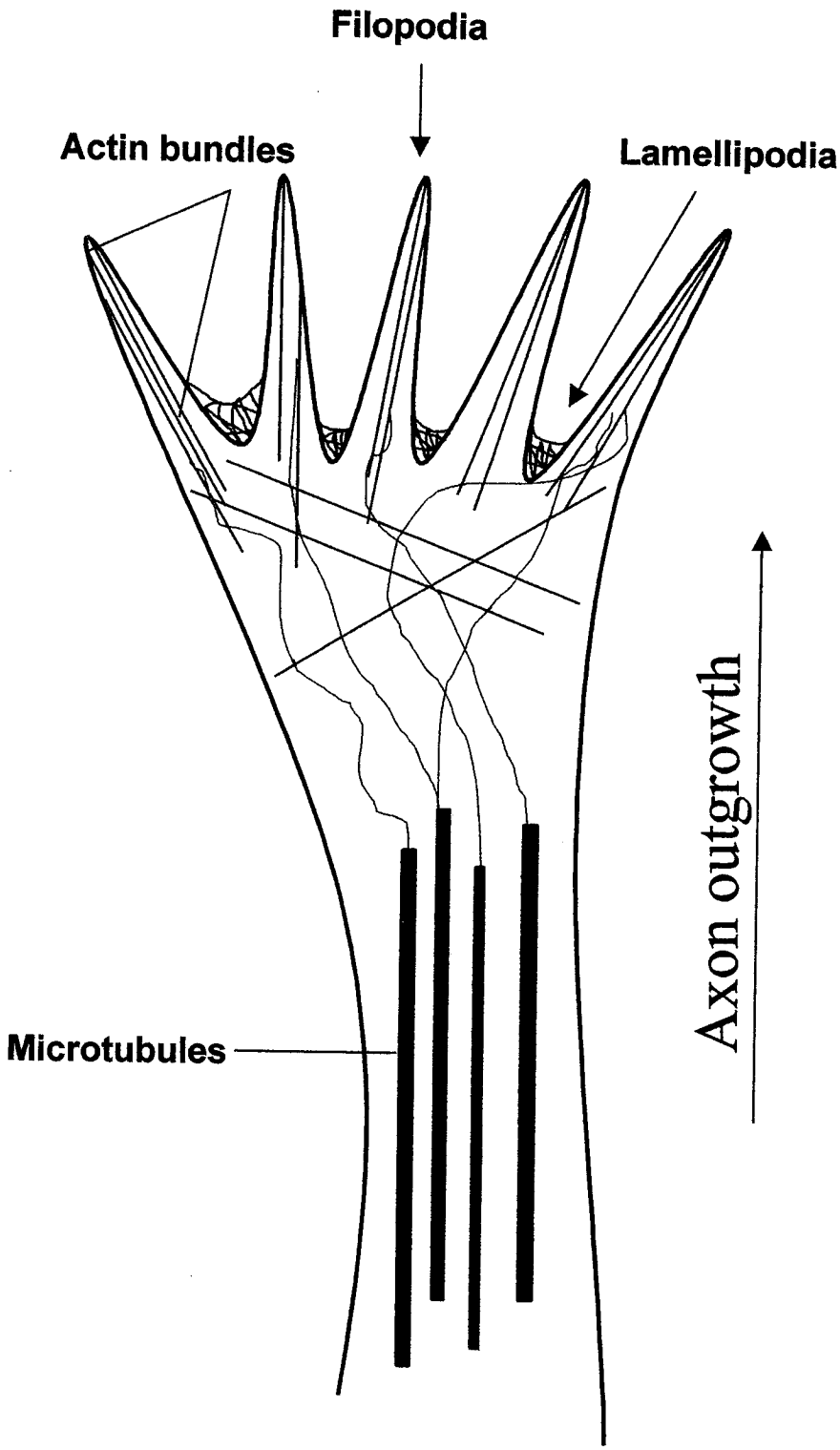
1.3 RHO GTPASES AND AXON GUIDANCE

1.3.1 The neuronal growth cone

During neural development, many neurons must project axons over relatively long distances in order to reach their final targets and make appropriate synaptic connections. Along the way, growth cones located at the leading edges of axons detect and respond to environmental cues that guide them to their final destination. These guidance cues include contact-mediated or secreted molecules, acting over short or long distances, respectively. It has been established that many individual guidance cues can function both as repellents and/or attractants (Tessier-Lavigne and Goodman, 1996). Growing axons must sense and respond to a number of axon guidance cues, and the mechanisms involved in the integration of information provided by guidance cues are now being unraveled. Like other cell types, the growth cone contains different actin structures such as lamellipodia and filopodia which probe the extracellular environment (Fig. 1.6). This peripheral actin network is associated in the proximal portion of the growth cone with microtubules located in the distal region of the axon shaft and contributes to the assembly and translocation of microtubules into more proximal regions of the growth cone (Mallavarapu and Mitchison, 1999; Mitchison and Kirschner, 1988; Suter and Forscher, 2000). In principle, extracellular guidance cues instruct the growth cone to advance, retract, or turn by regulating the actin cytoskeleton within the growth cone.

Figure. 1.6. Architectlure of the neuronal growth cone

The axon is an arm-like structure which contains a hand-like structure, the growth cone at its tip. The microtubules are mainly found in the arm and palm whereas actin structures like lamellipodia are located around the palm and filopodia compose the fingers.


Filopodia
Actin bundles
Lamellipodia
Axon outgrowth
Microtubules

1.3.2.RhoGTPases and neuronal morphogenesis

Given that Rho GTPases are key regulators of cell morphology and cell migration, it is not suprising that they have also been implicated in the regulation of neuronal morphogenesis, including migration, polarity, axon growth and guidance, dendrite elaboration and plasticity, and synapse formation (Govek et al., 2005). Rac1 and Cdc42 in particular, have been implicated in the regulation of axon outgrowth, guidance, and branching (Govek et al., 2005). It has been proposed that activation of Rac or Cdc42 by attractive guidance cues induce filopodia and lamellipodia formation leading to growth cone extension, whereas activation of Rho by repulsive cues induce filopodia and lamellipodia retraction leading to growth cone collapse (Mueller, 1999). At least four families of guidance cues were identified that provide directional information to growing axons: the ephrins, the netrins, the slit, and the semaphorins. For the purpose of this thesis, only the netrin family and its related signaling mechanisms will be described.

1.3.3. Netrins

1.3.3.1. The netrin family

For more than a century, developmental neurobiologists searched for the chemoattractive molecules that Ramón y Cajal described as fundamental axonal guidance mechanisms (Cajal, 1890). The first such molecules were identified in chick embryo through biochemical purification of growth-promoting factors for commissural axons in the spinal cord (Kennedy et al., 1994; Serafini et al., 1994). These were named Netrins ("one who guides" in Sanskrit). The netrins are a small family of phylogenetically conserved secreted proteins with amino acid sequence similarity to proteins of the laminin family. The first netrin cloned, UNC-6, was identified using a genetic screen for mutations affecting axon guidance in *C.elegans* (Ishii et al., 1992). So far, four members of the netrin gene family have been identified in mammals: netrin-1, netrin-3, netrin-G1 and netrin-4, also known as β-netrin (Koch, 2000; Nakashiba et al., 2000; Serafini, 1996; Wang et al., 1999a; Yin et al., 2000). Extensive characterization of netrin function in the worm, the fly and vertebrates has revealed the ability of netrins to function as both neuronal chemoattractants and repellents (Chisholm and Tessier-Lavigne, 1999). An example of netrin bifunctionality is observed in vertebrates, where netrin-1 functions *in*

vitro and *in vivo* to attract spinal cord commissural neuron axons to the floor plate and *in vitro* to repel trochlear motor axons (Kennedy et al., 1994; Serafini, 1996).

1.3.3.2. Phenotype of netrin-1 deficient mice

The generation of hypomorphic allele in mice that almost completely abolish netrin-1 expression has provided insight into the function of netrin-1 *in vivo* (Serafini, 1996). In these homozygous embryos, the corpus callosum, which joins the left and right cerebral cortices and the hippocampal commissure, which joins the left and right hippocampi are completely absent. In addition, the mice have severe defects in commissural axons. For example, many commissural axons are shortened and fail to invade the ventral part of the spinal cord. Moreover, some axons are misrouted and only a few reach the floor plate. The observation that some commissural axons do reach the floor plate raised the possibility that additional attractive cues were expressed by the floor plate. Recently, it was shown that the morphogen Sonic hedgehog (Shh) can mimic the additional chemoattractant activity of the floor plate *in vitro* and can act directly as a chemoattractant on isolated axons (Charron et al., 2003).

1.3.4. Netrin receptors

Members of the DCC (Deleted in colorectal cancer) family of netrin receptors, which include *C. elegans* UNC-40, *Drosophila* Frazzled, and vertebrate DCC have been shown to mediate netrin attraction (Chan et al., 1996; Keino-Masu et al., 1996b; Kolodziej, 1996). DCC proteins have large extracellular domains composed of five immunoglobulin (Ig) repeats, four fibronectin type III (FNIII) repeats, a small transmembrane region and a large cytoplasmic domain with three conserved motifs (P1, P2, and P3). Ligand-mediated multimerization of DCC proteins through their P3 regions is required for netrin-induced attraction events (Stein and Tessier-Lavigne, 2001). Studies of UNC-6-mediated repulsion in *C. elegans* show that some, but not all, repulsive events require both the DCC family member UNC-40 and the UNC-5 protein, and they suggest that different regions of UNC-6 are responsible for functional associations with these two receptors (Chan et al., 1996; Lim and Wadsworth, 2002; Merz et al., 2001; Wadsworth et al., 1996). The UNC-5 receptor family includes *C. elegans* UNC-5, *Drosophila* Dunc5,

and the vertebrate proteins UNC5H1, UNC5H2, and UNC5H3 (Ackerman, 1997; Keleman and Dickson, 2001; Leonardo, 1997; Leung-Hagesteijn et al., 1992). UNC5 proteins are also Ig super family members; however, they are structurally quite distinct from DCC proteins. Studies of netrin-mediated repulsion of vertebrate neurons demonstrated a requirement for direct association between the DCC and UNC-5 cytoplasmic domains (Hong et al., 1999). This association is ligand-dependent and is required for netrin-mediated long-range repulsion. There is current debate on the possibility that the adenosine A2b receptor may also act as a co-receptor for netrins. Some studies suggest that the A2b receptor is required for netrin-1-promoted outgrowth of dorsal spinal cord neurons and for netrin-1-induced growth cone attraction of Xenopus retinal ganglion cells (Corset et al., 2000; Shewan et al., 2002). However, these results have been challenged by studies showing that A2b receptor activation is not required for netrin attraction of commissural axons and that the A2b receptor is not expressed on commissural axons (Stein, 2001).

1.3.5. Phonotype of DCC deficient mice

Mice lacking DCC function (Fazeli, 1997) exhibit a phenotype relatively similar to that of netrin-1 deficient mice, including severe defects in the formation of the anterior commissure, absence of corpus callosum and pontine nuclei, reduction in size of the ventral spinal commissure, and errors in axonal pathfinding of commissural neurons in the spinal cord. Although the overall similarity in phenotypes in the *DCC* and *netrin-1* knockout mice is striking, spinal commissural axons appear more shortened in the dorsal spinal cord in *DCC* knockout mice. This difference could be due to the possibility that the studied *netrin-1* allele was not a complete null allele, and that some residual netrin-1 function was present in those animals. An alternative possibility is that DCC is required not only to mediate responses to netrin-1 but also to mediate the responses of commissural axons to other cues that collaborate with netrin-1 to guide these axons. Given that some commissural axons do reach the floor plate in the *DCC* knockout mice suggests the existence of a DCC-independent mechanism for guidance of commissural axons to the floor plate. In fact, evidence has been provided that Shh guides commissural

axons toward the floor plate through a DCC-independent mechanism (Charron et al., 2003)

1.3.6. Netrin-1 signaling

Recent studies have provided substantial information on the mechanisms by which netrin-DCC signaling can control the growth cone cytoskeleton. Ectopic expression of DCC in HEK 293T cells, or a neuroblastoma cell line, allows for netrin-induced increases in filopodial number and cell-surface area effects, which are blocked by dominant-negative forms of Cdc42 and Rac1, respectively (Shekarabi and Kennedy, 2002). Netrin-1 can activate Rac1 and Cdc42 in DCC-expressing cells and can also induce Rac-dependent actin reorganization in Swiss 3T3 fibroblasts expressing DCC (Li et al., 2002b; Shekarabi and Kennedy, 2002). The exact mechanism by which netrin modulates the activity of Rho GTPases is unknown but may involve the adapter protein Nck, which associates with DCC in commissural neurons (Li et al., 2002a). Moreover, a Nck protein lacking a functional SH2 domain acts in a dominant-negative fashion to prevent netrin-induced neurite extension in neuroblastoma cells and Rac1 activation in Swiss 3T3 fibroblasts (Li et al., 2002a). Thus, Nck may couple DCC signals to Rho GTPases.

1.3.7. Modulation of Netrin-1 signaling

One potential mechanism for modulating netrin signaling involves regulation of levels of cyclic nucleotides within the growth cones. Using the growth cone turning assay, it was observed that decreased cAMP levels convert DCC-mediated netrin attraction into repulsion, whereas increased cAMP levels resulted in attraction (Ming et al., 1997). Furthermore, multiple *in vitro* studies demonstrate that protein kinase A (PKA) activation modulates netrin signaling and that treatments affecting PKA activity can modulate netrin guidance (Ming et al., 2001; Ming et al., 1997). Moreover, it was reported that PKA potentiates netrin-1-dependent insertion of DCC into the neuronal plasma membrane and enhances axon outgrowth in response to netrin-1 (Bouchard et al., 2004). It is possible that netrin-1 may directly influence cAMP production through its association with both DCC and the adenosine A2b receptor. In fact, netrin-1 induces an increase in cAMP levels in HEK 293 cells transfected with the A2b receptor (Corset et

al., 2000), which suggests that this receptor might directly link netrin signaling to elevation of cAMP levels. While the role of A2b receptor in netrin-mediated attraction is controversial, it is nonetheless clear that regulation of cyclic nucleotide levels could be a convenient mechanism by which various extacellular cues might modulate netrin-mediated responses. Furthermore, it was reported that cyclic nucleotide signaling directly modulates the activity of L-type Ca2+ channels (LCCs) in axonal growth cones and alter the intracellular level of calcium (Nashiyama et al., 2003), which has been shown to be an important regulator of netrin-1-mediated attraction or repulsion (Hong et al., 2000). Recently, calcium-calmodulin-dependent protein kinase II (CaMKII) calcineurin (CaN) and phosphatase-1 (PP1) were reported to be implicated in the control of Ca^{2+} -dependent axonal guidance by netrin-1 (Wen et al., 2004).

Moreover, localized protein synthesis within the neuronal growth cone seems to be required for netrin-1-induced attraction and repulsion (Campbell and Holt, 2001). Netrin-1 stimulates phosphorylation of the translation initiation factor eIF4E, protein translation, and protein degradation in growth cones that have been separated from their cell bodies. Several translation initiation factors, such as eIF4E, are regulated by the mitogen-activated protein kinase (MAPK) signaling pathway. Therefore, the observation that extracellular signal-regulated kinase (ERK)-1/2 is recruited to DCC following netrin-1 stimulation and required for netrin-mediated attraction supports the idea that regulation of protein translation by MAPK signaling is crucial for growth cone guidance (Forcet et al., 2002). It was also reported that PI3-kinase and PLC-γ-dependent pathways may be involved in the turning responses induced by netrin-1 (Ming et al., 1999).

1.3.8. DCC and tumorigenesis

The gene encoding the receptor DCC was originally isolated as a candidate tumour-suppressor gene on human chromosome 18q21, one allele of DCC being frequently deleted in colorectal cancer (Fearon et al., 1990). Subsequently, many studies challenged the candidacy of DCC as a tumour-suppressor gene because of its low mutation frequency in cancer and the absence of a cancer-predisposition phenotype in *Dcc*-heterozygous mice (Fazeli, 1997), and the existence of other tumour-suppressor genes (*Smad* genes) at the 18q21 locus. Moreover, DCC was shown to be a netrin

receptor, thereby implicating it in axon guidance in neural tissues. However, several lines of evidence have previously indicated that DCC can function as a tumour suppressor (Arakawa, 2004). For instance, introduction of an intact copy of chromosome 18 into a colorectal cancer cell line lacking endogenous DCC expression yielded detectable levels of *DCC* transcripts, resulting in the suppression of growth in soft agar and the suppression of tumorigenicity in nude mice (Tanaka et al., 1991). Also, inhibition of DCC expression in normal rat cells by antisense RNA resulted in anchorage-independent growth of the cells *in vitro* and tumour formation in nude mice (Narayanan et al., 1992). Conversely, enforced expression of DCC in a tumorigenic keratinocyte cell line lacking endogenous DCC expression was shown to suppress tumorigenic growth of the cells in nude mice (Klingelhutz et al., 1995). These observations are consistent with the idea that *DCC* is a tumour-suppressor gene whose loss through deletion of chromosome 18q21.1 is functionally relevant. In addition, it was reported that DCC functions as a tumor suppressor by acting as a pro-apoptotic dependent receptor (Mehlen and Mazelin, 2003). Such receptors, which also include RET (rearranged during transfection), β-integrins, Patched and the p75 neurotrophin receptor (p75NTR), share the functional property of inducing cell death when disengaged from their ligands but not when bound by their ligands. These receptors thus create cellular states of dependence on their respective ligands. In the absence of netrin-1, DCC promotes apoptosis through activation of a novel pro-apoptotic caspase-dependent pathway (Forcet et al., 2001). UNC5H were also subsequently proposed to be dependence receptors, suggesting that netrin-1 may not only be a chemotropic factor for neurons but also a survival factor (Llambi et al., 2001).

RATIONALE AND OBJECTIVES

Rationale

RhoGTPases mediate a wide spectrum of biological effects in response to extracellular cues which interact with membrane receptors and modulate GAP or GEF activities. Many receptors and regulators involved in Rho GTPase signaling seem to be regulated through mechanisms involving phosphorylation. The biochemical regulation of CdGAP, a negative regulator of Cdc42 and Rac1 and the netrin-1 receptor DCC which is an upstream activator of these GTPases is poorly understood. Therefore, investigation of whether CdGAP and DCC are regulated through phosphorylation will enable us to better understand the molecular mechanisms that govern the activities of these proteins.

Objectives

There are three objectives in this thesis:

1. Understand the regulation of CdGAP at the biochemical level. Given that CdGAP contains a number of consensus phosphorylation sites for a variety of kinases and migrates higher than its predicted molecular weight on SDS-PAGE, we investigated whether CdGAP is phosphorylated *in vivo* and if CdGAP's activity is regulated through phosphorylation.

2. Characterize the GTPase specificity and biochemical regulation of the human ortholog of CdGAP.

3. Understand the biochemical events that link netrin-1 and DCC function to Rac activation in growth cone guidance. Given that a number of axon guidance receptors are regulated through phosphorylation, we investigated whether DCC was also a target for kinases.

PREFACE TO CHAPTER 2

A number of studies have established that RhoGAP proteins are regulated by a wide range of mechanisms including phosphorylation. Given that CdGAP contains many consensus phosphorylation sites for different kinases and that overexpressed CdGAP in cell lines migrates higher than its predicted molecular weight, we hypothesized that CdGAP's activity, localization, stability and interaction with other proteins could be modulated through phosphorylation. Using COS-7, Swiss 3T3 and HEK293 cells and classical biochemical approaches we investigated (a) the tissue distribution of CdGAP protein (b) whether overexpressed CdGAP is phosphorylated inside the cells, (c) which regions of CdGAP are phosphorylated (d) which kinases interact with and phosphorylate CdGAP (e) whether CdGAP phosphorylation is modulated by extracellular factors (f) the precise phosphorylation sites (g) whether mutation of these sites can effect CdGAP's activity.

CHAPTER 2

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

Extracellular signal-regulated kinase 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 (Burridge and Wennerberg, 2004; Van Aelst and D'Souza-Schorey, 1997). 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 (Cerione and Zheng, 1996); the GTPase-activating proteins (GAPs) which enhance the intrinsic GTPase activity, leading to the inactive state of the GTPase (Jenna and Lamarche-Vane, 2003); and the guanine nucleotide dissociation inhibitors (GDIs) which sequester Rho GTPases in their GDP-bound, inactive state (Olofsson, 1999).

Over 40 RhoGAP family members have been characterized in eucaryotes ranging from yeast to human (Moon and Zheng, 2003). Recent analysis of the human genome unraveled 66 different genes encoding potential RhoGAP domain-containing proteins, far outnumbering the existing 23 mammalian Rho GTPases (Bernards and Settleman, 2004; Peck et al., 2002). 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 (Jenna and Lamarche-Vane, 2003). 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 (Kozma, 1996) and TCGAP plays a direct role in insulin-stimulated glucose transport (Chiang et al., 2003). 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 (Ahmed et al., 1993; Jenna et al., 2002; Roof et al., 1998; Su et al., 2003).

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 (Lamarche-Vane, 1998). 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 (Jenna et al., 2002). 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.

EXPERIMENTAL PROCEDURES

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 (Jenna et al., 2002) 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 (Jenna et al., 2002). 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 (Jenna et al., 2002; Li et al., 2002b). 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 (Olson et al., 1995). 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-1 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 (Boyle et al., 1991). 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 (Li et al., 2002b).

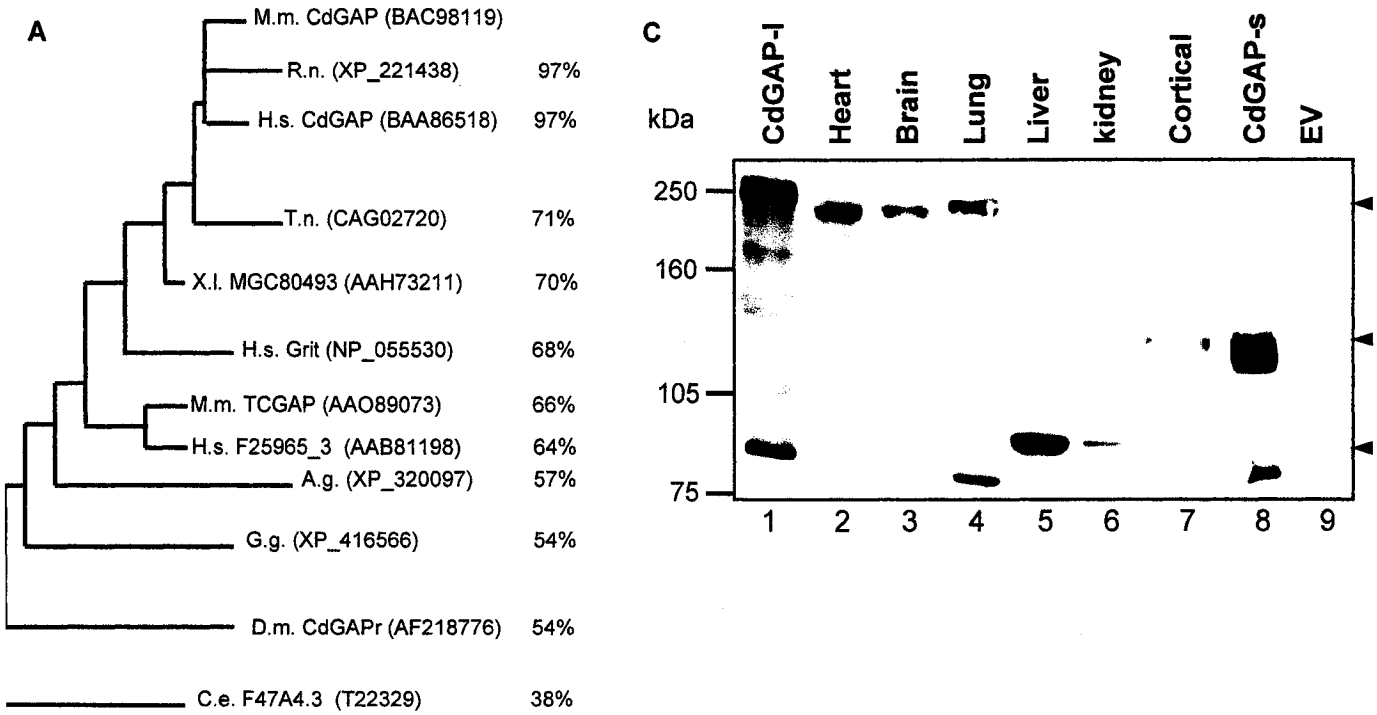
RESULTS

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* (Sagnier et al., 2000), 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) (Moon et al., 2003; Nakamura et al., 2002; Okabe et al., 2003; Taniguchi et al., 2003; Zhao et al., 2003) 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 (Chiang et al., 2003). 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™ 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, 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

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

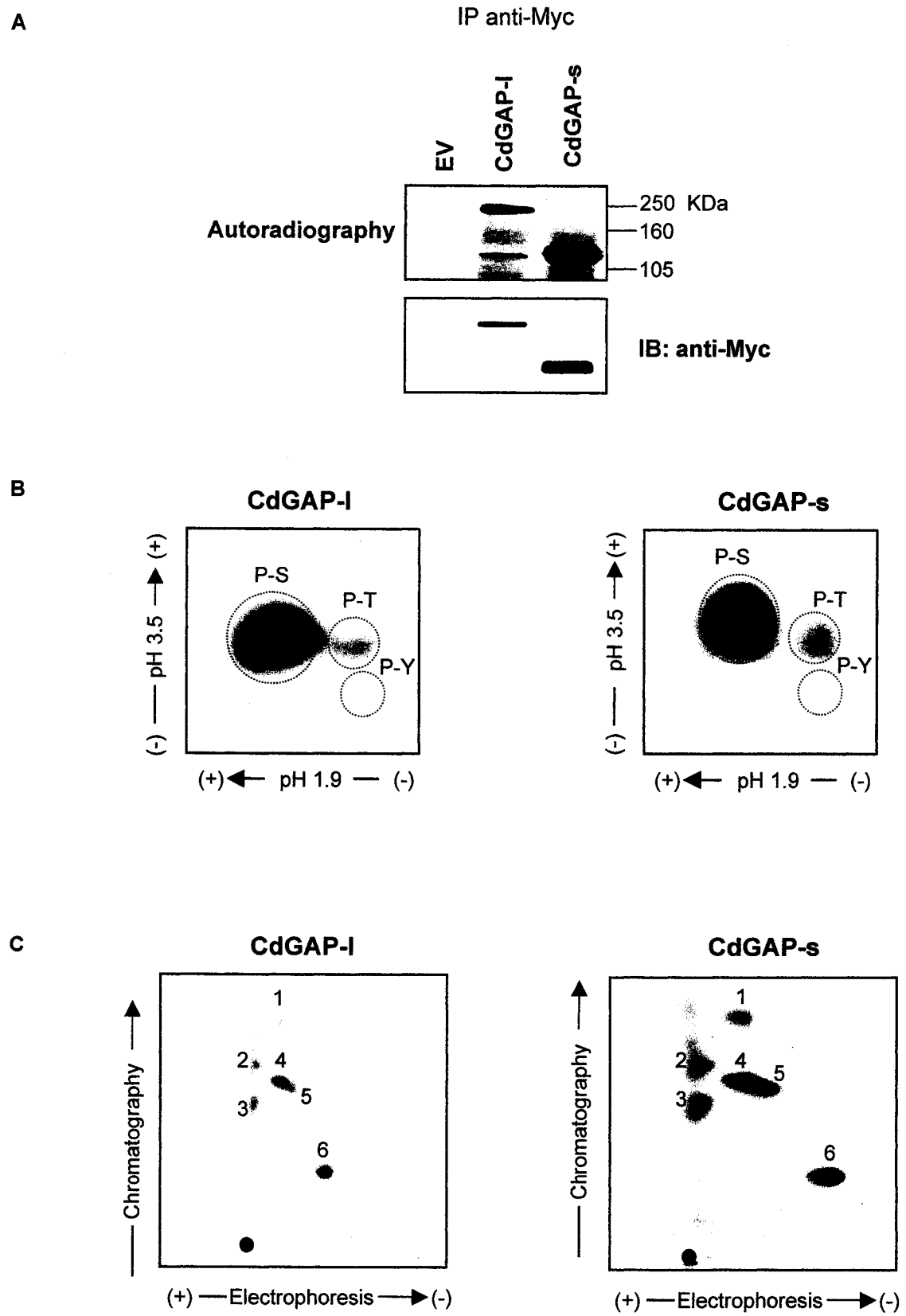
(Fig. 2.1C). These bands were not recognized in western-blots using preimmune sera (data not shown). The band of 250 kDa, which migrates at the same level as overexpressed CdGAP-l (lane 1), is present in the heart, brain and lung tissues (lanes 2, 3 and 4). The 90 kDa band which corresponds to the expected 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 mouse tissues contain at least three CdGAP proteins resulting from alternative splicing or post-tanslational modifications.

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.

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 → (-)

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 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 other groups using different proteins as substrates (Douville and Downward, 1997; Moor

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.

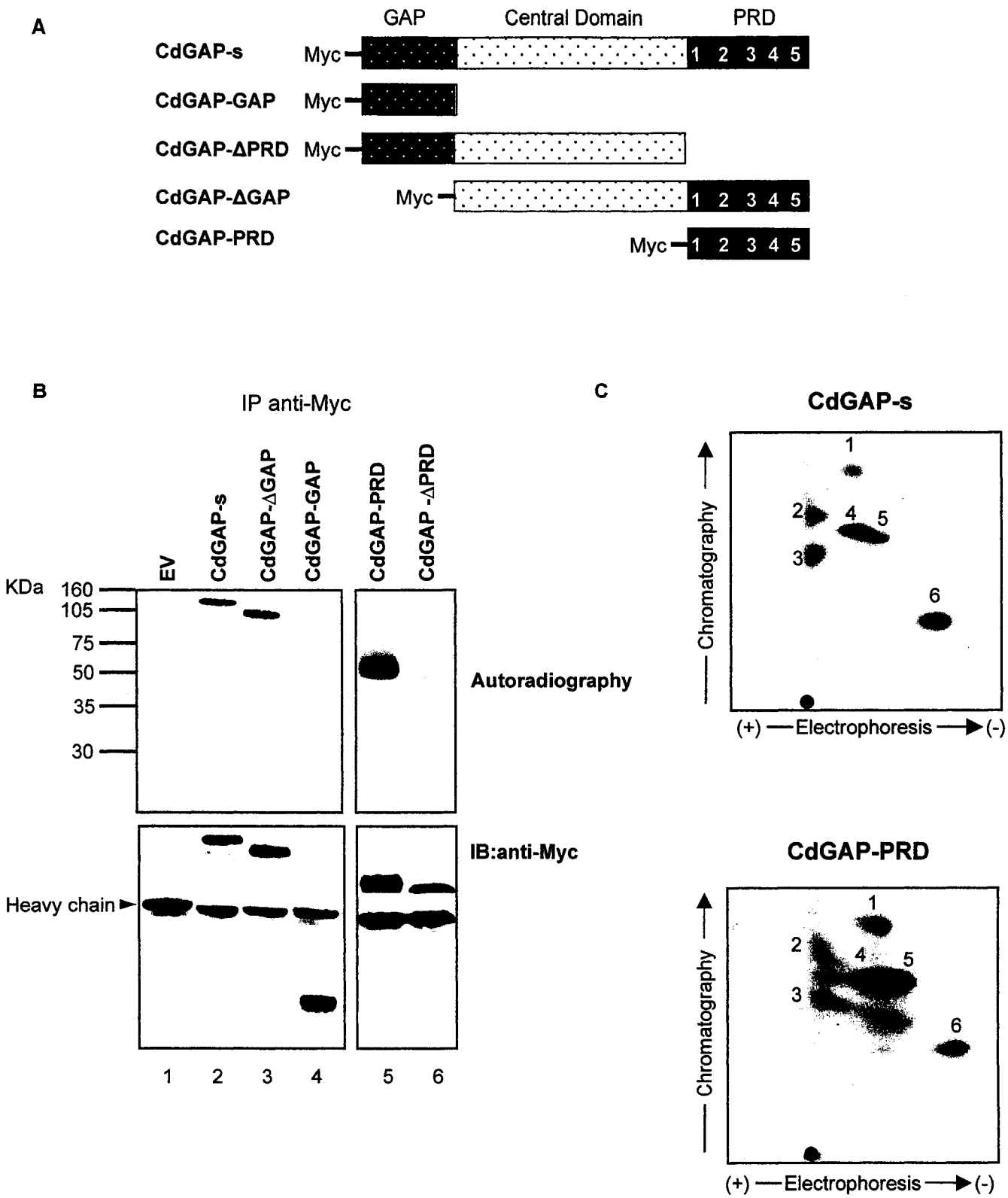
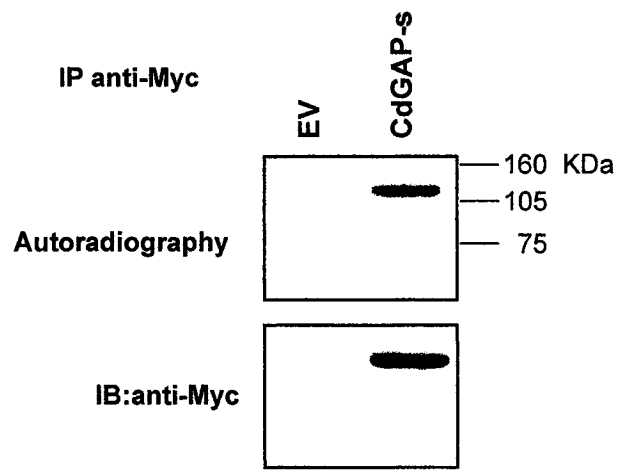

A
GAP
Central Domain
PRD
CdGAP-s
Myc
1 2 3 4 5
CdGAP-GAP
Myc
CdGAP-ΔPRD
Myc
CdGAP-ΔGAP
Myc
1 2 3 4 5
CdGAP-PRD
Myc
1 2 3 4 5
B
IP anti-Myc
EV
CdGAP-s
CdGAP-ΔGAP
CdGAP-GAP
CdGAP-PRD
CdGAP -ΔPRD
KDa
160
105
75
50
35
30
Autoradiography
IB:anti-Myc
Heavy chain
1 2 3 4 5 6
C
CdGAP-s
Chromatography
1
2
3
4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

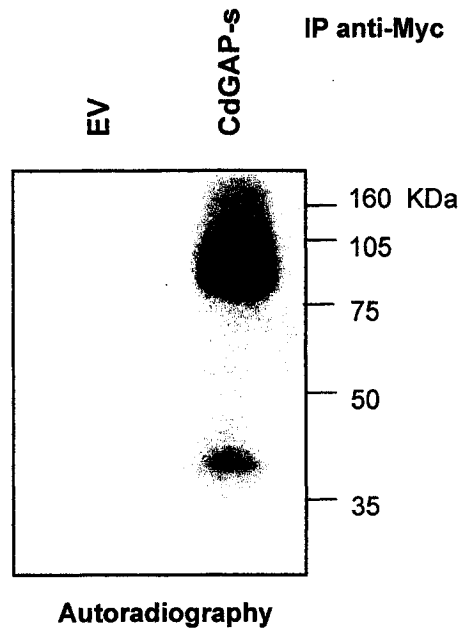
Figure 2.4. ERK1/2 and RSK interact with CdGAP-s

(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).

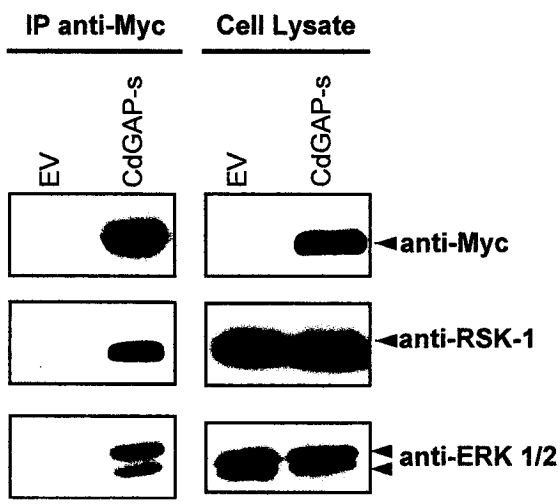
A


IP anti-Myc
EV
CdGAP-s
160 KDa
105
75
Autoradiography
IB:anti-Myc

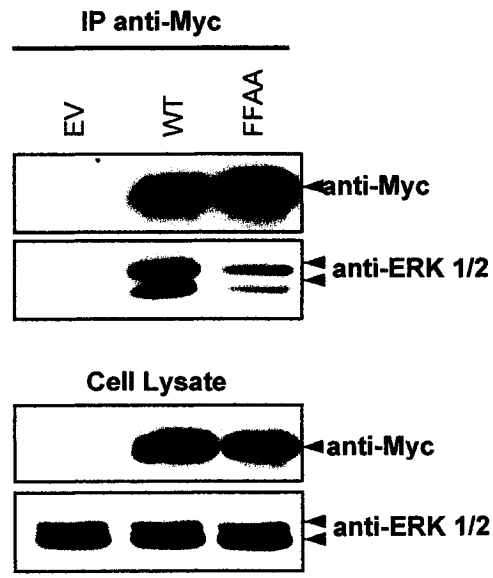
B


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

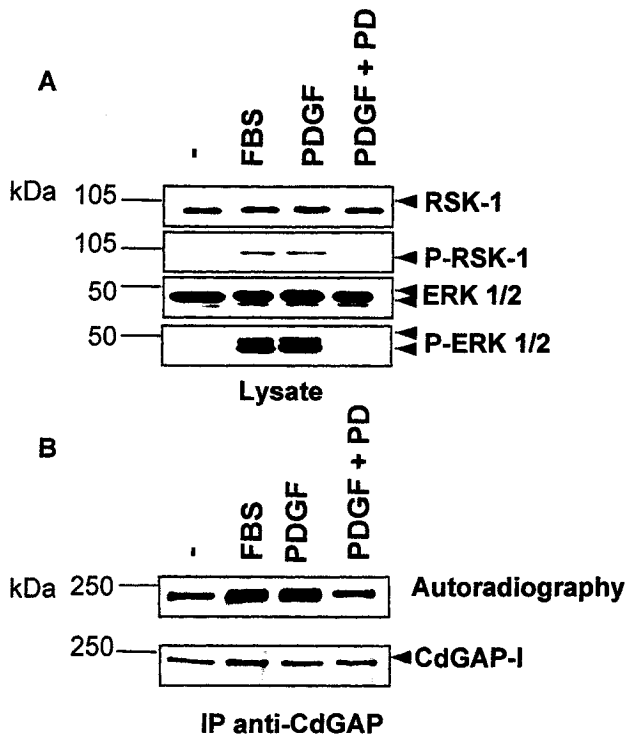
and Fliegel, 1999). In both cases, they showed that the 40 kDa kinases represented p42 ERK-2 and p44 ERK-1 whereas the 90 kDa kinase appeared to be p90 RSK-1. We have also investigated this possibility since the protein sequence of CdGAP contains a significant number of ERK1/2 and RSK-1 consensus phosphorylation sites PX(S/T)P and RXXS, respectively (Fig. 2.6A). As shown in Fig. 2.4C, both ERK 1/2 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.

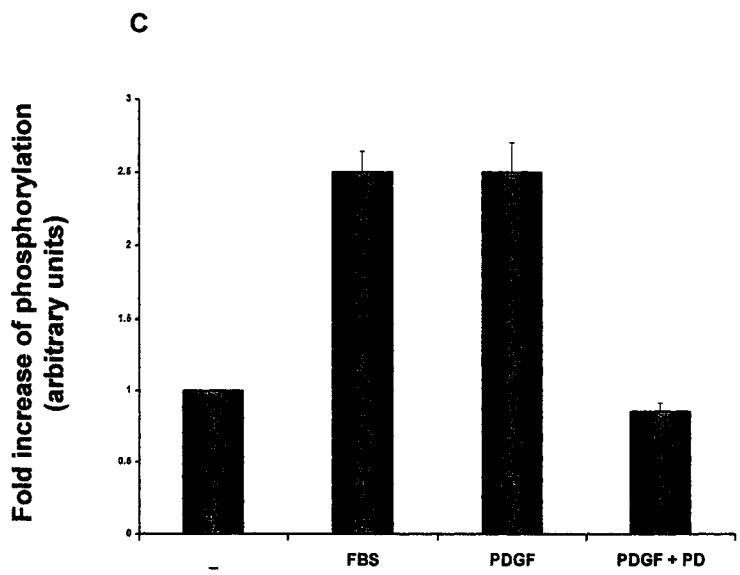
CdGAP-l is phosphorylated *in vivo* in response to serum and PDGF-stimulation via the MAPK pathway

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

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

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 (Erikson, 1991). The amino acid sequence of CdGAP contains three consensus phosphorylation motifs for ERK1/2, located within the proline-rich sequence 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*

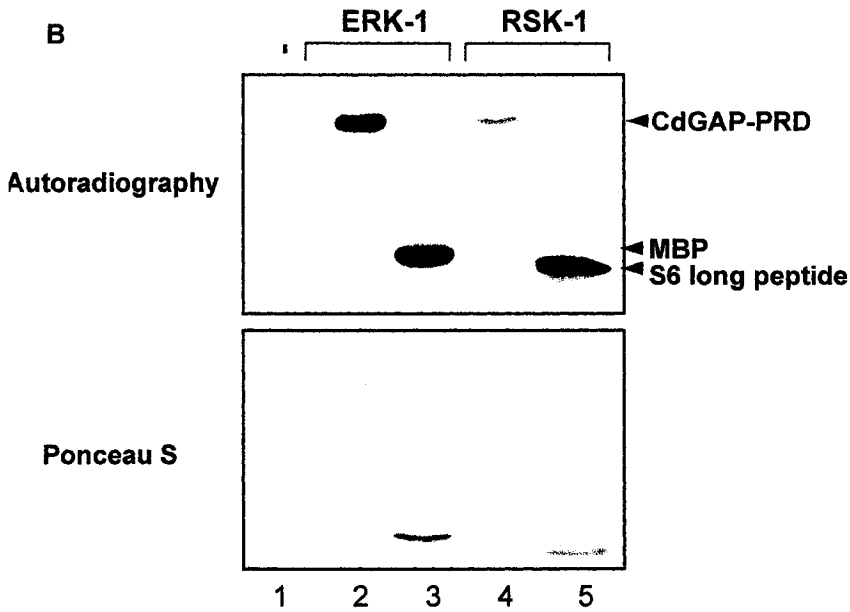
To identify the phosphorylation sites for ERK-1, each threonine of the three potential ERK-1 phosphorylation sites of CdGAP was substituted for an alanine residue. As shown in Fig. 2.7A, CdGAP-PRD containing the T612A amino acid substitution is

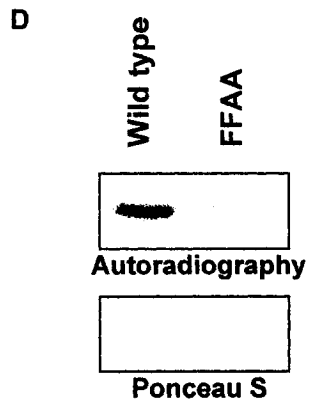
Figure 2.6 RSK-1 and ERK-1 phosphorylate CdGAP *in vitro*

(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





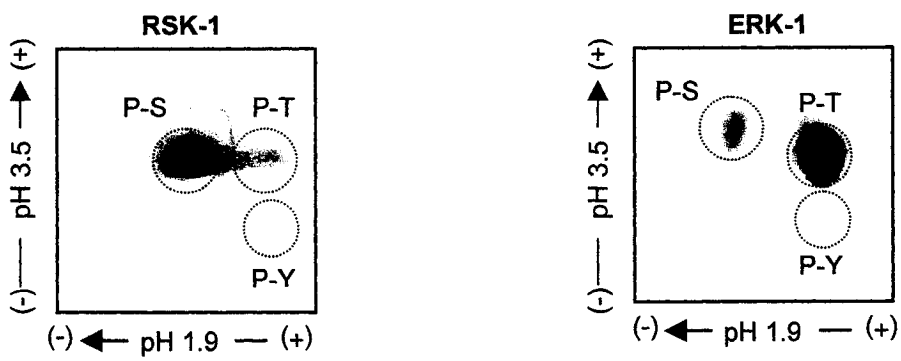
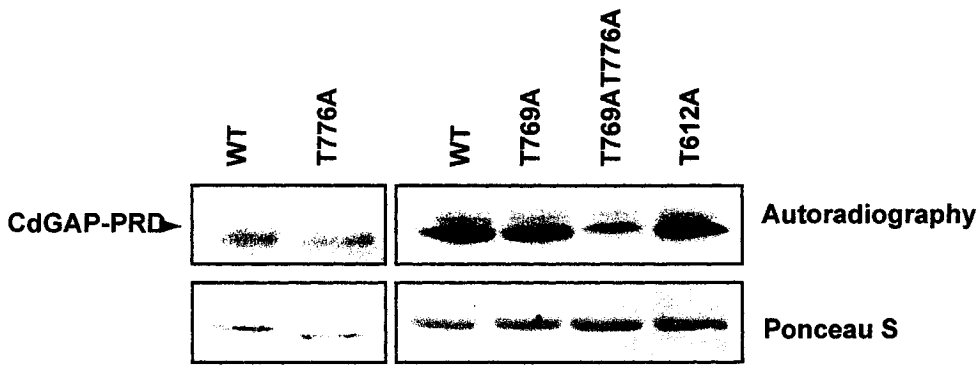


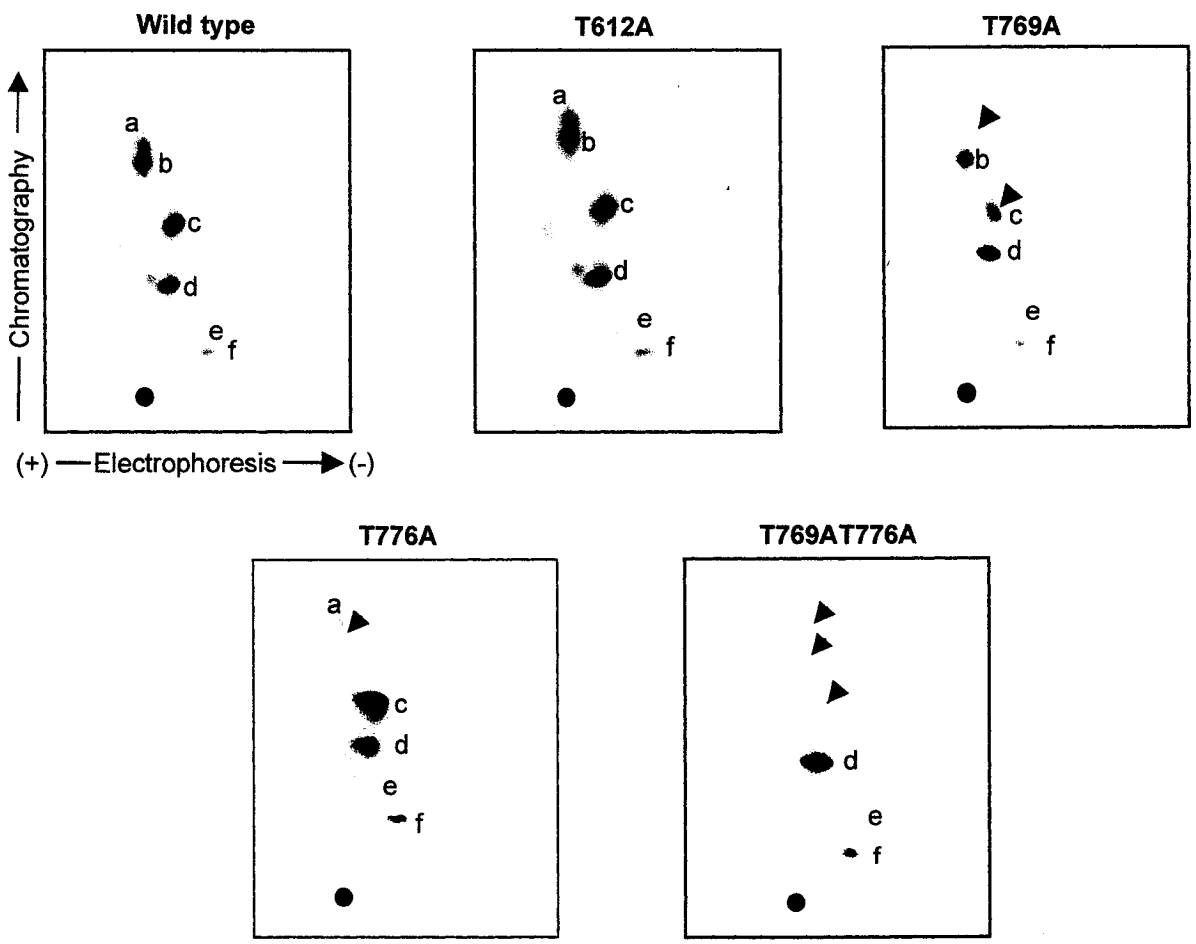
Figure 2.7 Thr^{769} and Thr^{776} in the proline-rich domain of CdGAP are phosphorylated *in vitro* by ERK-1

(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

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). 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. 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*.

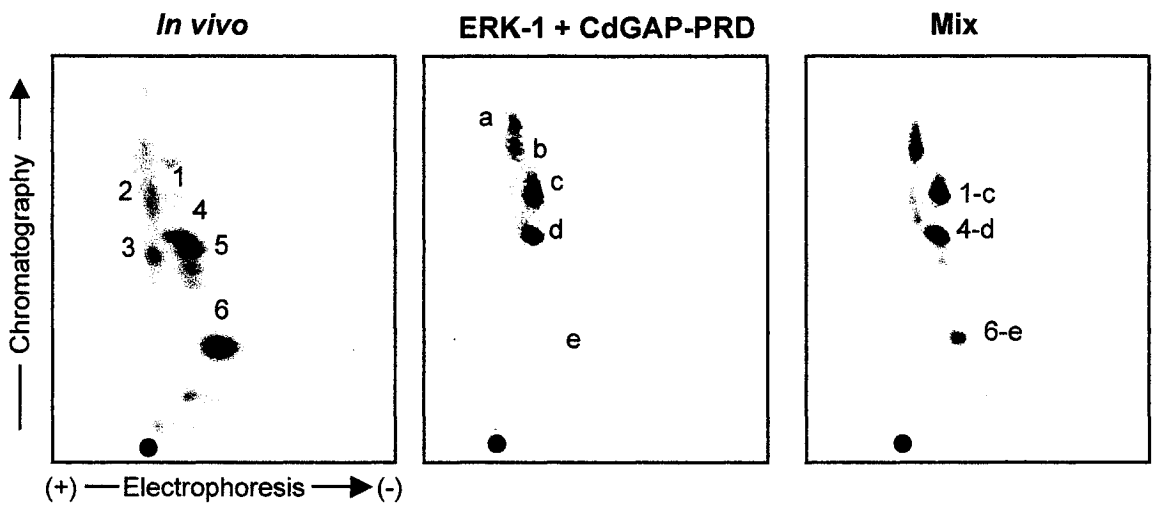
Thr^{776} is an *in vivo* phosphorylation site of CdGAP-s

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* 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*.

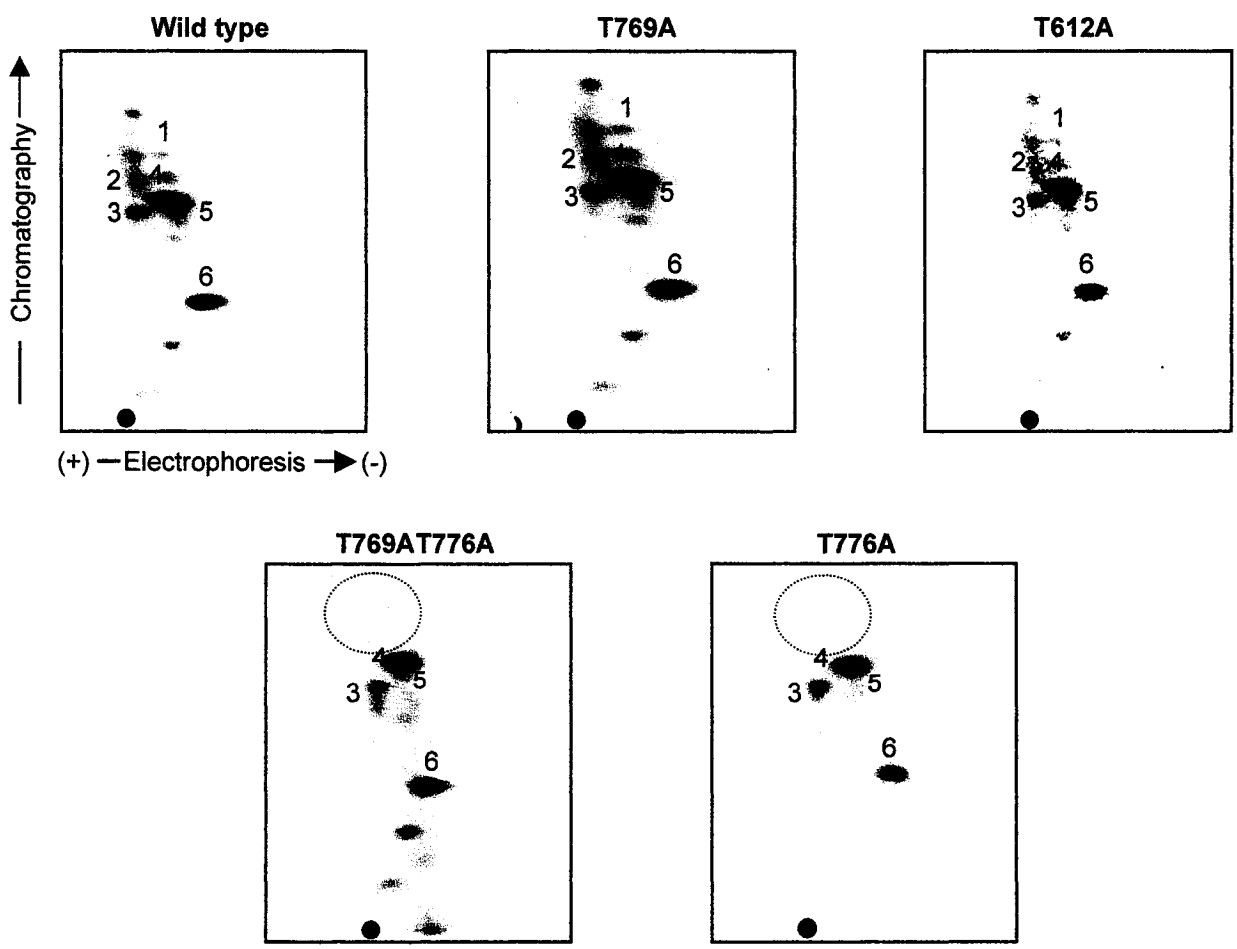
Figure 2.8 Thr^{776} is an *in vivo* phosphorylation site of CdGAP

(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

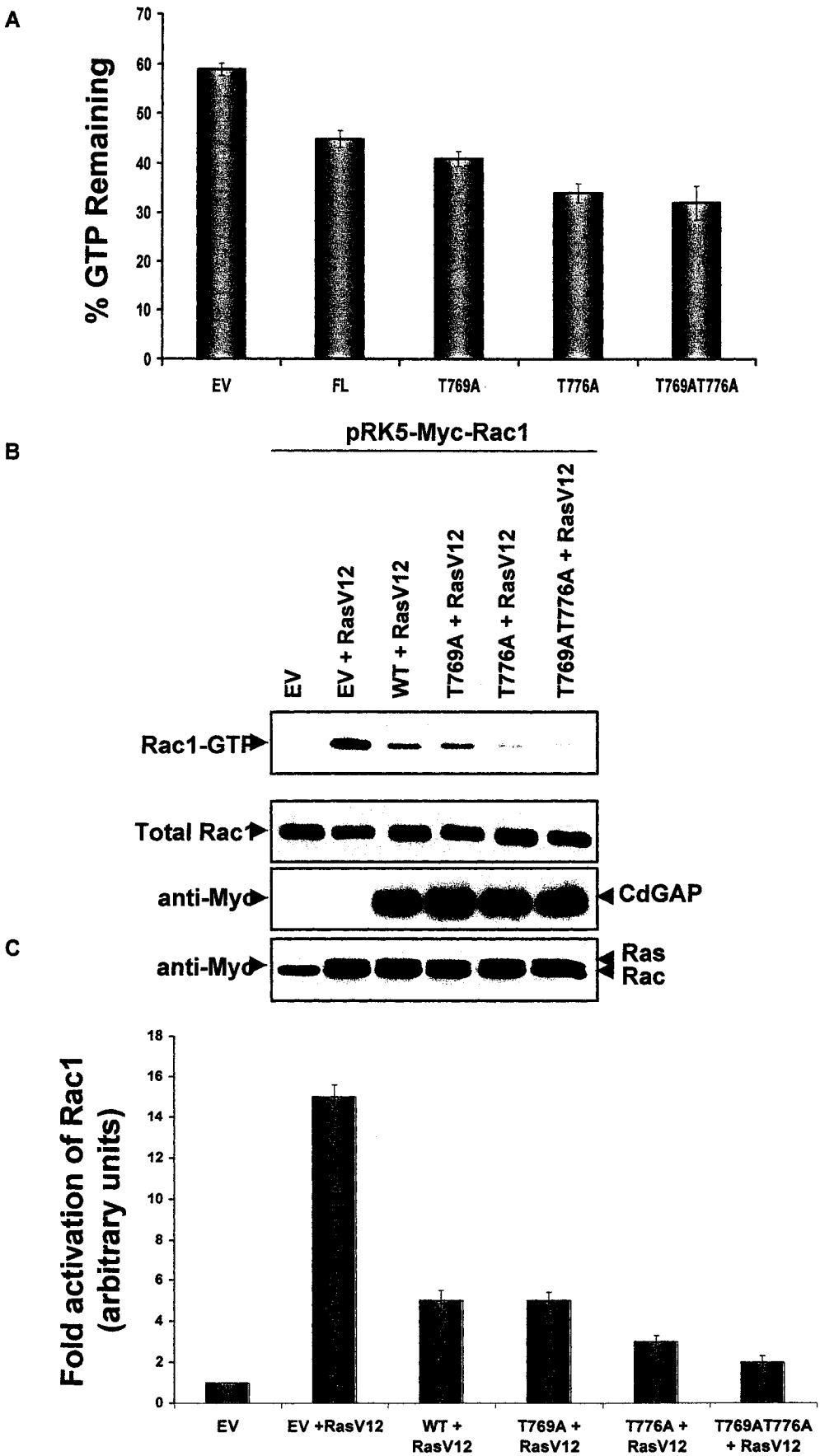

Wild type
T769A
T612A
T769AT776A
T776A
Chromatography
(+) Electrophoresis (-)
1
2
3
4
5
6

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 (Jenna et al., 2002). 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 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.

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

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* (Hu and Settleman, 1997; Roof et al., 1998). 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 (Okabe et al., 2003). 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 (Minoshima et al., 2003). 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 (Corbalan-Garcia et al., 1996).

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 (Douville and Downward, 1997; Moor and Fliegel, 1999). 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 (Gonzalez et al., 1991). 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 (Songyang et al., 1996). Interestingly, two of these sites are adjacent to a putative DEF domain containing the FPFP motif known to be an ERK docking site (Jacobs et al., 1999). Indeed, mutation of this motif alters CdGAP binding to ERK1/2 and leads to a loss of CdGAP phosphorylation by ERK-1 *in vitro*.

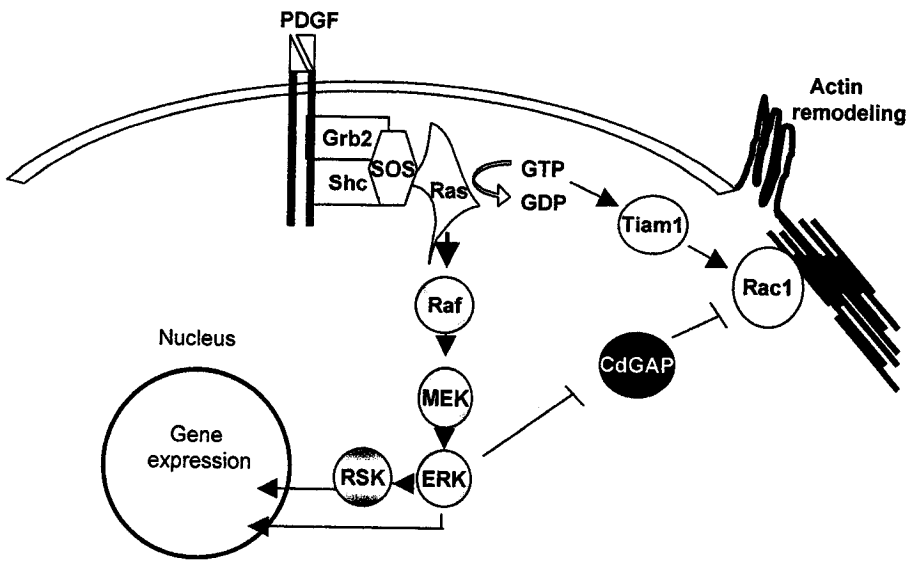
CdGAP belongs to a novel family of RhoGAP proteins that are phylogenetically well conserved among different species. Up to three human genes encode for CdGAP-related proteins which consist of a rhoGAP domain at the N-terminus and multiple SH3-binding motifs at the C-terminus of the proteins. Interestingly, 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-l (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 (Moran et al., 1991). 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 (Klemke et al., 1997; Miki et al., 1999; Mitsushima et al., 2004; Woo et al., 2004). 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

PREFACE TO CHAPTER 3

Although RhoGAP orthologs in human and mouse are usually structurally similar, they do not always display identical functionalities. For instance, while human and mouse ARAP3 share 97 % homology within their RhoGAP domain, their substrate preference is somewhat different. In fact, human ARAP3 displays GAP activity towards RhoA, Cdc42 and Rac1 whereas mouse ARAP3 is active exclusively on RhoA. Therefore, it is important not to presume that ortholog proteins behave the same, even if they are closely related. Using COS-7 and Swiss 3T3 fibroblasts as model systems, we investigated (a) the GTPase specificity of human CdGAP both *in vivo* and *in vitro* (b) whether human CdGAP is phosphorylated inside cells.

CHAPTER 3

The human ortholog of CdGAP is a phosphoprotein and a GTPase-activating protein for Cdc42 and Rac1 but not RhoA

The human orthologue of CdGAP is a phosphoprotein and a GTPase-activating protein for Cdc42 and Rac1 but not RhoA

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ABSTRACT

Rho GTPases regulate a wide range of cellular functions affecting both cell proliferation and cytoskeletal dynamics. They cycle between inactive GDP- and active GTP-bound states. This cycle is tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Mouse CdGAP (Cdc42 GTPase-activating protein) has been previously identified and characterized as a specific GAP for Rac1 and Cdc42 but not RhoA. It consists of an N-terminal RhoGAP domain and a C-terminal proline-rich region. In addition, CdGAP-related genes are present in both vertebrates and invertebrates. We have recently reported that two predominant isoforms of CdGAP (250 and 90 KDa) exist in specific mouse tissues. Here, we show the identification and characterization of human CdGAP (KIAA1204) which shares 76 % sequence identity to the long isoform of mouse CdGAP (mCdGAP-l). Similar to mCdGAP, it is active *in vitro* and *in vivo* on both Cdc42 and Rac1 but not RhoA and is phosphorylated *in vivo* on serine and threonine residues.

INTRODUCTION

Members of the Ras superfamily of small monomeric GTPases have been implicated in almost every fundamental cellular event. Currently, there are over 100 members in this ever growing family and based on sequence homology and function they have been grouped into six subfamilies: Ras, Rab, Arf, Rho, Ran, and Rad/Gem (Oxford and Theodorescu, 2003). RhoA, Rac1, and Cdc42, the best-characterized members of the Rho family of small GTPases regulate a wide spectrum of cellular processes such as cell growth, cell dynamics, intracellular membrane trafficking, gene transcription, cell cycle progression and apoptosis (Burridge and Wennerberg, 2004; Van Aelst and D'Souza-Schorey, 1997). One of the best-characterized cellular functions of the Rho proteins is to promote actin cytoskeleton reorganization. For instance, RhoA activation induces actin-myosin contractility by inducing the formation of stress fibers and focal adhesions (Ridley and Hall, 1992b), while Cdc42 and Rac1 promote actin reorganization at the cell periphery, leading to the formation of filopodia and lamellipodia respectively (Kozma et al., 1995; Nobes and Hall, 1995; Ridley and Hall, 1992a). All GTPases operate as molecular switches that cycle between an active GTP-bound and an inactive GDP-bound state. The guanine nucleotide exchange factors (GEFs) activate the GTPases by inducing the exchange of GDP for GTP (Cerione and Zheng, 1996). GTPase-activating proteins (GAPs) inactivate the Rho proteins by enhancing the intrinsic GTPase activity (Jenna and Lamarche-Vane, 2003) and the guanine nucleotide dissociation inhibitors (GDIs) sequester Rho proteins in their GDP-bound, inactive state (Olofsson, 1999).

p50rhoGAP was the first reported protein to have a GAP activity towards a member of the Rho family of GTPases (Olofsson, 1999). Since then, the number of RhoGAP members has reached epic proportions with over 40 characterized members in eukaryotes ranging from yeast to human (Moon and Zheng, 2003). Recent analysis of the human genome unraveled 66 different genes encoding potential RhoGAP domain-containing proteins, far outnumbering the existing 23 mammalian Rho GTPases (Bernards and Settleman, 2004; Peck et al., 2002). GAP proteins have been implicated in a wide range of cellular processes including neuronal morphogenesis, tumor suppression, cytokenesis, angiogenesis, and exocytosis (Brouns et al., 2000; Harada et al., 2000; Hirose et al., 2001; Su et al., 2004; Yuan et al., 2004). Alterations in genes coding for

RhoGAP proteins are involved in a number of human genetic disorders, in particular X-linked mental retardation, oculocerebrorenal syndrome of Lowe, leukemia, liver cancer, Bardet-Biedl syndrome (BBS) and, microphthalmia with linear skin defects syndrome (MLS) (Attree et al., 1992; Billuart et al., 1998; Borkhardt et al., 2000; Gorman et al., 1999; Schaefer et al., 1997; Yuan et al., 1998). The large number of RhoGAP proteins also suggests that they must be tightly regulated both in a spatial and temporal fashion, in order to selectively regulate a specific Rho GTPase pathway. In fact, accumulating evidence reveal that GAPs are regulated by phosphorylation, protein-protein interaction, lipid interaction, and proteolytic degradation (Ahmed et al., 1993; Jenna et al., 2002; Roof et al., 1998; Su et al., 2003)

CdGAP (Cdc42 GTPase-activating protein) is a serine- and proline-rich RhoGAP protein demonstrating GAP activity against both Cdc42 and Rac1 but not Rho A (Lamarche-Vane, 1998). 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. We have recently found that two predominant isoforms of CdGAP, CdGAP short (mCdGAP-s) and CdGAP long (mCdGAP-l) are present in mouse tissues. We have shown that both isoforms are phosphorylated *in vivo* and that CdGAP's activity is modulated through phosphorylation by ERK (Lamarche-Vane, 1998). In this paper, we characterize the human orthologue of mouse CdGAP that shares 76 % amino acid sequence identity in its entire protein sequence to mouse CdGAP-l. Like the mouse protein, it is active both *in vivo* and *in vitro* towards Cdc42 and Rac1 but not RhoA and is also phosphorylated *in vivo* on serine and threonine residues but not on tyrosine residues.

EXPERIMENTAL PROCEDURES

Reagents and antibodies

Human recombinant PDGF-BB was obtained from Calbiochem. Lysophosphatidic acid (LPA) and bradykinin were obtained from Sigma-Aldrich. Protease inhibitor cocktail tablets were from Roche Applied Science (Indianapolis, IN). Protein G-sepharose and [γ^{32}P]-GTP (6000 Ci/mmol) were obtained from Amersham Pharmacia Biotech. [^{32}P]-orthophosphate (3000 mci/ml) was purchased from Perkin Elmer. Myc-tagged proteins were detected using 9E10 anti-Myc monoclonal antibodies, which were kindly provided by Dr. Nicole Beauchemin (McGill University, Montreal, Canada).

DNA constructs

To produce pRK5myc-hCdGAP, the human cDNA clone NM_020754 of 6321 bp (coding region: 533-4867 bp) was obtained from the Kazuka DNA Research Institute (Chiba, Japan) and was cloned into pRK5myc vector. First, the clone was digested at the 3'-end (3477-5359 bp) with EcoRI and HindIII and ligated into pRK5myc vector also digested with EcoRI and HindIII. Next, the 533-3477 bp fragment was added into pRK5myc (containing the 3477-5359 bp fragment) vector by PCR using 5'-CTAGGATATCATATGAAGAACAAGGG-3' and 3'-CGATTAGAACTTTGTCTCTCCTTAAGACTGGG-5' as forward and reverse primers respectively and NM_020754 as template. The resulting PCR product was digested with EcoRV and EcoRI and ligated into pRK5myc (containing the 3477-5359 bp fragment) digested with SmaI and EcoRI. To produce pGex-2T-hCdGAP (amino acids 1-505), the following primers were used; 5'-CTAGGATATCCATGAAGAACAAGGG-3' and 3'-GGCGTCAGTAGTCGTGGTTGCTTAAGGATC-5' as forward and reverse primers respectively and pRK5myc-hCdGAP as template. The resulting PCR product was digested with EcoRV and EcoRI and ligated into pGEX-2T digested with SmaI and EcoRI.

Expression of recombinant proteins

Cdc42, Rac1, RhoA, mCdGAP (amino acids 3-662), and hCdGAP (amino acids 1-505) were produced in *E.coli* as glutathione S-transferase (GST) fusion proteins and purified on glutathione-Sepharose beads as described previously (Self. A.J. and Hall, 1995). The recombinant GTPases were released from the beads by cleavage with human thrombin (Calbiochem) and thrombin was removed by adding 10 µl of *p*-aminobenzamidine-agarose beads (Sigma) for 30 min at 4 °C. Purified proteins were dialyzed against 15 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT and concentrated by ultrafiltration using Centricon-10 (Amicon). Active protein concentrations were determined by the filter binding assay using [^{3}H]GTP as described previously (Ridley and Hall, 1992b). GST-hCdGAP (amino acids 1-505) and GST-mCdGAP (amino acids 3-662) were eluted from the beads with 5 mM reduced glutathione (Sigma) and concentrated by ultrafiltration using Centricon-10. Protein concentration was assayed following the method of Bradford, and purity of protein preparations was visualized on Coomassie Blue-stained SDS-polyacrylamide gels.

Northern Blot analysis

A human multiple tissue Northern Blot from CLONTECH was incubated with a radioactive DNA probe corresponding to a SmaI/EcoRI fragment (1216-1517 bp) of hCdGAP cDNA as recommended by the manufacturer. The SmaI/EcoRI fragment was labeled with [α^{32}P]dCTP using the multiprime DNA labeling systems from Amersham Pharmacia Biotech.

Cell transfection, immunoprecipitation and immunoblotting

COS-7 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 (Olson et al., 1995). Briefly, 5 µg of pRK5myc-mCdGAP-l, pRK5myc-hCdGAP 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 antibodies and revealed by ECL (Perkin Elmer).

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

COS-7 cells grown onto 100 mm- dishes were transfected with Myc-tagged mCdGAP-1 or Myc-tagged hCdGAP 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. Cells were incubated for 3 h in the presence of 0.5 mCi/ml of [^{32}P]-orthophosphate. CdGAP proteins were immunoprecipitated from cell lysates with anti-myc antibodies, 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.

In vitro GAP Assay

Recombinant RhoA, Rac1, and Cdc42 (5 µg) were preloaded with [γ-^{32}P]GTP (10 µCi, 6000 Ci/mmol) in 20 µl of 20 mM Tris-HCl (pH 7.5), 25 mM NaCl, 0.1 mM dithiothreitol, and 5 mM EDTA for 10 min at 30 °C. After the addition of $MgCl_2$ (final concentration of 20 mM), proteins could be kept on ice up to 1 h. 3 µl (0.75 µg) of the preloaded GTPases (to give a final concentration of 1.3 µM) were diluted with 20 mM Tris-HCl (pH 7.5), 0.1 mM DTT, 1 mM GTP, 0.86 mg/ml bovine serum albumin, and 0.2 µM of GST-hCdGAP (amino acids 1-505) or GST-mCdGAP (amino acids 3-662) or without GAP protein. The mixture was incubated at room temperature, and 5 µl samples were removed at 0, 5, 10, and 15 min, diluted in 1 ml of ice-cold buffer A (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$), and filtered through nitrocellulose filters (prewetted with buffer A). Filters were washed with 10 ml of cold buffer A, dried, and counted.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed as described (Boyle et al., 1991). Briefly, human and mouse CdGAP were overexpressed in COS-7 cells, radiolabeled and immunoprecipitated as described above. 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 MS film at -80 ^{0}C.

Cell culture and microinjection

Subconfluent, serum-starved Swiss 3T3 cells were prepared as described previously (Lamarche-Vane, 1998). Eukaryotic expression vector pRK5myc encoding the region of hCdGAP encompassing the GAP domain (amino acids 1-505) was microinjected at 0.1 mg/ml into the nucleus of ~100 cells over a period of 10 min. During microinjection, cells were maintained at 37 °C with an atmosphere of 10% CO_2. Cells were returned to the incubator for a further 2 h before treatment with extracellular factors.

Growth Factor Treatment and Immunofluorescence

Two hours after microinjection of pRK5myc-hCdGAP into subconfluent serum-starved Swiss 3T3 cells, cells were stimulated with 3 ng/ml platelet-derived growth factor for 12 min, 200 ng/ml lysophosphatidic acid for 30 min, or 100 ng/ml bradykinin for 5 min at 37 °C and fixed for 10 min in 4% (w/v) paraformaldehyde. All following steps were carried out at room temperature, and coverslips were rinsed in PBS between each step.

Cells were permeabilized for 5 min in 0.2% Triton X-100, and free aldehyde groups were reduced with 0.5 mg/ml $NaBH_4$ for 10 min. Cells were incubated for 60 min in the presence of anti-myc antibodies diluted in PBS, followed by incubation with a secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibodies and tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma). Coverslips were mounted by inverting them onto mowiol 4-88 mountant (Calbiochem) containing *p*-phenylenediamine (Sigma) as an antibleach agent. After 16 h at room temperature, cells were examined on a Zeiss 63 × 1.4 oil immersion objective. Fluorescence images were recorded using a digital camera (DVC) and analyzed with Northern Eclipse software (Empix imaging).

RESULTS

Identification of human CdGAP

We have previously identified and characterized in mouse tissues two isoforms of CdGAP that we have named mouse CdGAP-short (mCdGAP-s) and CdGAP –long (mCdGAP-l) (Lamarche-Vane, 1998). In addition, CdGAP-related genes are present in different organisms such as human, rat, fly, frog, chicken, worm and fish (Tcherkezian et al., 2005). In *D. melanogaster*, a single gene encoding a putative protein with high degree of homology to mCdGAP is present (Sagnier et al., 2000). At least three different genes encoding proteins that are highly homologous to mCdGAP-l are also found in *H. sapiens*. A protein named Grit (also known as p200RhoGAP, GC-GAP, RICS, and p250GAP) (Moon et al., 2003; Nakamura et al., 2002; Okabe et al., 2003; Taniguchi et al., 2003; Zhao et al., 2003) which shares 68 % homology to mCdGAP-l within the rhoGAP domain has been previously characterized. An uncharacterized cDNA, F25965_3 encoding a putative protein related to mouse TC-GAP (Chiang et al., 2003) also exists in human and shares 64% homology to mCdGAP-l within the rhoGAP domain. The cDNA KIAA1204 (Gen Bank™ accession number BAA86518) encodes a putative protein of 1,444 amino acids that shares 97% sequence indentity to mCdGAP-l within the rhoGAP domain and 76 % identity within the entire amino acid sequence. We have named this novel RhoGAP protein, hCdGAP (human CdGAP) based on its high amino acid sequence homology to mouse CdGAP (Fig. 3.1A). Similar to mCdGAP-l, hCdGAP contains an N-terminal RhoGAP domain followed by a long amino acid sequence enriched in both serine and charged residues. hCdGAP also contains two proline-rich sequences with a consensus Src homology 3 (SH3)-binding site, one of which is conserved between human and mouse CdGAP (fig 3.1B).

To examine the expression pattern of hCdGAP, we used a commercial human tissue Northern Blot. To ensure the specificity of the signal, a radiolabeled probe was designed outside of the conserved N-terminal RhoGAP domain. The Northern blot analysis revealed the presence of two transcripts of hCdGAP at approximately 7.5 kilobases and 1.35 kilobases. The 7.5 kb band is present in all tissues but is significantly enriched in heart and muscle tissues (Fig. 3.2). Brain, liver and kidney revealed little

Figure 3.1. Comparison of human and mouse CdGAP amino acid sequences

(A) Amino acid sequences of human (KIAA1204) and mouse CdGAP proteins. The human orthologue shares 76 % identity on its entire protein sequence to mouse CdGAP. The RhoGAP domain is underlined and the proline-rich sequences are in bold. (B) Sequence alignment of the proline-rich regions of human and mouse CdGAP with SH3 binding motif consensus sequence. The critical arginine (in class II consensus) and prolines are in bold; *p*, proline-preferred; *X*, non conserved residues. *, Conserved SH3-binding motif within human and mouse CdGAP protein sequences.

A

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Human   1    MKNKGAKQKLKRKGAASAFGCDLTEYLESSGQDVPYVLKSCAEFIETHGIVDGIYRLSGVTSNIQRLRQEFGSDQCPDLTREVYLQDIHCVGSLCKLYFR 100
Mouse   1    MKNKGAKQKLKRKGAASAFGCDLTEYLESSGQDVPYVLKSCAEFIETHGIVDGIYRLSGITSNIQRLRQEFGSDQCPDLTREVYLQDIHCVGSLCKLYFR 100

Human   101  ELPNPLLTYELYEKFTEAVSHCPEEGQLARIQNVIQELPPSHYRTLEYLIRHLAHIASFSSKTNMHARNLALVWAPNLLRSKEIEATGCNGDAAFLAVRV 200
Mouse   101  ELPNPLLTYELYEKFTEAVSHRPEEGQLARIQNVILELPPHYRTLEYLIRHLAHIASFSSKTNMHARNLALVWAPNLLRSKKIEATICNGDAAFLAVRV 200

Human   201  QQVVIEFILNHVDQIFNNGAPGSLENDENRPIMKSLTLPALSLPMKLVSLEEAQARSLATNHPARKERRENSLPEIVPPMGTLFHTVLELPDNKRKLSSK 300
Mouse   201  QQVVIEFILNHADQIFNGGAPGALQQDESRTITKSLTLPALSLPMKLVSLEEAQARSLATNHPARKERRENSLPEIVPP--P-FHTVLELPDNKRKLSSK 297

Human   301  SKKWKSIFNLGRSGSDSKSKLSRNGSVFVRGQRLSVEKATIRPAKSMDSLCSVPVEGKETKGNFNRTVTTGGFFIPATKMHSTGTGSSCDLTKQEGEWGQ 400
Mouse   298  SKKWKSIFNLGRSGSDSKSKLSRNGSVFVRGQRLSVEKATIRPAKSMDSLCSVPVEGKENKGNFSRTVTTGGFFIPATKMHASSTGSSCDLSK-EGEWGQ 396

Human   401  EGMPPGAEGGFDVSSDRSHLQGAQARPPPEQLKVFRPVEDPESEQTAPKMLGMFYTSNDSPSKSVFTSSLFQMEPSPRNQRKALNISEPFAVSVPLRVSA 500
Mouse   397  EGMPAGAEGG-------CEV-GGQIRPLPEQLKVFRPIGDPESEQSAPKLLGMFYTSSDSPGKSVFTSSLFQMEPSPRHQRKALNISEPFAVSVPLRVSA 488

Human   501  VISTNSTPCRTPPKELQSLSSLEEFSFHGSESGGWPEEEKPLGAETSAASVPKKAGLEDAKAVPEAPGTVECSKGLSQEPGAHLEEKKTPESSLSSQHLN 600
Mouse   489  VISTNSTPCRTPPKELQSLSSLEEFSFQGSESGGWPEEEKPLGAESFPGSVTKKAATEDTKPEPEVPGRAECSQSPPLDPGTQV-EKKTLHVSLGSQVSK 587

Human   601  ELEKRPNPEKVVEEGREAGEMESSTLQESPRARAEAVLLHEMDEDDLANALIWPEIQQELKIIESEEELSSLPPPALKTSPIQPILESSLGPFIPSEPPG 700
Mouse   588  EAEKRPKAEKVMEESQGASQPKPSTPQESLGAGTEPLILHEMDEEDLAQALIWPEIQQELKIIESEEEFSSLPPAAQKTSPI-P--ESSPAPFFPFEAPG 684

Human   701  SLPCGSFPAPVSTPLEVWTRDPANQSTQGASTAASREKPEP---------EQGLHPDLASLAPLEIVPFEKASPQATVEVGGPGNLSPPLPPAPPPPTPL 791
Mouse   685  SL-----PSS-SAPREVWTRDAANQSIQEAAILTDREKLEPVCSLLESESQQELSPDPASLAPLEMLLFEKVSSPARIEIGGPRNLSPPLTPAPPPPTPL 778

Human   792  EESTPVLLSKGSPEREDSSRKLRTDLYIDQLKSQDSPEISSLCQGEEATPRHSDKQNSKNAASEGKGCGFPSPTREVEIVSQEEEDVTHSVQEPSDCDED 891
Mouse   779  EEEPEVLLSKEGPDREDAARDSRTDVYTEQPTPKESPGIPTPCQREEAIASPNEKQNARHAVPENKGPGLPSPTKEVDIIPQEEGGAPHSAQEPSDCDED 878

Human   892  DTVTDIAQHGLEMVEPWEEPQWVTSPLHSPTLKDAHKAQVQGLQGHQLEKRLSHRPSLRQSHSLDSKPTVKSQWTLEVPSSSSCANLETERNSDPLQPQA 991
Mouse   879  DTVTDPAQHGLEMVEPWEEPQWVTSPLHSPTLKEVQESQTQGSQGHRLERRLCHRPSLRQSHSLDSKTTGNSHWTLEAPFSSSCANLETERNYEPLQPPA 978

Human   992  PRREITGWDEKALRSFREFSGLKGAEAPPNQKGPSGVQPNPAETSPISLAEGKELGTHLGHSSPQIRQGGVPGPESSKESSPSVQDSTSPGEHPAKLQLK 1091
Mouse   979  ARTKIAGLEEKALKAFREFSGLKGLEVLPSQKGPSGIQPKPVETNFMGLAEGKEQEPQLELSNRQMKHSDVPGPDSSKESSPRAQDSTLPGEHPLQLQLK 1078

Human   1092 STECGPPKGKNRPSSLNLDPAIPIADLFWFENVASFSSPGMQVSEPGDPKVTWMTSSYCKADPWRVYSQDPQDLDIVAHALTGRRNSAPVSVSAVRTSFM 1191
Mouse   1079 NTECGPSKGKHRPSSLNLDSATPIADLFRLENGAPFSSPGIELSELGDTKVTWMSSSHCKAAPWN--SQDTQDLDIVAHTLTGRRNSAPVSVSAVRTSFM 1176

Human   1192 VKMCQARAVPVIPPKIQYTQIPQPLPSQSSGENGVQPLERSQEGPSSTSGTTQKPAKDDSPSSLESSKEEKPKQDPGAIKSSPVDATAPCMCEGPTLSPE 1291
Mouse   1177 VKMCQAKAVPVIPPKIQYTQIPQPLPSQSTGEGGAQPLERSQEEPGSTPEIPQKSTKDDSPSSLGSPEEEQPKQETGASASRRQASITSCMYEGSSCSPE 1276

Human   1292 PGSSNLLSTQDAVVQCRKRMSETEPSGDNLLSSKLERPSGGSKPFHRSRPGRPQSLILFSPPFPIMDHLPPSSTVTDSKVLLSPIRSPTQTVSPGLLCGE 1391
Mouse   1277 PSASTLASTQDAVVQCRKRTSETEPSGDNLLSSKLERASGGPKAFHRSRPGRPQSLIL----FPIMDHLPSSPTVIDSKVLLSPIRSPTQTVSPGLLCGE 1372

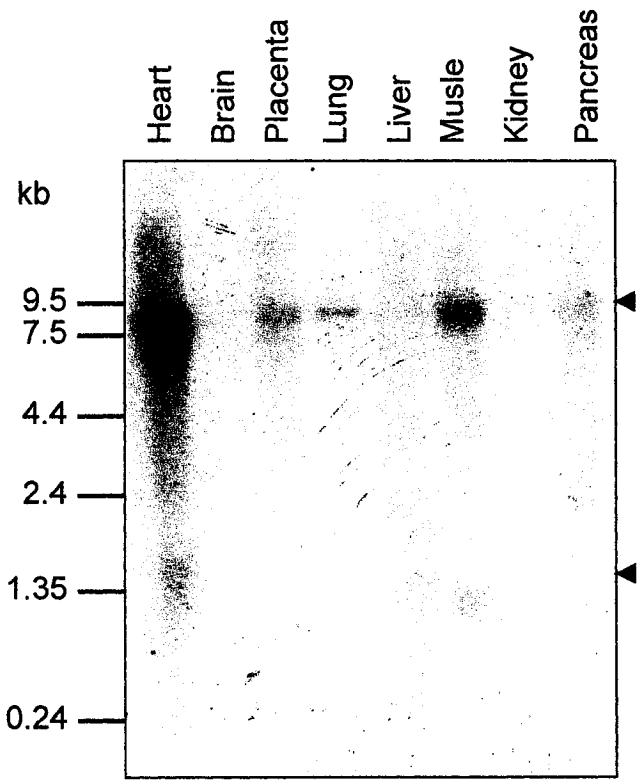
Human   1392 LAENTWVTPEGVTLRNKMTIPKNGQRLETSTSCFYQPQRRSVILDGRSGRQIE 1444
Mouse   1373 LAENTWITPEGVTLRNKMTIPKNGQRLETSTSCFYQPQRRSVILDGRSGRQIE 1425
```

B

	CLASS II CONSENSUS		X	**P**	p	X	**P**	X	**R**
	p47PHOX (362-370)		Q	**P**	A	V	**P**	P	**R**
	Grit (1060-1068)		A	**P**	L	P	**P**	E	**R**
	Grit (1628-1636)		P	**P**	P	K	**P**	E	**R**
	Sos1 (1153-1161)		P	**P**	P	V	**P**	P	**R**
	Human (988-994)		Q	**P**	Q	A	**P**	R	**R**
*	Human (783-791)	P	A	**P**	P	P	**P**		
	Mouse (550-557)		E	**P**	E	V	**P**	G	**R**
	Mouse (563-568)	S	P	**P**	L	D	**P**		
	Mouse (608-613)	P	K	**P**	S	T	**P**		
	Mouse (678-683)	P	F	**P**	E	A	**P**		
*	Mouse (770-775)	P	A	**P**	P	P	**P**		
	p50RhoGAP (230-235)	P	M	**P**	P	R	**P**		
	3BP-1 (270-275)	P	P	**P**	L	P	**P**		
	SH3 BINDING MOTIF CONSENSUS	P	X	**P**	P	X	**P**		

Figure 3.2. Tissue distribution of human CdGAP mRNA by Northern Blot analysis

Poly $(A)^+$ mRNA from human tissues (CLONTECH) was subjected to Northern Blot analysis using a radioactively labeled SmaI/EcoRI fragment (1216-1517 bp) from human CdGAP cDNA as a probe. Arrows indicate the two predominant hCdGAP mRNA.


Heart
Brain
Placenta
Lung
Liver
Musle
Kidney
Pancreas
kb
9.5
7.5
4.4
2.4
1.35
0.24

detectable signal, however, at longer exposure low levels of mRNA were observed (data not shown). Only heart and muscle showed detectable levels of the 1.35 kb transcript. We conclude that similar to mCdGAP, hCdGAP is expressed in most tissues although at different levels of expression and hCdGAP has its highest levels of expression in the heart.

In vitro and *In vivo* activities of human CdGAP

To determine whether human CdGAP encodes a functional GAP activity towards Rho GTPases, amino acids 1-505 of human CdGAP were subcloned into the pGEX-4T3 *E.coli* expression vector as described under "Experimental procedures". Fig. 3.3 shows that similar to mouse CdGAP, the GAP domain of human CdGAP stimulates the intrinsic GTPase activity of both Cdc42 and Rac1 but not RhoA. These results indicate that the RhoGAP domain of both mouse and human CdGAP behaves in a similar fashion *in vitro*. To assess the *in vivo* specificity of human CdGAP towards the Rho GTPases, a eukaryotic expression vector pRK5 encoding Myc-tagged full length human CdGAP was microinjected into serum-starved, subconfluent Swiss 3T3 fibroblasts, and its effects on actin changes induced by the addition of extracellular factors was examined by staining actin filaments with TRITC-conjugated phalloidin. Fig. 3.4 shows that expression of human CdGAP inhibits Rac-dependent, platelet-derived growth factor-induced membrane ruffling and Cdc42-dependent, bradykinin-induced filopodia. However, it does not affect Rho-dependent, lysophosphatidic acid-induced stress fiber formation. Similar to mouse CdGAP, these results show that human CdGAP is able to down-regulate both Cdc42 and Rac1 *in vivo*.

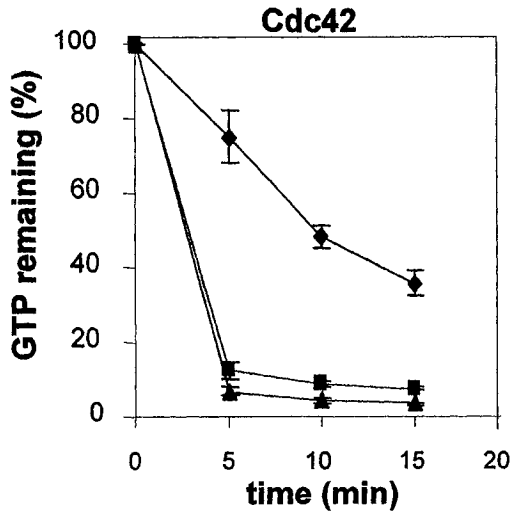
Human CdGAP is phosphorylated on serine and threonine residues *in vivo*

We have previously found that mouse CdGAP-s and –l are highly phosphorylated on serine and threonine residues in fibroblast cells (Lamarche-Vane, 1998). Since the amino acid sequence of human CdGAP also contains many putative phosphorylation sites for different protein kinases such as ERK, RSK, CKII and PKC, we hypothesized that hCdGAP may also be phosphorylated. To examine whether human CdGAP is phosphorylated *in vivo*, Myc-tagged human CdGAP and mouse CdGAP–l were expressed

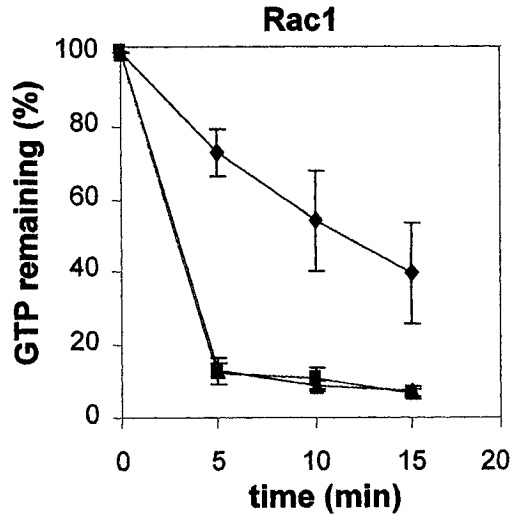
Figure 3.3. Analysis of recombinant human CdGAP activity *in vitro*

RhoA, Rac1, Cdc42, mCdGAP and hCdGAP were produced using the pGEX expression vector as described previously. Equal concentrations (0.2μM) of recombinant GST-hCdGAP (amino acids 1-505) or GST-mCdGAP (amino acids 3-662) were added to a GAP assay using 1.3 μM [γ-^{32}P]GTP-loaded Cdc42 (A), Rac1 (B) or RhoA (C). (■) indicates the intrinsic GTPase activities, (▲) mouse CdGAP, (◆) human CdGAP.

A


Cdc42
GTP remaining (%)
100
80
60
40
20
0
0
5
10
15
20
time (min)

B


Rac1
GTP remaining (%)
100
80
60
40
20
0
0
5
10
15
20
time (min)

C

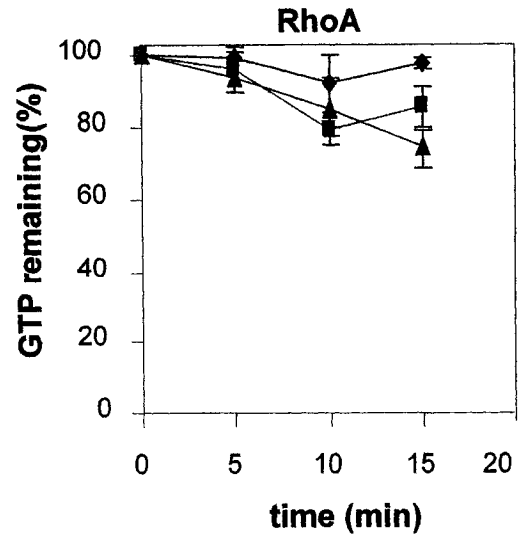
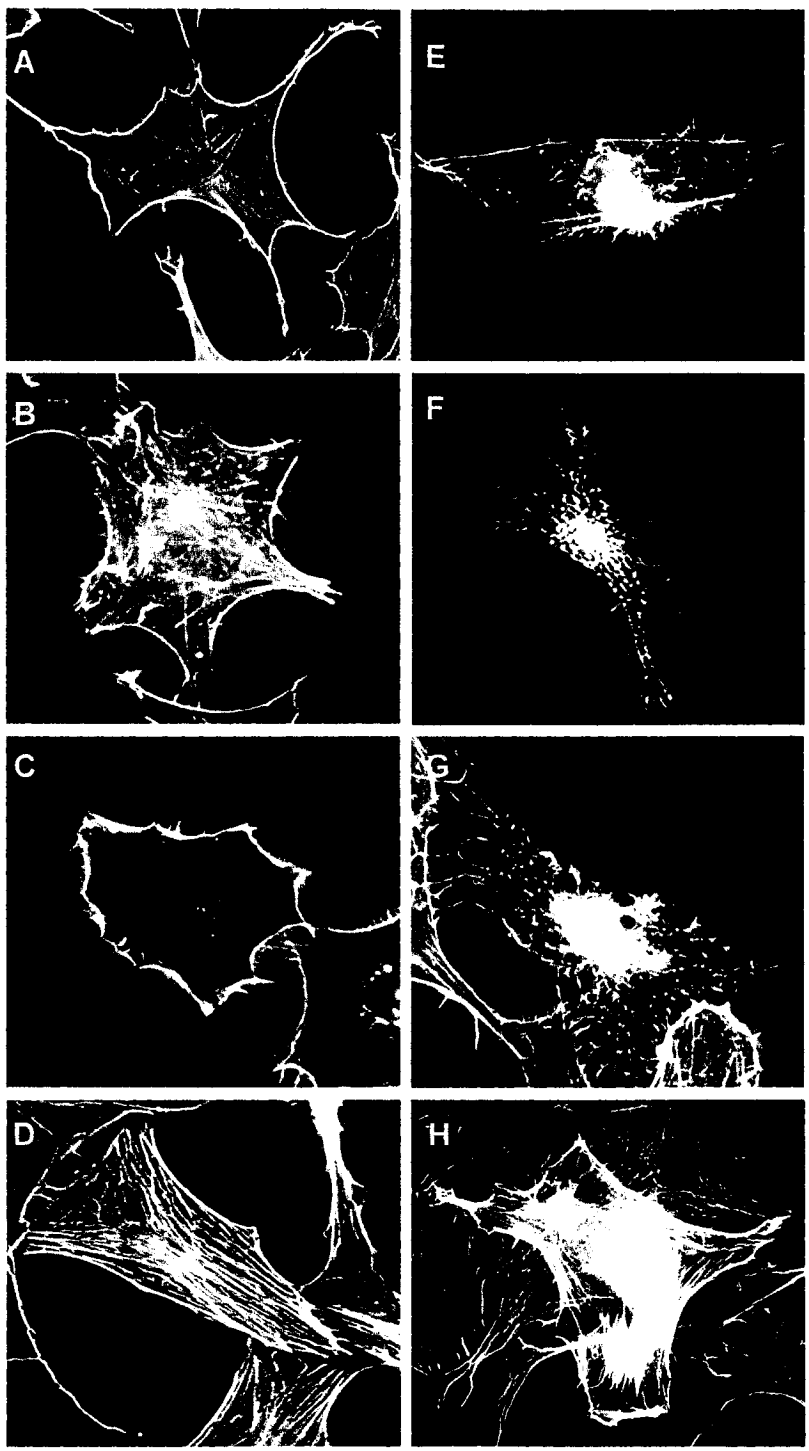

RhoA
GTP remaining(%)
100
80
60
40
20
0
0
5
10
15
20
time (min)

Figure 3.4. Human CdGAP inhibits actin remodeling induced by PDGF and bradykinin but not LPA.

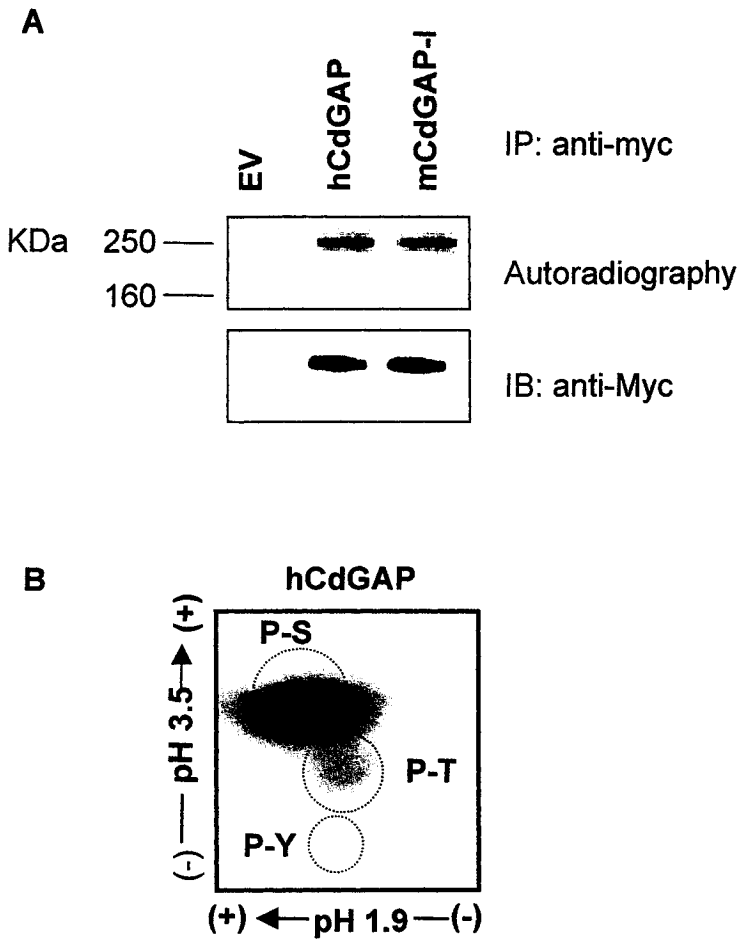
Serum-starved, subconfluent Swiss 3T3 cells were fixed after stimulation with 3 ng/ml PDGF for 12 min (C and G), 200 ng/ml LPA for 30 min (D ang H), 100 ng/ml bradykinin for 5 min (B and F) or no stimulation (A and E). In panels E to H, cells were microinjected with pRK5myc-encoding human CdGAP (0.1 mg/ml) 2 h before stimulation. Filamentous actin was visualized by TRITC-conjugated phalloidin, and injected cells were localized by co-staining with an anti-Myc antibody and by indirect immunofluorescence. Approximately 100 cells were microinjected per coverslip.


A
E
B
F
C
G
D
H

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. 3.5A, both immunoprecipitated CdGAP proteins are phosphorylated *in vivo* to a similar extent in COS-7 cells. To assess the content of phosphorylated residues on human CdGAP, a phosphoamino-acid analysis of immunoprecipitated hCdGAP was performed and revealed that like mouse CdGAP, the human protein is also phosphorylated on serine residues and to a lesser extent on threonine and not on tyrosine residues (Fig. 3.5B). The absence of tyrosine phosphorylation was also confirmed by immunoblotting using anti-phosphotyrosine antibodies (data not shown). Thus, these results demonstrate that human CdGAP is also phosphorylated on serine and threonine residues in fibroblast cells.

Figure 3.5. Human CdGAP is phosphorylated *in vivo* on serine and threonine residues.

(A) COS-7 cells expressing Myc-tagged hCdGAP or mCdGAP-1 were metabolicaly 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) The phosphorylated protein band corresponding to hCdGAP was cut and subjected to phosphoamino acid analysis. Phosphoamino acids were resolved by thin layer chromatography and detected by autoradiography. Migration of phosphoamino acid standards is indicated with dashed circles: phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y).


A
EV
hCdGAP
mCdGAP-I
IP: anti-myc
KDa
250
160
Autoradiography
IB: anti-Myc
B
hCdGAP
P-S
P-T
P-Y
(-) — pH 3.5 — (+)
(+) — pH 1.9 — (-)

DISCUSSION

In this study, we identify and characterize the human orthologue (KIAA1204) of mouse CdGAP that shares 97% sequence identity to mCdGAP within the rhoGAP domain and 76 % identity within the entire amino acid sequence. According to Northern Blot analysis, both mCdGAP (Lamarche-Vane, 1998) and hCdGAP mRNAs are ubiquitously expressed with the highest level of expression in heart. However, mCdGAP but not hCdGAP transcript is highly present in lung tissues. We have recently shown the differential expression of at least two major isoforms of mCdGAP in specific mouse tissues (Lamarche-Vane, 1998). The presence of two CdGAP transcripts in human tissues (heart and muscle) also suggests the expression of different isoforms of CdGAP possibly resulting from alternative splicing of the transcripts.

Similar to mCdGAP, human CdGAP contains an N-terminal rhoGAP domain, several charged amino acids and serine residues, and two proline-rich sequences containing SH3-binding sites. The PAPPPP motif is well conserved in CdGAP from both species, suggesting that interaction with putative SH3-containing protein (s) may be important for CdGAP cellular functions. However, SH3-containing proteins binding to CdGAP via these proline-rich sequences remain to be identified.

We have previously shown that mouse CdGAP is active both *in vitro* and *in vivo* on Cdc42 and Rac1 but not RhoA (Lamarche-Vane, 1998). Here we demonstrate that the GAP activity of human CdGAP behaves in a similar fashion showing *in vitro* and *in vivo* GAP specificity towards Rac1 and Cdc42, as expected from the high degree of homology between the two proteins. Similar to mCdGAP, human CdGAP also migrates at 250 KDa, which is higher than its expected molecular weight of 155 KDa. As proposed for mCdGAP, post-translational modifications of the proteins may be in part responsible for this mobility shift. Indeed, we have recently shown that mCdGAP contains three ERK consensus phosphorylation sites but that only T776 is an *in vivo* phosphorylation site and an important regulatory site for CdGAP (Lamarche-Vane, 1998). Here we find that hCdGAP is phosphorylated *in vivo* on serine and threonine residues. Interestingly, only the ERK phosphorylation site T776 is conserved in hCdGAP suggesting an important role for this regulatory site of CdGAP function. It will be of interest to investigate whether

hCdGAP also interacts with RSK-1 and ERK1/2. However, unlike mCdGAP, the human protein does not have a putative DEF domain containing the FPFP motif known to be an ERK docking site. Provided that ERK is still able to bind hCdGAP, this could lead to the identification of a novel ERK binding site. In the case of RSK-1, the two consensus phosphorylation motifs present in the central domain of mCdGAP are fully conserved in human. Phosphorylation of one of these motif RRENSLP by RSK could possibly create a binding site for 14-3-3, a scaffolding protein that mediates interaction between many signaling proteins and that can also sequester proteins in specific subcellular compartments (Fu et al., 2000). It will be of interest to determine the functional role of human CdGAP phosphorylation.

In conclusion, CdGAP belongs to a novel family of RhoGAP proteins that are well conserved among different species. Here we demonstrate that the GAP substrate specificity of human CdGAP is very similar to mouse CdGAP. Future studies on human CdGAP phosphoregulation and protein interaction will provide a better understanding of the functional role of this protein.

PREFACE TO CHAPTER 4

Several lines of evidence indicate that phosphotyrosine signaling is implicated in neuronal morphogenesis (Desai et al., 1997). Recent studies have illustrated that a number of guidance receptors although lacking intrinsic kinase activity may nonetheless serve as substrates for tyrosine kinases and could be regulated by tyrosine phosphorylation (Bashaw and Goodman, 1999; Schmucker et al., 2000; Tamagnone et al., 1999). In the case of the netrin receptors, *C. elegans* UNC-5 and its mouse ortholog UNC-5H3/RCM (Rostral Cerebellar Malformation) have been shown to be phosphorylated on tyrosine *in vivo* in response to Netrin-1 (Tong et al., 2001). More importantly, phosphorylation of cytoplasmic tyrosine 482 appears to be critical for UNC-5 function *in vivo* (Killeen et al., 2002). Although DCC's role in axon guidance was established over a decade ago, very little is known about its biochemical regulation. By combining the dissection and isolation of primary commissural neurons (CN) with classical biochemical technics we investigated (a) whether endogenous DCC is phosphorylated *in vivo* in response to netrin-1 stimulation (b) which residues were phosphorylated (Ser, Thr or Tyr) (c) which kinases are responsible for DCC phosphorylation (d) the effect of kinase inhibitors on netrin-1 induced neurite outgrowth and turning (e) which precise sites are phosphorylated and the effect of mutating these sites on DCC function and (f) the phosphorylation state of DCC in Fyn kinase deficient mice.

CHAPTER 4

Phosphorylation of DCC by Fyn mediates Netrin-1 signaling in growth cone guidance

Phosphorylation of DCC by Fyn mediates Netrin-1 signaling in growth cone guidance

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ABSTRACT

Netrin-1 acts as a chemoattractant molecule to guide commissural neurons (CN) towards the floor plate by interacting with the receptor deleted in colorectal cancer (DCC). The molecular mechanisms underlying Netrin-1/DCC signalling are still poorly characterized. Here we show that DCC is phosphorylated *in vivo* on tyrosine residues in response to Netrin-1 stimulation of CN and that the Src family kinase inhibitors PP2 and SU6656 block both Netrin-1-dependent phosphorylation of DCC and axon outgrowth. PP2 also blocks the reorientation of *Xenopus* RGC in response to netrin-1 suggesting an essential role of the Src kinases in netrin-1-dependent orientation. Fyn but not Src is able to phosphorylate the intracellular domain of DCC *in vitro* and we demonstrate that Y1418 is crucial for DCC axon outgrowth function. Both DCC phosphorylation and Netrin-1-induced axon outgrowth are impaired in Fyn -/- CN and spinal cord explants, respectively. We propose that DCC is regulated by tyrosine phosphorylation and that Fyn is essential for the response of axons to Netrin-1.

INTRODUCTION

During the development of the CNS, axons are guided to their appropriate targets in response to extracellular cues. At the leading edge of the axons, the growth cone acts as a sensory-motor structure to detect and respond to attractive and repulsive cues (Mehlen and Mazelin, 2003; Tessier-Lavigne and Goodman, 1996). These guidance cues can be either membrane-bound factors or secreted molecules, acting over short or long distances respectively to guide the growth of axons. They initially include members of the Netrin, ephrin, semaphorin, slit, receptor protein tyrosine phosphatases and neurotrophin families of protein (Dickson and Senti, 2002; Grunwald and Klein, 2002; Guan and Rao, 2003; Huber et al., 2003; Tessier-Lavigne and Goodman, 1996, Cook et al, 1998). More recently, morphogens for embryonic patterning have also been implicated in axon guidance (Charron et al., 2003, Salinas et al, 2003). Netrins belong to a small family of bifunctionally and phylogenetically conserved secreted proteins (Chisholm and Tessier-Lavigne, 1999; Dickson, 2002). In the developing spinal cord, Netrin-1 attracts commissural axons towards the ventral midline but also repels different classes of neurons (Kennedy et al., 1994; Serafini et al., 1994; Tessier-Lavigne and Goodman, 1996). The conserved transmembrane proteins belonging to the DCC family of Netrin receptors mediate the chemoattractant effect of Netrin-1 (Keino-Masu et al., 1996a; Serafini, 1996) but they can also participate in repulsion (Chan et al., 1996; Hong et al., 1999; Merz et al., 2001). The UNC-5 family of receptors seems to be involved exclusively in the repulsive effects mediated by Netrins, either alone or in combination with DCC (Keleman and Dickson, 2001; Kennedy, 2000; Leonardo et al., 1997; Merz et al., 2001).

The actin cytoskeleton plays a prominent role in the response of axons to guidance cues (Guan and Rao, 2003; Luo, 2002). Recent evidence has implicated the Rho family of small GTPases, in particular RhoA, Rac1 and Cdc42 as important signalling molecules downstream of most, if not all, guidance cue receptors (Dickson, 2001; Lundquist, 2003; Luo, 2000). Indeed, the Rho family of proteins mediates a cascade of responses from the guidance cue receptors to actin remodeling (Huber et al., 2003). We and others have demonstrated that Rac1 is an important mediator of the signalling response to the Netrin-1 receptor DCC (Li et al., 2002b; Shekarabi and Kennedy, 2002) and that the adaptor

protein Nck-1 couples DCC to the activation of Rac1 through a mechanism that still remains to be determined (Li et al., 2002a).

Several lines of evidence also indicate that phosphotyrosine signalling is implicated in the development of the nervous system (Desai et al., 1997). Recent studies have illustrated that a number of guidance receptors although lacking intrinsic kinase activity may nonetheless serve as substrates for tyrosine kinases and could be regulated by tyrosine phosphorylation (Bashaw and Goodman, 1999; Schmucker et al., 2000; Tamagnone et al., 1999). In the case of the Netrin receptors, *C. elegans* UNC-5 and its mouse ortholog UNC-5H3/RCM (Rostral Cerebellar Malformation) have been shown to be phosphorylated on tyrosine *in vivo* in response to Netrin-1 (Tong et al., 2001). More importantly, phosphorylation of cytoplasmic tyrosine 482 appears to be critical for UNC-5 function *in vivo* (Killeen et al., 2002).

To investigate whether protein phosphorylation is implicated in the regulation of the netrin-1/DCC signalling pathways, we combined the dissection and isolation of primary CN with *in vivo* metabolic radiolabeling techniques to examine the phosphorylation status of DCC in embryonic spinal CN. In the present study, we provide the first evidence that Netrin-1 stimulates phosphorylation of DCC on serine, threonine and tyrosine residues *in vivo*. Netrin-1-dependent phosphorylation of DCC is completely abolished in the presence of tyrosine kinase inhibitors, suggesting that tyrosine phosphorylation of DCC is a prerequisite step for DCC phosphorylation on serine and threonine residues. Furthermore, treatment of E13 rat dorsal spinal cord explants with the Src family kinase inhibitor PP2 or SU6656 inhibits axon outgrowth induced by Netrin-1, suggesting that Src family kinases participate to Netrin-1 signalling. Indeed, we show here that the intracellular domain of DCC is phosphorylated by Fyn and that phosphorylation of Y1418 is required for DCC function. We therefore propose that Fyn initiates Netrin-1 signalling via the phosphorylation of Y1418 of DCC.

EXPERIMENTAL PROCEDURES

DNA constructs and site-directed mutagenesis

The tyrosine residues located at the positions 1261, 1272, 1361 and 1418 of the intracellular domain of full-length DCC in pRK5 vector (Li et al., 2002a) were substituted to phenylalanine residues using a PCR oligonucleotide-directed mutagenesis according to standard protocols. The following primers were used:

Y1261F (5' GAAAGTGCCCAGTTCCCAGGAATCCTCCCG-3');

Y1272F(5'-CCCACATGTGGATTTCCGCATCCACAGTTC-3');

Y1361F(5'-CAAAAGTCCTTTTACACCGCTTTTGTCG-3');

Y1418F(5'GCCAGTGTATTCGAACAGGATGACTTG-3').

PCR fragments were inserted using Hind III into pRK5-DCC to create the mutants. The intracellular domain of DCC mutants (DCC-C) were subcloned into the pGEX vector in order to produce GST fusion proteins (Li et al., 2002a).

Ligand and Drug treatments

Recombinant Netrin-1 was produced and purified as described (Serafini et al., 1994). Netrin-1 was used at a final concentration of 500ng/ml to stimulate dissociated CN and Cos-7 cells and at 160ng/ml to treat spinal cord explants. Genistein (Sigma) and PP_2 (Calbiochem) were used at final concentrations of 100 μM and 10 μM respectively. SU6656 (Calbiochem) was used at 0.5, 1 and 2 μM final concentrations.

Cell culture

E13 rat or E11.5 mouse CN were dissected from dorsal spinal cords as described (Tessier-Lavigne et al., 1988); (Shekarabi and Kennedy, 2002). Isolated CN and N1E-115 were plated on 60mm dishes or on coverslips coated with 20 μg/ml of laminin-1 (Canlab) and grown as described (Li et al, 2002b).

Cell transfection

N1E-115 cells were transfected using the LipofectAMINE reagent (Invitrogen) according to the manufacturer's protocol with 1 μg of the following constructs: pRK5 empty vector, DCC, DCC-Y1261F, DCC-Y1272F, DCC-Y1361F, DCC-Y1418F, Fyn, Fyn-K299A

(Fyn-DN), Fyn-Y531F (Fyn-CA), Src-Y538F (Src-CA) and Src-K298R (Src-DN). Cos-7 cells were co-transfected with 4 μg of the following constructs: pRK5 empty vector, DCC, DCC-Y1261F or DCC-Y1418F constructs together with 1μg of pRK5-myc-Rac1 using the DEAE-dextran method as described (Olson et al., 1995). 24h later, the cells were serum-starved overnight before a 5min treatment with Netrin-1.

Immunofluorescence

Transfected N1E-115 cells and E13 rat CN plated on laminin-1-coated coverslips were fixed for 10 min in 3.7% formaldehyde/PBS. The cells were permeabilized for 5 min in 0.25% TritonX-100 in PBS and incubated for 30 min in 0.1% BSA. The cells were incubated with anti-DCC antibodies (Pharmingen, G97-449) and rhodamine-conjugated phalloidin (Sigma) followed by incubation with FITC-coupled secondary antibodies (Sigma). The coverslips were mounted on Mowiol (Calbiochem) and cells were examined on a Zeiss Axiovert 135 microscope using a Zeiss oil immersion 63X planapochromat lens. Images were recorded using a digital camera (DVC) and analyzed using Northern Eclipse software (Empix Imaging Inc.).

Immunoprecipitation

Transfected N1E-115 cells, E13 rat or E11.5 mouse CN treated with Netrin-1 for different periods of time were lysed in 20 mM Hepes, pH 7.5, 100mM NaCl, 10% glycerol, 1% Triton-X100, 20 mM NaF, 1 mM sodium orthovanadate, 1mM PMSF, 10 μg/ml aprotinin and leupeptin. The supernatants were pre-cleared for 2h with protein G-sepharose beads before being incubated overnight at 4°C with 20μl of protein G-sepharose beads and 2.5 μg of anti-DCC antibodies. Protein samples were resolved on SDS-PAGE followed by transfer on nitrocellulose. The membranes were immunoblotted with the following antibodies: anti-pY (4G10; Upstate); anti-Fyn, kindly provided by Dr Veillette (IRCM, Montreal); anti-DCC.

Explant assays

E13 rat and E11.5 mouse dorsal spinal cords were dissected as described (Tessier-Lavigne et al., 1988). Dorsal explants embedded in 3D-collagen type I gels (BD

Biosciences) were treated either with Netrin-1 alone or both with Netrin-1, PP_2 or SU6656. The images were captured after 36h using a digital camera (DVC) on a Zeiss Axiovert 135 microscope with a 40X phase contrast objective lens. The total length of the axon bundles was calculated using Northern Eclipse software (Empix Imaging Inc.).

Fyn knock-out mice

Male and female homozygous null breeding pairs for Fyn-/- mice (B6; 129S-Fyn ^{tm1sor}) were purchased from Jackson Laboratory (Bar Harbor, Me). Genomic DNA was made from tail tips clipped at weaning in order to genotype the mice using a PCR assay as described on the Jackson Laboratory web site.

Retinal Cultures and Growth Cone Turning Assays

Eye primordia were dissected from stage 24 X.laevis embryos and plated as explant tissue on coverslips coated with 50 μg/ml fibronectin (Roche). 24h cultures were used in the growth cone turning assay as described (de la Torre et al., 1997). Growth cones that actively grew in a straight line for 30 min before the beginning of the experiment were chosen. The responses of these growth cones to an applied concentration gradient were recorded for 45 min using a Cohu CCD video camera and Scion Image capture software (shareware). Stable netrin-1 gradients were formed by pulsatile ejection of a concentrated solution of 1 μg/μl Netrin-1 from a 0.5-1.0 μm tip glass capillary pipette placed at a 45° angle from the actively extending growth cone. Control solutions consisted of 1% BSA in PBS. Upon completion of the experiment, only actively extending growth cones (>5 μm growth) were analyzed. Experiments were performed in a blinded fashion so that only after the analysis of the recorded video was the identity of the pipette solution revealed to the experimenter. The trajectories of the growth cones were traced onto a graph and the turning angles measured.

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

E13 rat CN grown for 72h on laminin-coated 60mm dishes were starved in a phosphate and serum-free medium 2h before being radiolabeled. CN were then incubated for 2h in the presence of 0.5 mCi/ml of [^{32}P]-orthophosphate. The cells were stimulated with

purified Netrin-1 for 5, 10 and 30 min. The endogenous DCC was immunoprecipitated from the cell lysates and proteins were separated on SDS-PAGE followed by transfer onto PVDF membranes. The radiolabeled proteins were visualized by autoradiography at -80°C.

Phosphoamino-acid analysis

The bands corresponding to phosphorylated DCC obtained after 5 or 30 min of Netrin-1 stimulations were subjected to a phosphoamino-acid analysis as previously described (Boyle et al., 1991). Radiolabeled amino acids were detected by autoradiography at -80^{0}C and the signals obtained were quantified by densitometry.

Protein purification

Cultures of E.coli strain DH5α transformed with pGEX-DCC-C, pGEX-DCC-C-Y1261F, pGEX-DCC-C-Y1272F, pGEX-DCC-C-Y1361F or pGEX-DCC-C-Y1418F were subjected to protein production and purification as described (Li et al., 2002a).

***In vitro* kinase assay**

20 μg of either GST or GST fusion proteins containing the cytoplasmic domains of DCC (DCC-C) or DCC-C-Y1261F, DCC-C -Y1272F, DCC-C -Y1361F and DCC-C -Y1418F as well as 5 μg of MBP were incubated for 10 min at 30°C without or with 20ng of active Fyn (Upstate) in buffer A (200mM Tris-HCl, pH7.5, 0.4mM EGTA, 0.4mM sodium orthovanadate) in presence of 10μCi/μl [γ ^{32}P]-ATP. 20 μg of GST and GST-DCC-C as well as 5μg of Src substrate peptide (KVEKIGEGTYGVVYK) were incubated 10min at 30°C with 10ng/μl of Src active kinase (Upstate) in buffer B (100mM Tris, pH7.2, 125mM $MgCl_2$, 25mM $MnCl_2$, 2mM EGTA, 0.25mM Sodium orthovanadate) in presence of 10μCi/μl [γ^{32}P]-ATP. The phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography at -80 °C. A range of DCC-C concentrations from 25-100 nM was used to estimate the Km value using the Lineweaver-Burk equation.

Rac1 Activation Assay

The amounts of GTP-loaded Rac1 in Cos-7 cells control or expressing DCC, DCC-Y1261F, DCC-Y1272F, DCC-Y1361F or DCC-Y1418F mutant proteins together with

myc-Rac1 and treated for 5min with Netrin-1 were measured using a pull-down assay as described (Li et al., 2002a).

RESULTS

Netrin-1 induces DCC phosphorylation *in vivo* in embryonic spinal CN

We first examined whether endogenous DCC is phosphorylated *in vivo* upon Netrin-1 stimulation in dissociated CN. For this purpose, we dissected the first half of embryonic day 13 rat dorsal spinal cords that contain the cell bodies of the CN (Tessier-Lavigne et al., 1988). Dissociated CN exhibit long and fasciculated axons enriched in growth cones at the tips of the axons after 72 hours in culture (Fig. 4.1A). As shown in Fig. 4.1B, a high level of DCC expression is observed in the cell bodies and along the neurite extensions as previously described (Li et al., 2002b; Shekarabi and Kennedy, 2002). Thus, we have chosen this time point to examine the phosphorylation of DCC *in vivo* in response to Netrin-1. 72 hours after plating on laminin-1-coated dishes, the CN were serum-starved and then incubated in phosphate-free medium supplemented with [^{32}P]-orthophosphate for 2 hours prior to stimulation with Netrin-1 for different periods of time. Phosphorylation of endogenous DCC is increased 5 min after stimulation with Netrin-1 and a 12-fold increase in the level of DCC phosphorylation is observed after 30 min of stimulation (Fig. 4.1C and 4.1D). To assess the content of phosphorylated residues on DCC upon Netrin-1 stimulation, a phosphoamino-acid analysis of immunoprecipitated DCC was performed after 5 and 30 min incubation with Netrin-1. Interestingly, DCC is mainly phosphorylated, *in vivo,* on serine and threonine residues and to a lesser extent on tyrosine residues (Fig. 4.1E and 4.1F). The 8-fold increase in tyrosine phosphorylation of DCC after 30 min of Netrin-1 stimulation (Fig. 4.1F and 4.1G) is confirmed by western blotting using anti-phosphotyrosine (anti-PY) antibodies on immunoprecipitated DCC from embryonic commissural neuron lysates (Fig. 4.1C). Therefore, Netrin-1 induces DCC phosphorylation *in vivo* on serine, threonine and tyrosine residues in embryonic CN.

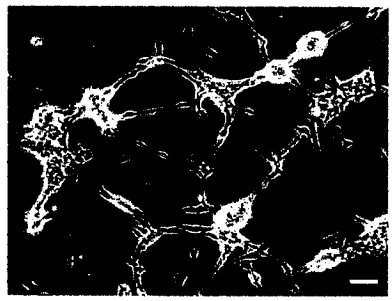
Netrin-1-induced phosphorylation of DCC requires Src family tyrosine kinase activity

Because the Src family kinases, particularly the Src and Fyn members, play pivotal roles in neuronal signalling cascades (Arnaud et al., 2003; Maness et al., 1996; Morse et al., 1998; Sperber et al., 2001), we examined the implication of the Src family in

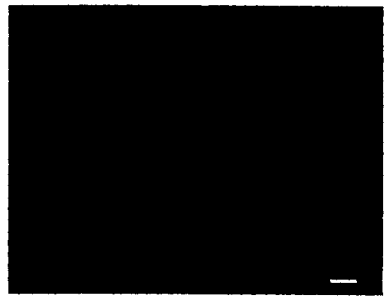
Figure 4.1. DCC is rapidly phosphorylated *in vivo* upon Netrin-1 stimulation in embryonic rat CN

(A) Phase contrast image (10X) of E13 rat CN 72h after plating on laminin. (B) Immunofluorescence of CN with anti-DCC antibodies showing DCC expression in the cell bodies and along the axons. Bar, 20 μm. (C) E13 rat CN labeled with [^{32}P]-orthophosphate for 2h were either not stimulated or stimulated with Netrin-1 for 5, 10 and 30 min. Endogenous DCC was immunoprecipitated (IP) from the cell lysates. The radiolabeled proteins were subjected to SDS-PAGE and identified by autoradiography. The membrane was immunoblotted with anti-pY and anti-DCC antibodies to show the total amount of DCC. (D) Quantitative analysis of the phosphorylation level of DCC after Netrin-1 stimulation of rat CN. Fold increase in total phosphorylation of DCC was determined by densitometry (n=3). (E and F) The bands corresponding to phosphorylated DCC obtained after 5min (E) or 30min (F) of Netrin-1 stimulation were subjected to a phosphoamino-acid analysis.

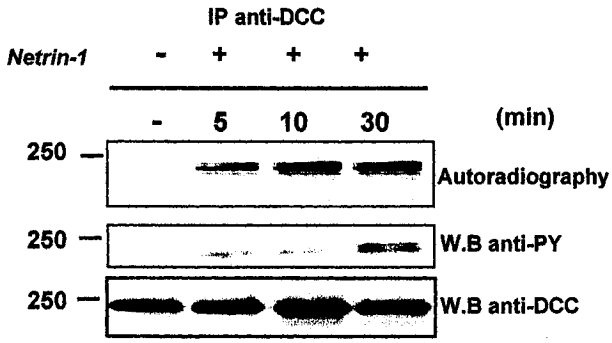
A



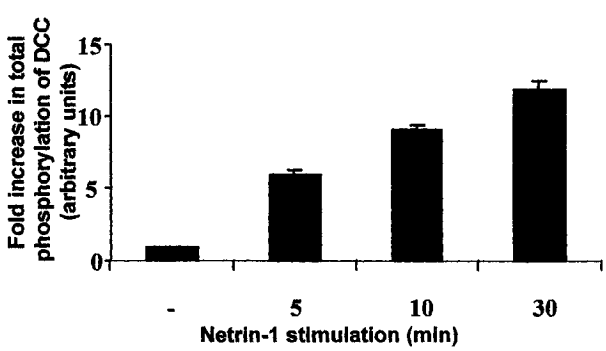
B



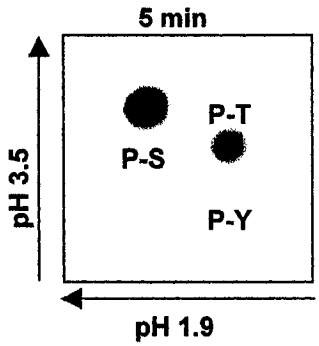
C


IP anti-DCC
Netrin-1
- + + +
- 5 10 30
(min)
250
Autoradiography
250
W.B anti-PY
250
W.B anti-DCC

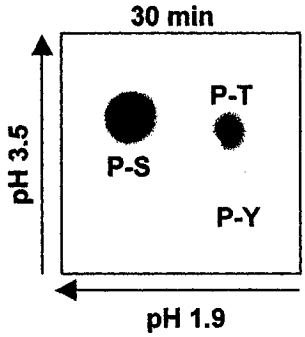
D


Fold increase in total phosphorylation of DCC (arbitrary units)
15
10
5
0
-
5
10
30
Netrin-1 stimulation (min)

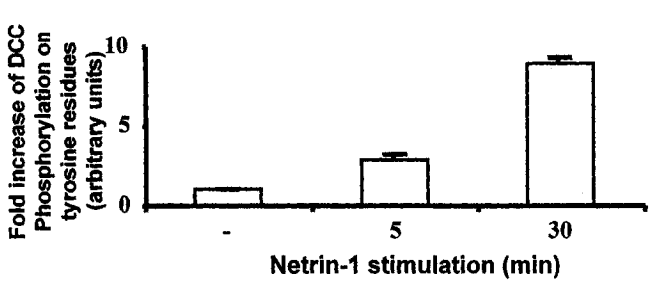
E


5 min
P-T
P-S
P-Y
pH 3.5
pH 1.9

F


30 min
P-T
P-S
P-Y
pH 3.5
pH 1.9

G


Fold increase of DCC Phosphorylation on tyrosine residues (arbitrary units)
10
5
0
-
5
30
Netrin-1 stimulation (min)

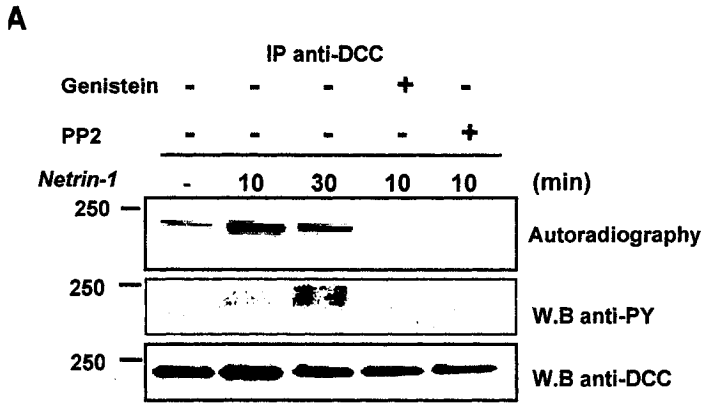
the phosphorylation of DCC. *In vivo* [^{32}P]-orthophosphate-labeled CN were left untreated or treated with either the wide-spectrum tyrosine kinase inhibitor Genistein (Akiyama et al., 1987) or with two different Src family kinase specific inhibitors PP2 (Hanke et al., 1996) and SU6656 (Blake et al., 2000) prior to a 10 min stimulation with Netrin-1. By western blotting using anti-pY antibodies, we show that tyrosine phosphorylation of DCC is completely inhibited when the cells were treated with Genistein (Fig.4.2A and B). PP2 as well as SU6656 treatments of the CN also lead to the inhibition of DCC tyrosine phosphorylation, indicating that the Src kinases are implicated in this phosphorylation event (Fig.4.2A, C and D). Interestingly, immunoprecipitation of [^{32}P]-radiolabelled DCC reveals that both Genistein and PP2 significantly decreased the total phosphorylation of DCC in response to Netrin-1 (Fig. 4.2A and 4.2B). Because serine residues account for most of the phosphorylation sites in DCC (Fig. 4.1E and 4.1F), these data suggest that Src family kinase activity is critical to initiate the molecular events leading to Netrin-1-dependent phosphorylation of DCC.

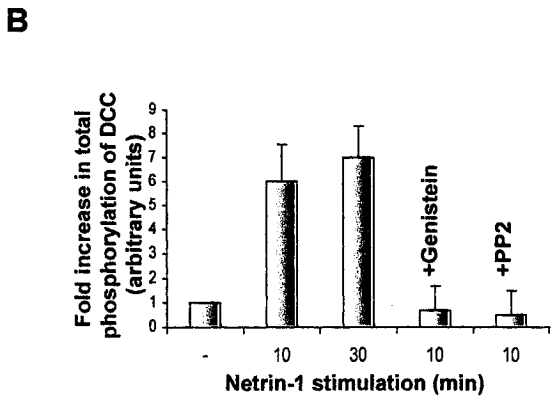
Src family kinases are required for axon outgrowth and attraction induced by Netrin-1

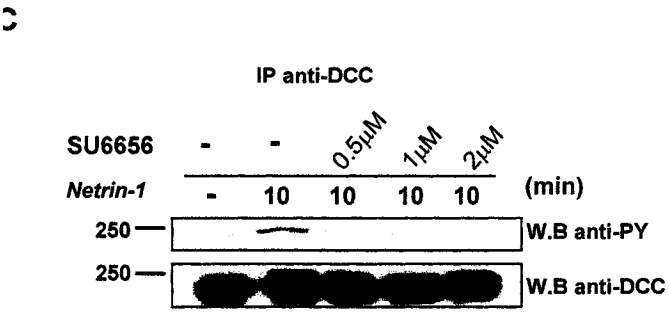
To address the physiological significance of phosphorylation of DCC, we examined whether Src family kinases are involved in mediating the axon outgrowth-promoting effects of Netrin-1. Explants of E13 rat dorsal spinal cord cultured in a 3D collagen gel in presence of Netrin-1 (160 ng/ml) show maximal commissural axon outgrowth (Fig. 4.2E and 4.2F) as previously reported (Serafini, 1996). When PP2 or SU6656 were added in presence of Netrin-1 to the explants, the increase in axon outgrowth was completely abolished similar to the control (Fig. 4.2E and 4.2F). These results demonstrate that inhibition of the Src kinases interferes with the effect of Netrin-1 to mediate commissural axon outgrowth, suggesting a critical role for the Src kinases in netrin-1/DCC signalling. To determine whether Src kinases are required for netrin-1 to mediate axon attraction, we used the *in vitro* turning assay in which *X.laevis* retinal ganglion cells (RGC) turn towards a source of netrin-1 (de la Torre et al.1997). As shown in Fig. 4.3B, a retinal growth cone extending from a 24-hour, stage 24 explant culture turned towards the source of netrin-1 in the control bath. In contrast, the retinal growth

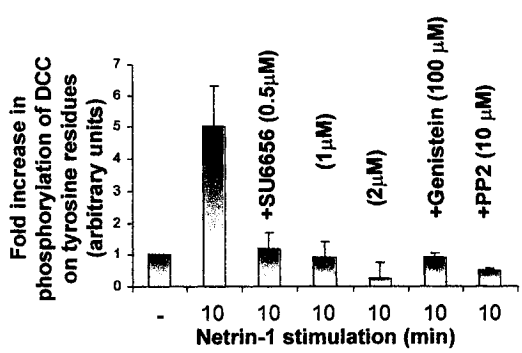
Figure 4.2. DCC phosphorylation on tyrosine residues is Src family kinase-dependent and is critical for Netrin-1-mediated axon outgrowth

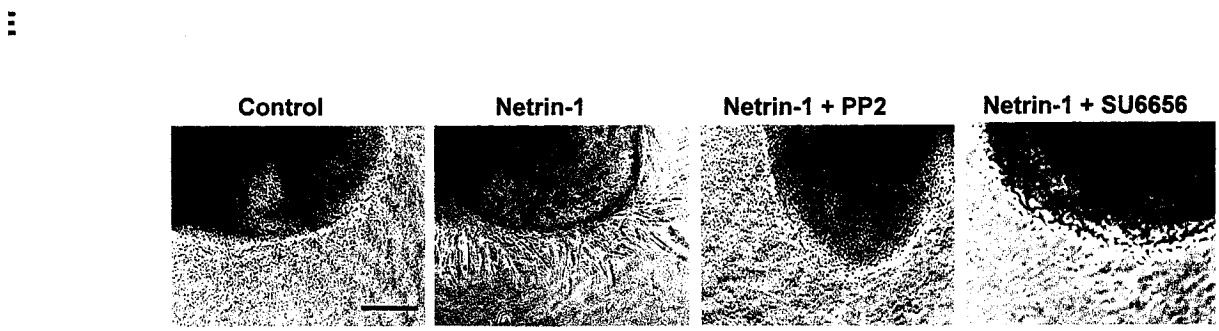
(A) Labeling of CN was followed by stimulation with Netrin-1 for 0, 10 and 30 min. The neurons were also treated or not, for 2h, with either Genistein or PP2 prior to a 10 min Netrin-1 stimulation. (C) Non- labeled rat CN were also treated for 2h with different concentrations of SU6656 prior to a 10 min Netrin-1 stimulation. DCC was immunoprecipitated (IP) from the cell lysates and the products were analyzed by SDS-PAGE and autoradiography. The membrane was immunoblotted with anti-PY and anti-DCC antibodies to analyze the total amount of DCC. (B) Quantitative analysis of the total DCC phosphorylation after Genistein or PP2 treatments (n=4). (D) Quantitative analysis of DCC phosphorylation on tyrosine residues after Genistein, PP2 or SU6656 treatments. (E) E13 rat dorsal spinal cord explants were cultured for 36h, alone (control); in presence of Netrin-1 or with both Netrin-1 and PP2 or SU6656. Magnification: 40X. Bar, 100 μm. (F) Quantification of the total length of axon bundles per explant in μm.


A
IP anti-DCC
Genistein - - - + -
PP2 - - - - +
Netrin-1 - 10 30 10 10 (min)
250
Autoradiography
250
W.B anti-PY
250
W.B anti-DCC


B
Fold increase in total phosphorylation of DCC (arbitrary units)
+Genistein
+PP2
Netrin-1 stimulation (min)


C
IP anti-DCC
SU6656 - - 0.5μM 1μM 2μM
Netrin-1 - 10 10 10 10 (min)
250
W.B anti-PY
250
W.B anti-DCC


D
Fold increase in phosphorylation of DCC on tyrosine residues (arbitrary units)
+SU6656 (0.5μM)
(1μM)
(2μM)
+Genistein (100 μM)
+PP2 (10 μM)
Netrin-1 stimulation (min)


Control
Netrin-1
Netrin-1 + PP2
Netrin-1 + SU6656

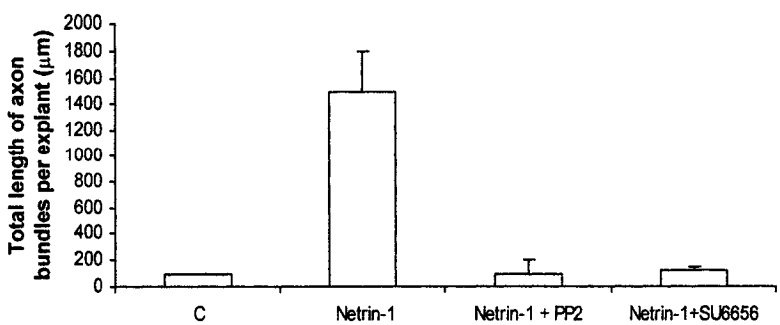
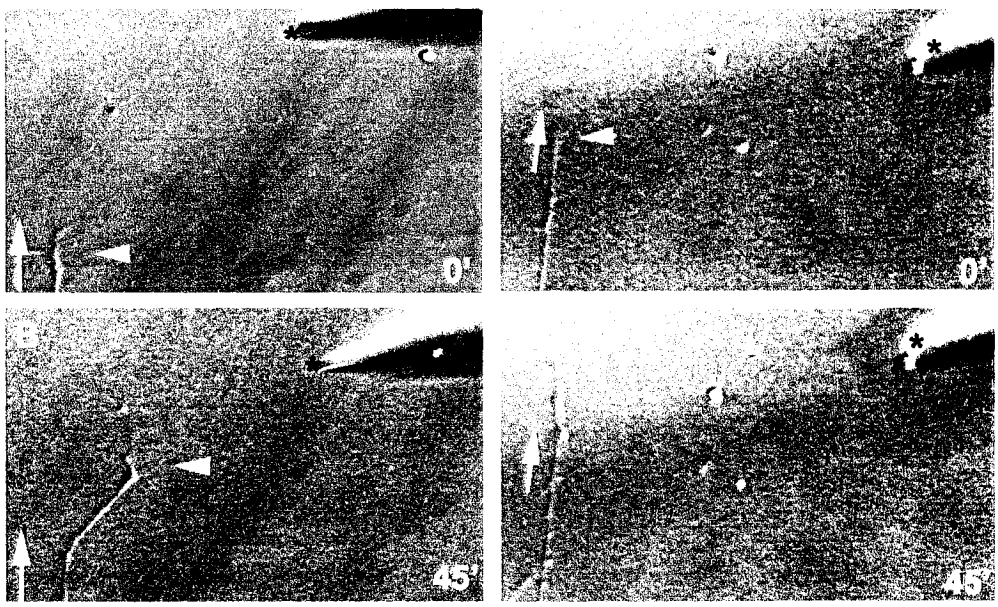
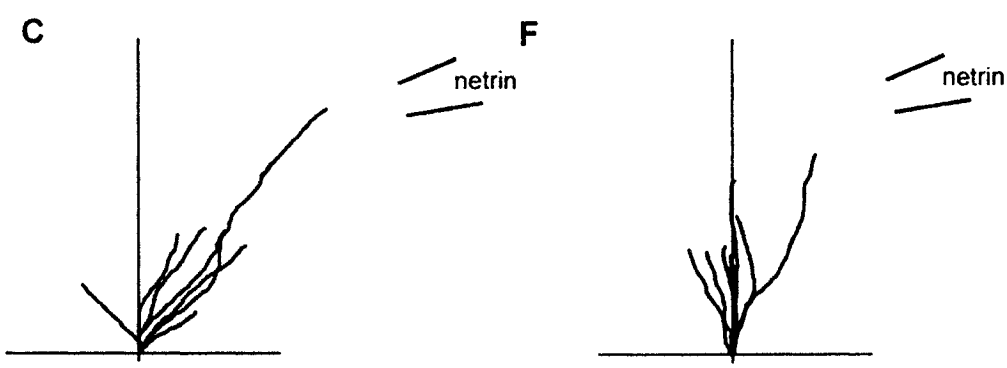

Total length of axon bundles per explant (μm)
C
Netrin-1
Netrin-1 + PP2
Netrin-1+SU6656

Figure 4.3. Inhibition of Src family kinases abolishes netrin-1-induced turning response of RGC growth cones

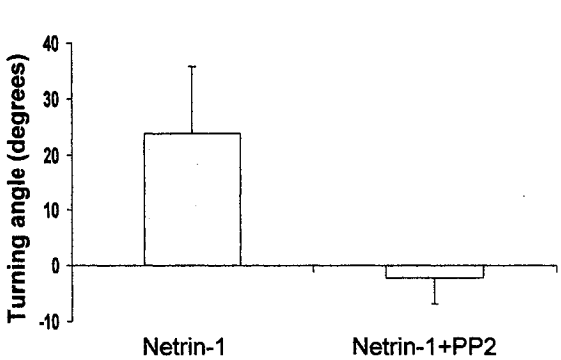
Xenopus RGC growth cones extending from a 24h stage 24 explant culture. Pulsatile ejection of netrin-1 from a pipette (*) with a 0.5-1.0 μm opening was used to set up a concentration gradient next to an extending RGC growth cone (arrowheads). (A, D) Trajectories of growth cones prior to applying the pipette solution (arrows). (B, E) Growth cones 45 min after continuous exposure to the netrin-1 concentration gradient. The growth cone in the control bath (A-B) turned towards the netrin-1 source, whereas the growth cone bathed in 0.25 μM Src kinase inhibitor PP2 (D-E) ignored the netrin-1 source. (C, F) Superimposed neurite trajectories of growth cones exposed to netrin-1 source in a control medium (C), and growth cones exposed to netrin-1 in the presence of PP2 (F). (G) Representation of the mean turning angles of the growth cones in C and F ($p<0.05$; unpaired two-tailed student's t-test).





control bath
n=7

Src kinase inhibitor bath
(0.25 uM PP2)
n=8



cone ignored the source of netrin-1 in the presence of PP2 (Fig. 4.3E). A trace of the paths taken by all growth cones is represented in Fig. 4.3C and F. The turning angles taken by the retinal growth cones were quantified in Fig. 4.3G These results reveal the implication of Src family kinases in the turning response of growth cones to Netrin-1.

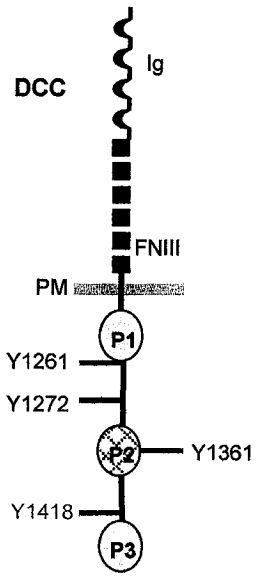
DCC is phosphorylated *in vitro* by Fyn tyrosine kinase

DCC comprises a large extracellular domain of four immunoglobulin repeats followed by six fibronectin type III repeats, a single transmembrane domain and a cytoplasmic tail with three conserved motifs (P1, P2, and P3) (Grunwald and Klein, 2002) (Fig. 4.4A). The P3 region is involved in the ligand-gated multimerization of DCC required to mediate Netrin-1-induced attraction response (Giger and Kolodkin, 2000; Stein, 2001). The P1 region interacts with the intracellular domain of the UNC5 receptor family inducing heterodimerization between DCC and UNC5 receptors that has been shown to be involved in some, but not all, repulsive events induced by Netrin-1 (Merz et al., 2001; Seeger and Beattie, 1999). The cytoplasmic tail of DCC contains four tyrosine residues highly conserved across rat, mouse, human and *X. laevis* species (Fig. 4.4B). Only tyrosine 1272 is conserved in Frazzled, the *Drosophila* ortholog of mammalian DCC, and only Y1418 is conserved in neogenin, a member of the DCC family of proteins. Surprisingly, none of the four-tyrosine residues is conserved in UNC-40, the *C. elegans* ortholog of DCC. The four tyrosine residues in the intracellular domain of the rat DCC are located within the limits of the P1, P2 and P3 regions suggesting that they may play a critical role in DCC function (Fig. 4.4A). Interestingly, only the motif pYEQD containing Y1418 is a likely phosphorylation target site of Src tyrosine kinases as it resembles the known consensus sequence pYEEI for Src kinases (Songyang and al, 1993). We thus examined whether purified Src or Fyn directly phosphorylates *in vitro* the cytoplasmic domain of DCC. Truncated DCC lacking the majority of the extracellular domain (DCC-C) was expressed as a GST fusion protein and purified as described in *Material and Methods*. As shown in Fig. 4.4C, purified Fyn but not Src phosphorylates DCC-C after a 10min incubation. A small range of DCC-C concentrations was used to roughly estimate a Km value of 70nM, suggesting that DCC is a good substrate for Fyn. To determine which tyrosine residue(s) are phosphorylated by Fyn, each tyrosine of the

Figure 4.4. The intracellular domain of DCC is phosphorylated, *in vitro* by Fyn tyrosine kinase

(A) The extracellular domain of DCC is characterized by 4 amino-terminal Immunoglobulin (Ig)-like domains and 6 fibronectin-type III (FNIII) repeats, the transmembrane domain and the cytodomain that contains 3 regions according to the rat amino-acid sequence: P1, P2 and P3. Protein mutants of rat DCC were created by substituting each tyrosine in the cytodomain at 1261, 1272, 1361 and 1418 positions into a phenylalanine residue. (B) Amino acid sequence alignment of DCC proteins from different species and human neogenin. The tyrosine residues corresponding to the rat amino acid sequence of DCC are shown in red. (C) 20 μg of GST fusion proteins containing the cytodomains of the wild type DCC (DCC-C) and DCC-C- Y1261F and DCC-C- Y1418F mutants were incubated without or with the active Fyn or Src kinases in presence of [γ ^{32}P]-ATP. As a positive control Fyn was incubated with 5μg of MBP and Src with 5μg of a specific substrate peptide. The products were analyzed on a SDS-PAGE followed by autoradiography and the total amount of GST fusion proteins is determined by western-blotting (W.B) with anti-DCC antibodies. Autophosphorylated bands of Fyn and Src are also shown as positive controls of their autoactivation. (D) Quantitative analysis of the % of DCC phosphorylated by Src kinases *in vitro*. 100% corresponds to the phosphorylation of wild-type DCC-C protein (n=3).

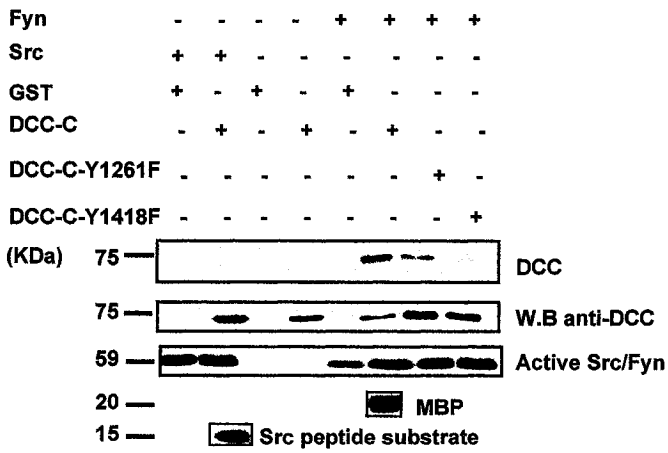
A



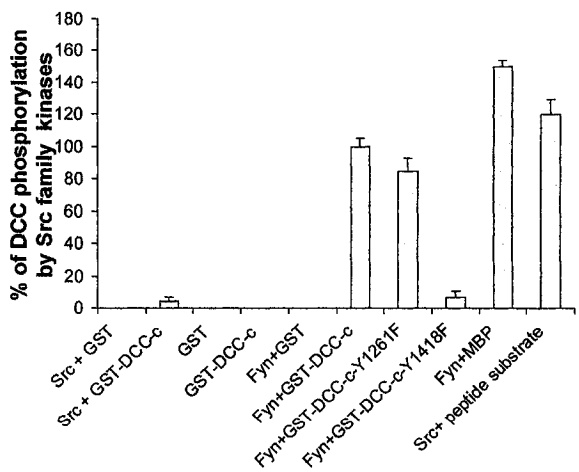
B

		1261 1272	
R. norvegicus	rDCC	AQY---PGILPSPTCGYPHPQFTLRPVP	1283
M. musculus	mDCC	AQY---PGILPSPTCGYPHPQFTLRPVP	1283
H. sapiens	hDCC	AQY---PGILPSPTCGYPHPQFTLRPVP	1283
X. laevis	xDCC	AQY---PGILPSPTCGYPHPQFTLRPVP	1266
H. sapiens	Neogenin	PHHHFHSSSLASPARSHLYHPGSPWPIG	1288
D. melanogaster	Frazzled	PQS---NSMAQTPEHPYGGYDAN-----	1404
		1361	
R. norvegicus	rDCC	KVPYTPLLSQPGPTLPKTHVKTASLGLAGKA	1388
M. musculus	mDCC	KVPYTPLLSQPGPTLPKTHVKTASLGLAGKA	1390
H. sapiens	hDCC	KVPYTPLLSQPGPTLPKTHVKTASLGLAGKA	1390
X. laevis	xDCC	KVPYTTLLSQTGSGLSKAQVKTASLGLAGKA	1370
H. sapiens	Neogenin	ALPSTPLLSQQALNHHIHSVKTASIGTLGRS	1408
		1418	
R. norvegicus	rDCC	VYEQDDLSEQMASLEGLMKQLNAITGSAF--	1445
M. musculus	mDCC	VYEQDDLSEQMASLEGLMKQLNAITGSAF--	1447
H. sapiens	hDCC	VYEQDDLSEQMASLEGLMKQLNAITGSAF--	1447
X. laevis	xDCC	VYEQDDLSEQMASLEGLMKQLNAITGSAF--	1427
H. sapiens	Neogenin	SYEPDELTKEMAHLEGLMKDLNAITTA----	1461

C



D



intracellular domain of DCC was substituted by a phenylalanine residue. Three DCC mutant proteins containing either the Y1261F, Y1272F or Y1361F amino acid substitutions are phosphorylated by Fyn at a similar level as DCC-C (Fig. 4.4C, 4D and data not shown). However, phosphorylation of DCC-C -Y1418F by Fyn is significantly reduced compared to DCC-C (Fig. 4.4C and 4D) indicating that this Y1418 is a phosphorylation target site of Fyn *in vitro*.

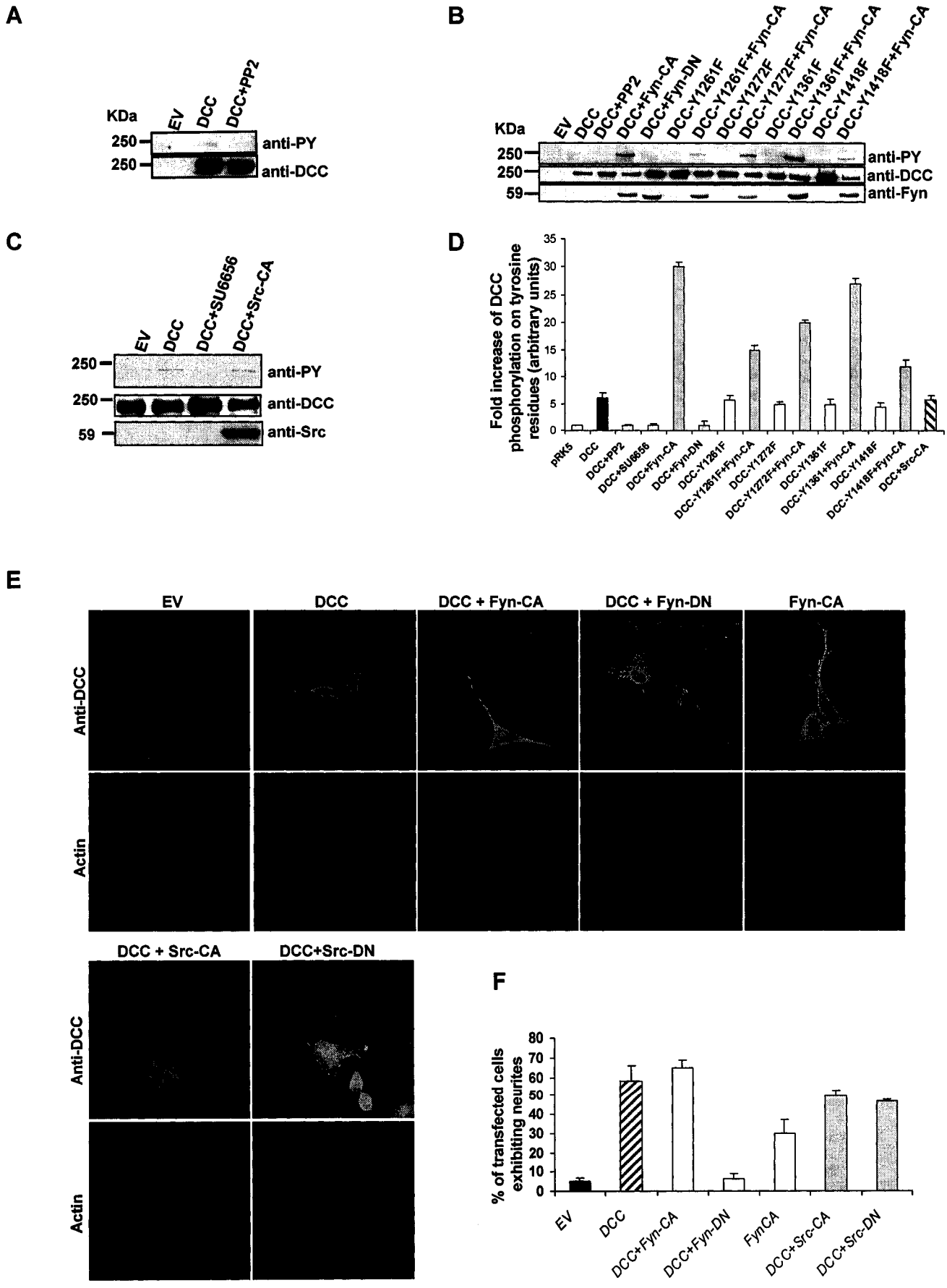
Fyn tyrosine kinase but not Src regulates the phosphorylation of DCC in N1E-115 neuroblastoma cells

To determine which tyrosine residue(s) of DCC are phosphorylated *in vivo*, we investigated the phosphorylation status of the various DCC mutant proteins expressed in N1E-115 mouse neuroblastoma cells. We have previously shown that N1E-115 cells constitutively produce Netrin-1 but do not express DCC. In the presence of serum, N1E-115 cells are round and show lamellipodia formation and multiple filopodia but they do not extend neurites. The expression of DCC in these cells induces neurite extension (Li et al., 2002b). As shown in Fig. 4.5A, DCC is phosphorylated on tyrosine residues in N1E-115 cells, consistent with the tyrosine phosphorylation of DCC observed in rat primary CN. To confirm that phosphorylation of DCC is regulated by Fyn tyrosine kinase in N1E-115 cells, we co-expressed constitutively active Fyn (Fyn-CA) or dominant negative Fyn (Fyn-DN) with DCC in N1E-115 cells (Fig. 4.5B). Indeed, Fyn-CA shows a 30-fold increase in the level of tyrosine phosphorylation of DCC, whereas Fyn-DN inhibits the basal level of tyrosine phosphorylation of DCC similar to the results observed when cells are treated with PP2 or SU6656 (Fig. 4.5A, 4.5B, 4.5C and 4.5D). In comparison, the expression of constitutively active Src (Src-CA) in N1E-115 cells did not affect the basal level of DCC phosphorylation (Fig. 4.5C and 4.5D).

To further characterize the tyrosine residue(s) phosphorylated by Fyn *in vivo,* DCC tyrosine mutants Y1261F, Y1272F, Y1361F and Y1418F were co-expressed with Fyn-CA in N1E-115 cells. As shown in Fig. 4.5B and 4.5D, the tyrosine phosphorylation status of DCC mutant proteins Y1272F and Y1361F is moderately decreased compared to wild-type DCC. The level of tyrosine phosphorylation of DCC mutant proteins containing Y1261F or Y1418F, however, shows a 2-fold decrease compared to the wild-type protein.

Figure 4.5. Fyn tyrosine kinase regulates the phosphorylation of DCC and is critical for DCC-induced neurite outgrowth in N1E-115 cells

(A) N1E-115 cells were transfected with empty vector (EV) or pRK5-DCC (DCC). Cells expressing DCC were treated with PP2. DCC was immunoprecipitated from the lysates and the total amount of DCC was determined using anti-DCC antibodies. Anti-pY antibodies were used to show the level of DCC phosphorylation on tyrosines. (B) The empty vector (EV), DCC, DCC-Y1261F, DCC-Y1272F, DCC-Y1361F, or DCC-Y1418F constructs were transfected either alone or together with Fyn-CA in N1E-115 cells. DCC was also co-transfected with Fyn-DN. The expression of these proteins was analyzed by western-blot using anti-DCC and anti-Fyn antibodies and the phosphorylation level of these proteins was assessed using anti-pY antibodies. (C) N1E-115 cells were transfected with empty vector (EV), DCC, alone or with Src-CA. DCC transfected cells were also treated with SU6656. After DCC immunoprecipitation, the phosphorylation level of these proteins was assessed using anti-pY antibodies and the total amount of the expressed proteins was determined using anti-DCC and anti-Src antibodies. (D) Quantitative analysis of the tyrosine phosphorylation of DCC and DCC mutant proteins in N1E-115 cells (n=3). (E) N1E-115 cells were co-transfected with empty vector (EV) and DCC either alone or together with Fyn-CA, Fyn-DN, Src-CA or Src-DN. The cells were co-stained with anti-DCC antibodies and rhodamine conjugated phalloidin to visualize the actin filaments. Fyn or Src expression was visualized using anti-p62 antibodies (Santa-Cruz) (data not shown). (F) Quantitative analysis of the % of transfected N1E-115 cells exhibiting neurites shown in (E) (n=3) was performed in a blinded fashion.


A
KDa
EV
DCC
DCC+PP2
250
250
anti-PY
anti-DCC
B
KDa
EV
DCC
DCC+PP2
DCC+Fyn-CA
DCC+Fyn-DN
DCC-Y1261F
DCC-Y1261F+Fyn-CA
DCC-Y1272F
DCC-Y1272F+Fyn-CA
DCC-Y1361F
DCC-Y1361F+Fyn-CA
DCC-Y1418F
DCC-Y1418F+Fyn-CA
250
250
59
anti-PY
anti-DCC
anti-Fyn
C
EV
DCC
DCC+SU6656
DCC+Src-CA
250
250
59
anti-PY
anti-DCC
anti-Src
D
Fold increase of DCC phosphorylation on tyrosine residues (arbitrary units)
35
30
25
20
15
10
5
0
pRK5
DCC
DCC+PP2
DCC+SU6656
DCC+Fyn-CA
DCC+Fyn-DN
DCC-Y1261F
DCC-Y1261F+Fyn-CA
DCC-Y1272F
DCC-Y1272F+Fyn-CA
DCC-Y1361F
DCC-Y1361F+Fyn-CA
DCC-Y1418F
DCC-Y1418F+Fyn-CA
DCC+Src-CA
E
EV
DCC
DCC + Fyn-CA
DCC + Fyn-DN
Fyn-CA
Anti-DCC
Actin
DCC + Src-CA
DCC+Src-DN
Anti-DCC
Actin
F
% of transfected cells exhibiting neurites
80
70
60
50
40
30
20
10
0
EV
DCC
DCC+Fyn-CA
DCC+Fyn-DN
FynCA
DCC+Src-CA
DCC+Src-DN

Consistent with the data obtained in the *in vitro* kinase assay, these results show that DCC is phosphorylated by Fyn but not Src in N1E-115 cells and that tyrosines 1261 and 1418 are the major phosphorylation sites of Fyn *in vivo*.

Fyn tyrosine kinase activity but not Src is required to mediate neurite outgrowth induced by DCC

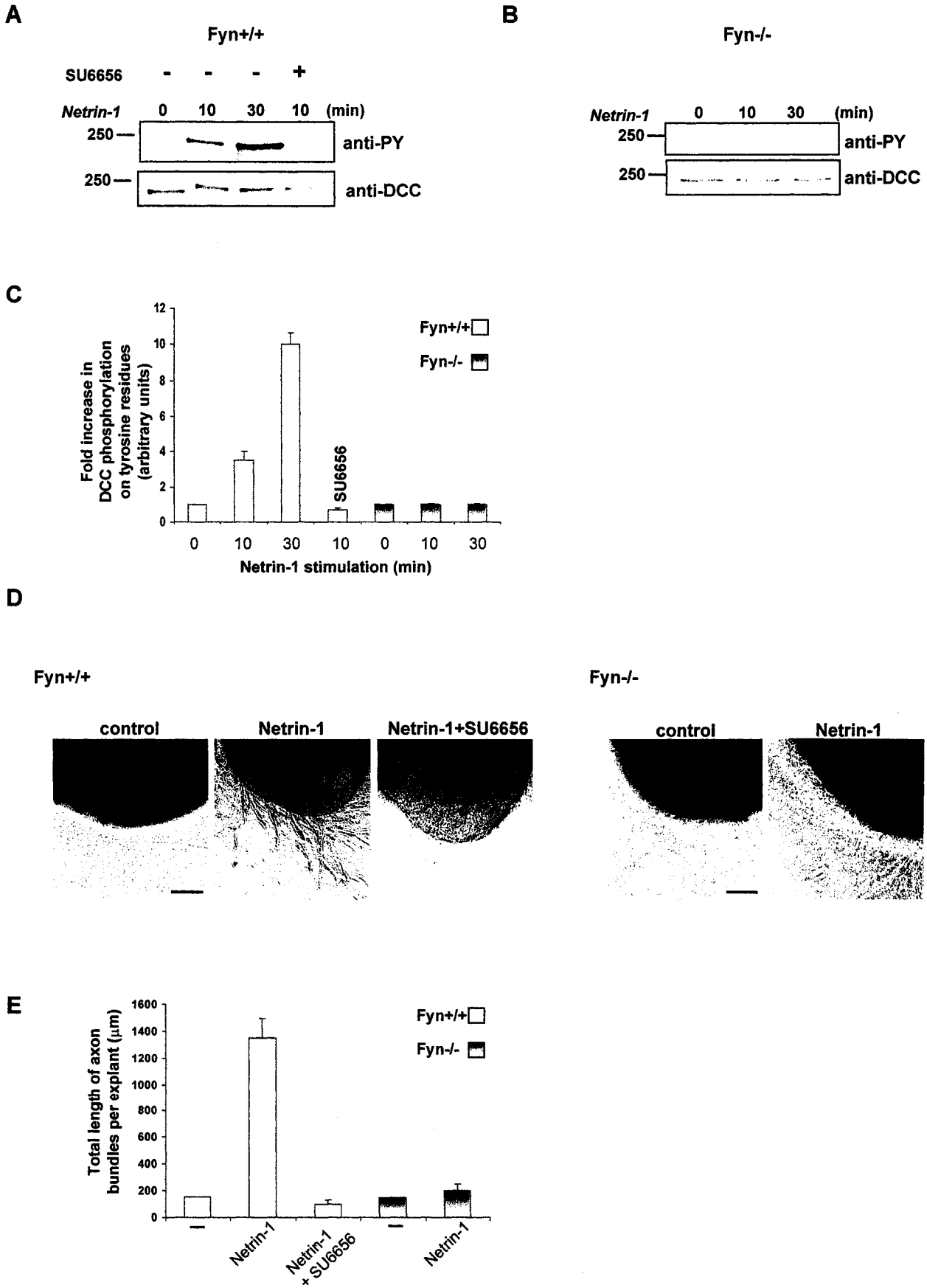
To further demonstrate the importance of the tyrosine kinase activity of Fyn on the neurite outgrowth function of DCC, N1E-115 cells were co-transfected with DCC and with either Fyn-CA or Fyn-DN. Indeed, we show that the expression of Fyn-DN completely inhibits neurite extensions induced by DCC whereas the expression of Fyn-CA leads to a small increase in cells exhibiting neurites compared to cells expressing DCC alone (Fig. 4.5E and 4.5F). Likewise, treatment of N1E-115 cells expressing DCC with PP2 or SU6656 abolish the ability of DCC to induce neurite extensions, demonstrating the essential role of Src family kinases in the neurite outgrowth function of DCC (Fig. 4.7A and 4.7B). The expression of Fyn-CA by itself induces the formation of neurites in N1E-115 cells as previously demonstrated (Suetsugu et al., 2002) but at a lesser extent than induced by DCC expression (Fig. 4.5E and 4.5F). In comparison, the expression of Src-CA or Src-DN in N1E-115 cells did not affect the neurite outgrowth function of DCC (Fig. 4.5E and 4.5F). These results show that the kinase activity of Fyn but not of Src is required for the neurite outgrowth function of DCC.

Fyn tyrosine kinase activity is essential for DCC phosphorylation and netrin-1-dependent axon outgrowth function *in vivo*

To confirm that Fyn tyrosine kinase is implicated *in vivo* in the regulation of the phosphorylation of DCC, we examined the phosphorylation level of DCC in embryonic CN from Fyn deficient mice (Fyn-/-) in response to Netrin-1. As shown in figure 4.6A and 4.6C, a 10-fold increase in DCC phosphorylation on tyrosine residues is observed after 30 min of Netrin-1 stimulation in wild-type E11.5 CN. However, no tyrosine phosphorylation band is detected when DCC is immunoprecipitated from Fyn-/- CN, after 30 min of Netrin-1 stimulation, or from wild-type CN pretreated with SU6656 before a 10 min Netrin-1 stimulation (Figure 4.6A, 4.6B and 4.6C). Then, we examined the ability of

Figure 4.6. Netrin-1-induced DCC phosphorylation on tyrosine residues and neurite outgrowth are impaired in Fyn -/- mice

(A) E11.5 Fyn+/+ mouse CN were stimulated or not for 10 min with Netrin-1. The neurons were also pre-treated with SU6656 prior to a 10 min stimulation with netrin-1. DCC was immunoprecipitated from the cell lysates and the products were analyzed by SDS-PAGE. The membrane was immunoblotted with anti-pY and anti-DCC antibodies. (B) E11.5 CN dissected from Fyn -/- mice were stimulated or not for 10 or 30 min with Netrin-1. DCC immunoprecipitates were analyzed by western-blot using anti-pY and anti-DCC antibodies. (C) Quantitative analysis of the phosphorylation level of DCC on tyrosine residues after Netrin-1 stimulation in E11.5 wild-type or Fyn -/- CN, determined by densitometry (n=3). (D) E11.5 dorsal spinal cord explants from Fyn+/+ or Fyn-/- mice were cultured for 20h, alone (control) or in presence of Netrin-1. Fyn+/+ explants were also treated with both Netrin-1 and SU6656. Magnification: 40X. Bar, 100 μm. (E) Quantification of the total length of axon bundles per explant in μm (n=36).


A
Fyn+/+
SU6656 - - - +
Netrin-1 0 10 30 10 (min)
250
anti-PY
250
anti-DCC
B
Fyn-/-
Netrin-1 0 10 30 (min)
250
anti-PY
250
anti-DCC
C
Fold increase in DCC phosphorylation on tyrosine residues (arbitrary units)
Fyn+/+
Fyn-/-
SU6656
0 10 30 10 0 10 30
Netrin-1 stimulation (min)
D
Fyn+/+
control
Netrin-1
Netrin-1+SU6656
Fyn-/-
control
Netrin-1
E
Total length of axon bundles per explant (μm)
Fyn+/+
Fyn-/-
Netrin-1
Netrin-1 + SU6656
Netrin-1

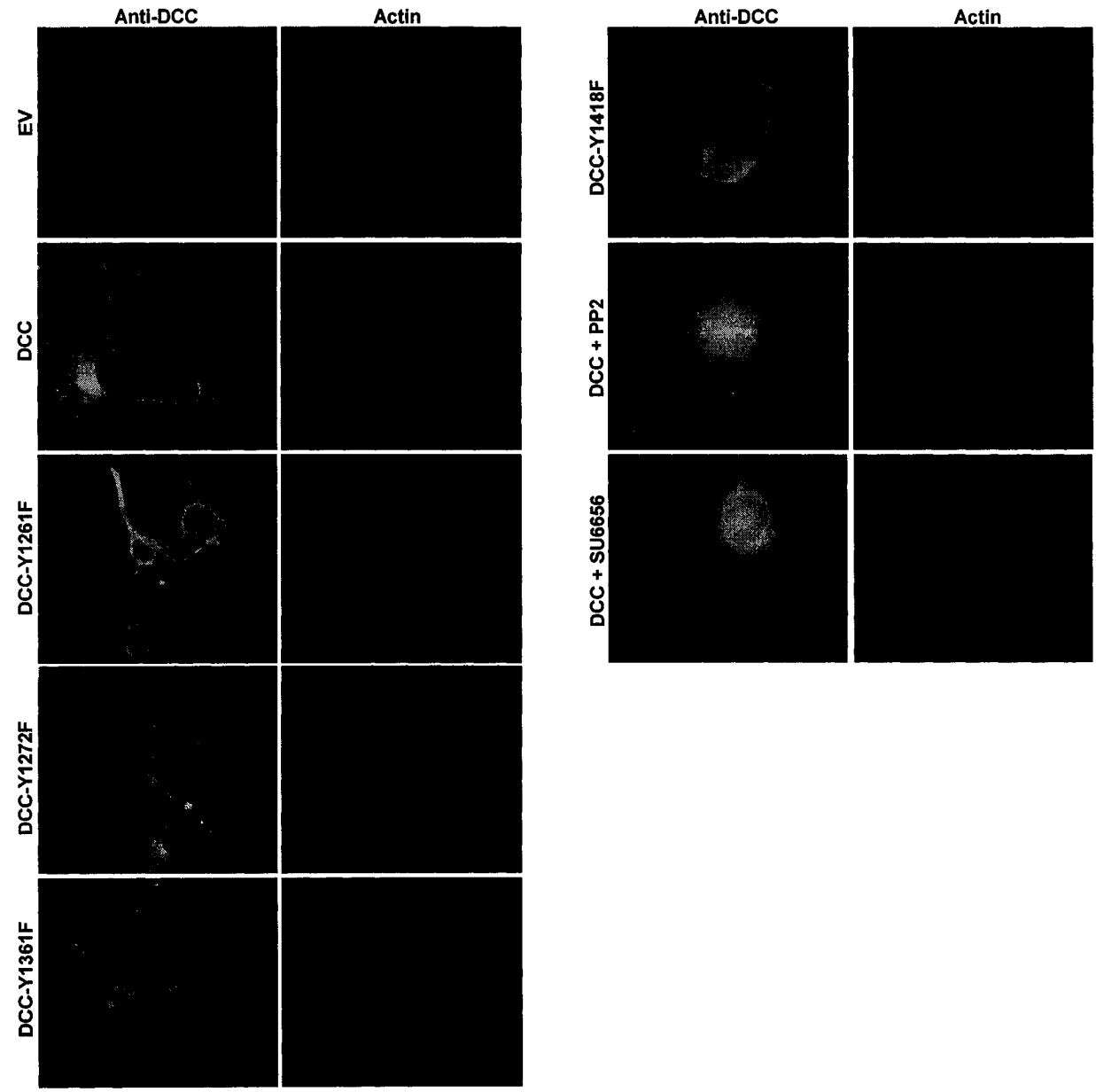
dorsal spinal cord explants from Fyn -/- mouse embryos to extend neurites in response to Netrin-1 in comparison to Fyn +/+ explants. As shown in figure 4.6D, explants from Fyn+/+ mice, treated 20h with Netrin-1, showed maximal axon outgrowth compared to the non-treated control explants. When the Src family kinase inhibitor SU6656 was added with Netrin-1 to the Fyn +/+ explants, the axon outgrowth was completely abolished (Figure 4.6D and 4.6E), as observed with rat dorsal spinal cord explants. However, when explants from Fyn -/- dorsal spinal cords were cultured in presence of Netrin-1 for 20h, the axon outgrowth was completely abolished in up to 90% of the total number of explants examined (n=36) (Figure 4.6D and 4.6E). These results are consistent with the data obtained in vitro showing the crucial role of Fyn tyrosine kinase in DCC phosphorylation and Netrin-1-induced outgrowth function *in vivo*.

Phosphorylation of tyrosine 1418 is critical for DCC-induced neurite outgrowth in N1E-115 cells

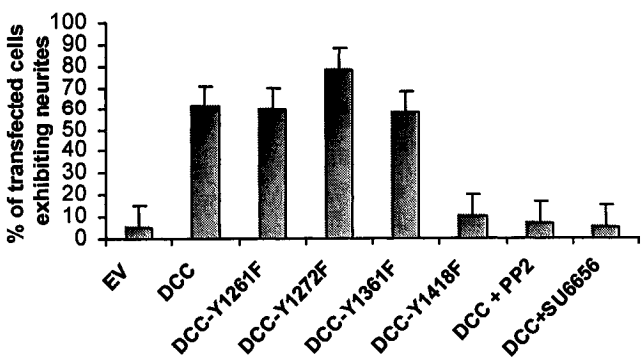
To define the importance of tyrosine phosphorylation of DCC in neurite outgrowth, we examined whether N1E-115 cells expressing the various DCC mutants can extend neurites. In the presence of 5% serum, control cells are round with some lamellipodia structures and filopodia (Fig. 4.7A). In contrast, cells expressing DCC exhibit long neurite extensions with thin filopodia along the neurites (Fig. 4.7A and 4.7B). The expression of Y1261F, Y1272F or Y1361F -DCC mutant proteins induces neurite extensions in N1E-115 cells similar to the levels obtained with the expression of DCC. However, expression of Y1418F mutant protein completely inhibited the ability of DCC to induce neurite outgrowth (Fig. 4.7A and 4.7B). Cells expressing Y1418F mutant are flat and round with no specific actin structures, similar to cells treated with PP2 or SU6656. Although both Y1261 and Y1418 are phosphorylated by Fyn in N1E-115 cells, these findings indicate that only phosphorylation of Y1418 is critical for DCC to induce neurites in response to netrin-1.

Figure 4.7. DCC phosphorylation on tyrosine 1418 is critical for neurite outgrowth in N1E-115 cells

(A) Immunocytochemistry of N1E-115 cells expressing DCC, Y1261F, Y1272F, Y1361F or Y1418F –DCC mutant proteins. The cells were co-stained with anti-DCC antibodies and rhodamine conjugated phalloidin to stain F-actin. Cells expressing DCC were treated either with PP2 or SU6656. Bar, 20 μm. Magnification: 63X. (B) Quantitative analysis of the % of transfected N1E-115 cells exhibiting neurites shown in (A) (n=4). The % of transfected N1E-115 cells exhibiting neurites was determined by counting more than 100 expressing cells exhibiting at least one neurite per cell. A neurite is defined as a process that measured at least the length of one cell body.


Anti-DCC
Actin
EV
DCC
DCC-Y1261F
DCC-Y1272F
DCC-Y1361F
Anti-DCC
Actin
DCC-Y1418F
DCC + PP2
DCC + SU6656

B


% of transfected cells exhibiting neurites
100
90
80
70
60
50
40
30
20
10
0
EV
DCC
DCC-Y1261F
DCC-Y1272F
DCC-Y1361F
DCC-Y1418F
DCC + PP2
DCC+SU6656

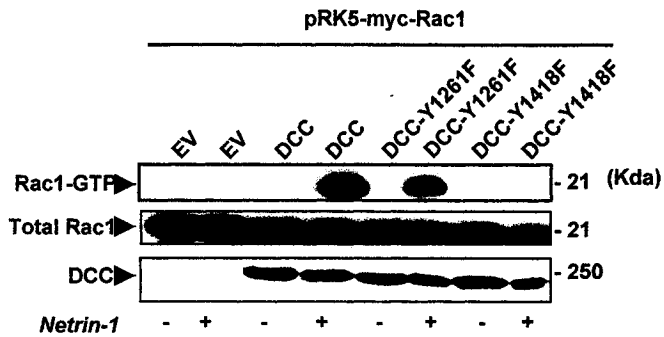
Phosphorylation of tyrosine 1418 is essential for DCC-induced activation of the small GTPase Rac1

We have previously demonstrated that expression of DCC in fibroblasts specifically activates the small GTPase Rac1 in a Netrin-1-dependent manner and that Rac1 activity is essential for DCC-induced neurite outgrowth in N1E-115 cells (Li et al., 2002b). To address whether substitution of Y1418 by a phenylalanine residue interferes with the signalling pathways leading to Rac1 activation after Netrin-1 stimulation, we performed a pull-down assay in which GTP-loaded Rac1 was trapped by specific binding to the Cdc42/Rac interactive binding domain (CRIB) of $p65^{PAK}$ fused to glutathione S-transferase (GST-PAK). DCC or the various DCC mutants were co-expressed with myc-tagged Rac1 in COS-7 cells for 24h and serum-starved overnight. After 5 min of Netrin-1 stimulation, protein lysates were prepared and incubated with GST-PAK, and the amount of Rac1-GTP precipitated by GST-PAK was determined by Western-blot analysis. As shown in Fig. 4.8A and 4.8B, Netrin-1 stimulates an 8-fold increase in the level of activated Rac1. The expression of Y1261F, Y1272F or Y1361F -DCC mutant protein shows a slight decrease in the level of activated Rac1 induced by Netrin-1 compared to DCC (Fig. 4.8A, 4.8B and data not shown). In contrast, activation of Rac1 by Netrin-1 was completely abolished when Y1418F mutant protein was expressed in COS-7 cells (Fig. 4.8A and 4.8B). Thus, phosphorylation of Y1418 seems to be crucial for the activation of Rac1 by DCC. Together with the results obtained in N1E-115 cells, these data provide evidence for an important role of the phosphorylation of Y1418 in the signalling pathways mediated by the Netrin-1 receptor DCC leading to neurite outgrowth.

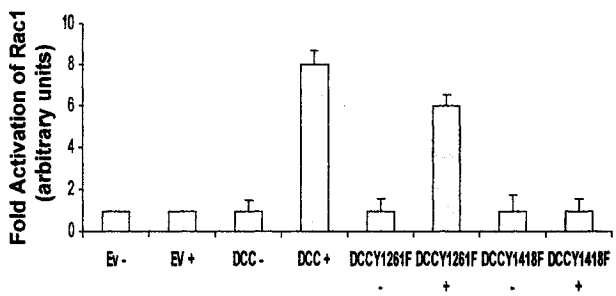
Figure 4.8. DCC phosphorylation on tyrosine 1418 is critical for Netrin-1- induced Rac1 activation

(A) Cos-7 cells co-expressing DCC, Y1261F or Y1418F –DCC mutants together with myc-Rac1 protein were treated or not with Netrin-1 for 5min. The GTP loaded Rac1 were pulled-down from the cell lysates using GST-CRIB-Pak1 fusion protein. The proteins from the pull-down and from the total cell lysate were analyzed by western-blot using anti-DCC antibodies and anti-myc antibodies to detect both Rac1-GTP and the total Rac1. (B) Quantitative analysis of Rac1 activation by DCC and DCC tyrosine mutants after Netrin-1 treatment (n=5).

A


pRK5-myc-Rac1
EV
EV
DCC
DCC
DCC-Y1261F
DCC-Y1261F
DCC-Y1418F
DCC-Y1418F
Rac1-GTP
- 21 (Kda)
Total Rac1
- 21
DCC
- 250
Netrin-1
- + - + - + - +

B


Fold Activation of Rac1 (arbitrary units)
10
8
6
4
2
0
Ev -
EV +
DCC -
DCC +
DCCY1261F -
DCCY1261F +
DCCY1418F -
DCCY1418F +

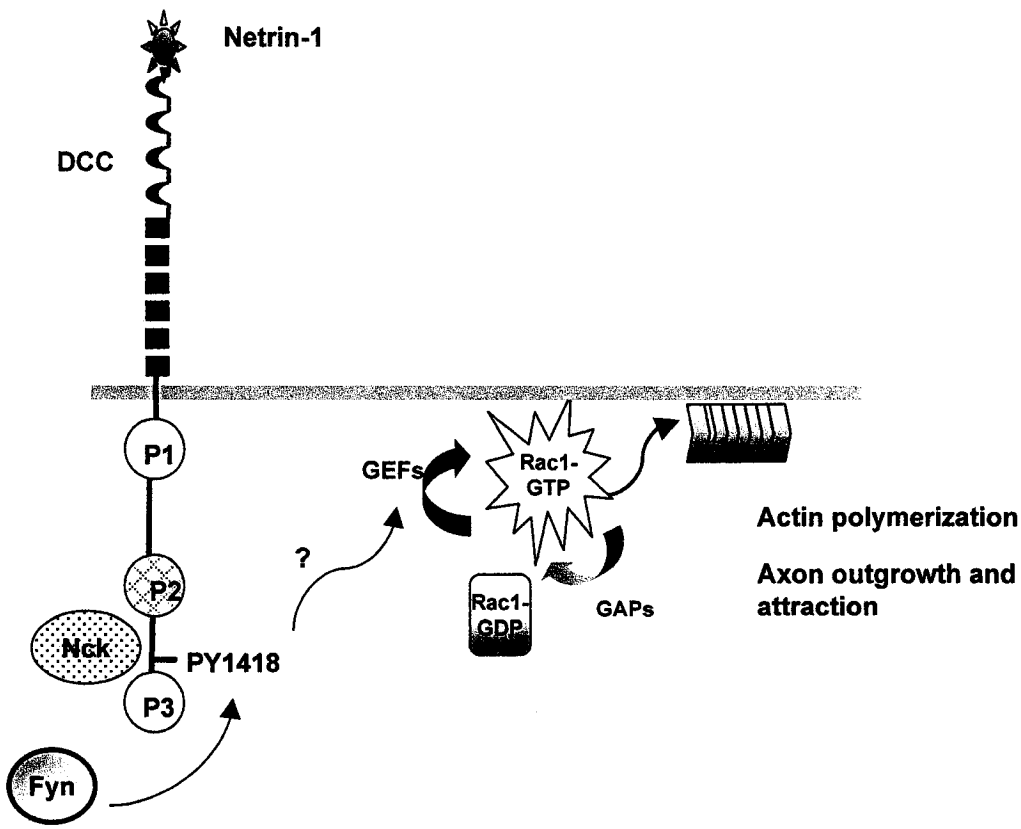
DISCUSSION

In the present study, we provide the first evidence that the transmembrane receptor DCC is regulated by phosphorylation in a Netrin-1-dependent manner. In dissociated CN, DCC is highly phosphorylated on serine, threonine and tyrosine residues in response to Netrin-1. Fyn tyrosine kinase regulates DCC phosphorylation and is required to mediate axon outgrowth and attraction induced by Netrin-1. *In vitro* studies suggest that Y1418 in the vicinity of the P3 region of the intracellular tail of DCC is a likely phosphorylation site for Fyn but not Src tyrosine kinase. Phosphorylation of this tyrosine residue is essential for DCC to promote neurite outgrowth in N1E-115 cells, and to mediate downstream signals leading to the activation of the small GTPase Rac1 (Fig. 4.9).

Biochemical and genetic studies have demonstrated the essential role of tyrosine phosphorylation in neurite outgrowth and growth cone guidance (Bashaw et al., 2000; Desai et al., 1997; Flanagan and Vanderhaeghen, 1998; Killeen et al., 2002; Tong et al., 2001). In the case of netrin signalling, tyrosine phosphorylation of the mammalian UNC5H3 receptor has been observed in mouse brain. In addition, Netrin-1 was able to stimulate tyrosine phosphorylation of UNC5H3 expressed in HEK-293 cells (Tong et al., 2001), however, the physiological role of UNC5H3 tyrosine phosphorylation remains to be demonstrated. On the other hand, mutations of nine tyrosine residues in the cytoplasmic domain of *C. elegans* UNC-5 severely compromise UNC-5 function *in vivo* (Killeen et al., 2002). Tyrosine phosphorylation of UNC-40 *in vivo* has also been reported in *C. elegans* although the role of this phosphorylation has not been determined (Tong et al., 2001). In this study, we demonstrated that Netrin-1 stimulates the *in vivo* tyrosine phosphorylation of DCC in metabolically radiolabeled CN dissociated from the developing spinal cord. Interestingly, we also observed that DCC is highly phosphorylated on ser/thr residues as well. The role of ser/thr phosphorylation of DCC is still unknown and will require further investigation. The interaction of DCC with the MAP kinases ERK1/2 and MEK1/2 proteins and their contribution to Netrin signalling in axon outgrowth and guidance (Campbell and Holt, 2003; Forcet et al., 2002) suggest that

Figure 4.9. *The model.* Netrin-1 binding to DCC induces a rapid phosphorylation of Y1418 in the vicinity of the P3 region by Fyn. This primary event might initiates directly or indirectly the activation of a specific unknown GEF that will activate Rac1 leading subsequently to actin assembly at the plasma membrane, axon outgrowth and attraction.

The model


Netrin-1
DCC
P1
P2
Nck
PY1418
P3
Fyn
?
GEFs
Rac1-GTP
Rac1-GDP
GAPs
Actin polymerization
Axon outgrowth and attraction

ERK1/2 and MEK 1/2 are interesting candidates to investigate as potential Ser/Thr kinases for DCC.

When DCC phosphorylation is completely inhibited by the Src family kinase inhibitor PP2, Netrin-1 is no longer able to induce commissural axon outgrowth or to reorientate retinal growth cones. These findings suggest that phosphorylation of DCC is essential to mediate the intracellular neuronal responses leading to axon outgrowth and attraction. Moreover, it shows that Src family kinases are crucial to Netrin-1-mediated axon outgrowth and guidance. This is consistent with several studies that have implicated Src and Fyn tyrosine kinases as pivotal players in CNS development (Arnaud et al., 2003; Brouns et al., 2001; Maness et al., 1996; Morse et al., 1998; Sperber et al., 2001). Indeed, Src, Fyn, Yes and Lyn tyrosine kinases are expressed in the developing vertebrate CNS, where they are enriched in growth cone membrane fractions, suggesting that they may play a role in neurite outgrowth and guidance (Bare et al., 1993; Bixby and Jhabvala, 1993; Maness et al., 1988). However, studies from the single knock-out mutants of the tyrosine kinases Src, Fyn, Yes and Lyn revealed that only Fyn -/- mice display any obvious neuronal defects and reduced tyrosine phosphorylation of proteins in the brain (Grant et al., 1992). Interestingly, the inhibition of Src family kinase activity decreases the total phosphorylation of DCC below the basal level, suggesting that blocking Src family kinase activities also impairs DCC phosphorylation on ser/thr residues. One possibility is that phosphorylation of specific tyrosine residue(s) is a prerequisite step to facilitate subsequent phosphorylation on ser/thr residues of DCC. One can also hypothesize that the activity of Src family tyrosine kinases is required as an initiation event to activate the ser/thr kinase(s) leading to full phosphorylation of the receptor DCC. In the present study, we found that tyrosine phosphorylation of DCC is highly increased in the presence of Fyn-CA but not Src-CA when both proteins are expressed in N1E-115 cells. As well, the basal level of tyrosine phosphorylation of DCC is also inhibited in the presence of PP2 or SU6656. In addition, we demonstrated that Fyn but not Src phosphorylates the intracellular domain of DCC *in vitro* and that DCC tyrosine phosphorylation is completely impaired in Fyn-/- CN. Therefore, we propose that Fyn is the major kinase involved in the regulation of DCC phosphorylation.

We identified that Y1418 is the major phosphorylation site by Fyn tyrosine kinase *in vitro*, however, when each of the DCC tyrosine mutant proteins are co-expressed with Fyn in N1E-115 cells, tyrosine phosphorylation of both Y1261F and Y1418F –DCC mutants shows a 2-fold decrease compared to DCC. These findings suggest that both Y1261 and Y1418 are phosphorylation target sites of Fyn *in vivo*. Interestingly, only mutation of Y1418 impairs the ability of DCC to induce neurite outgrowth in N1E-115 cells similar to inhibition of neurite outgrowth by PP2 and SU6656. Therefore, our data suggest that more than one tyrosine of the cytoplasmic tail of DCC is phosphorylated *in vivo* by Fyn but only phosphorylation of DCC Y1418 by Fyn is required to mediate neurite outgrowth in N1E-115 cells. Consistent with our data, more than one tyrosine residue in the cytodomain of *C. elegans* UNC-5 is phosphorylated *in vivo* but only Y482 in the juxtamembrane region is critical for UNC-5 axon guidance function *in vivo* (Killeen et al., 2002). Y1418 in the cytoplasmic tail of DCC is well conserved among the vertebrate species and *X. laevis*, whereas it is not conserved in *C. elegans* UNC-40 and *D. melanogaster* Frazzled. There are however other tyrosine residues that may serve a similar function as Y1418 in UNC-40 and Frazzled. Interestingly, Y1418 is also conserved within the cytoplasmic tail of neogenin, a member of the DCC family of proteins. Netrin-1/neogenin interaction has been recently shown to play a role in mammary gland morphogenesis, a non-neural tissue (Srinivasan et al., 2003). It will be of interest to address whether the conserved tyrosine in neogenin is phosphorylated and play a similar role in cell migration and non-neural organogenesis.

We and others have previously shown that DCC induces the activation of the small GTPase Rac1 in response to Netrin-1 stimulation (Li et al., 2002b; Shekarabi and Kennedy, 2002). Rac1 activity is required for DCC to induce neurite outgrowth in N1E-115 neuroblastoma cells, however, the cascade of molecular events from DCC leading to Rac1 activation is still poorly understood. Here we show that phosphorylation of Y1418 in DCC is required to trigger Rac1-GTP loading in a Netrin-1-dependent manner. Interestingly, mutation of the other tyrosine residues in the intracellular domain of DCC did not interfere with Rac1 activation. Consistent with the data obtained in N1E-115 cells, these results emphasize the essential role that Rac1 plays in Netrin-1-mediated neurite outgrowth.

Based on these data, we propose the following model (Fig. 49). Netrin-1 binding to DCC induces rapid phosphorylation of Y1418 in the vicinity of the P3 region in the intracellular domain of DCC by Fyn. This primary event will initiate directly or indirectly the activation of a specific unknown GEF that will lead to the GTP loading of Rac1. Activation of Rac1 results in actin assembly at the plasma membrane that leads to axon outgrowth and attraction. The adapter molecule Nck-1 is constitutively bound to DCC and its role in the regulation of phosphorylation of DCC remains elusive. It is however possible that Nck-1 serves to recruit Fyn in close proximity to DCC via its free SH2 domain in response to Netrin-1.

Recent data have shown that signalling through Calcineurin and NFAT proteins is implicated in regulating embryonic axon outgrowth in response to neurotrophins and netrins. It has been proposed that Netrin-1 induces a calcineurin/NFAT molecular cascade controlling the axon outgrowth function of DCC but not the growth cone attraction (Graef et al., 2003). Here, we suggest that phosphorylation of DCC and the subsequent intracellular events leading to Rac1 activation are involved in axon outgrowth and in Netrin-1-mediated axon attraction. It will be of great interest to determine in future studies which proteins interact with the specific phosphorylation sites of DCC and how they affect the outgrowth and chemotropic responses of axons to Netrin-1.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 MAJOR FINDINGS

1. Previous to the findings in this thesis, very little was known about the biochemical regulation of CdGAP and the DCC receptor. We have demonstrated that phosphorylation of CdGAP inside cells is enhanced by extracellular factors which activate components of the Ras-MEK-ERK pathway (Tcherkezian et al., 2005). This increase in CdGAP phosphorylation was abrogated by a pharmacological inhibitor against the MEK enzyme. Moreover, we have identified two kinases which are involved in Ras signaling, ERK and Rsk-1 that interact with and phosphorylate CdGAP. We have also shown that phosphorylation of CdGAP on T776 by ERK negatively regulates its activity both *in vitro* and *in vivo*. These findings provide compelling evidence that oncogenic Ras can activate Rac not only through activation of a RhoGEF but also by inhibiting a RhoGAP.

2. We have also demonstrated that the human ortholog of CdGAP is also active both *in vitro* and *in vivo* on Cdc42 and Rac1 but not RhoA. Furthermore, we found that the human protein is also highly phosphorylated inside cells on serine and threonine residues but not tyrosine (Tcherkezian et al., 2005). Given that the ultimate goal of studying mouse proteins is to eventually be able to correlate some of the findings to human biology, these results suggest that at least for CdGAP, some of the results obtained in mice could also be true in humans.

3. We have provided for the first time the evidence that DCC function is regulated through phosphorylation (Meriane, Tcherkezian et al., 2004). Our results clearly show that netrin-1 induces phosphorylation of DCC on serine, threonine and tyrosine residues, in dissociated spinal cord commissural neurons. Tyrosine phosphorylation of DCC is mainly achieved by the Src family tyrosine kinases that also seem to be required for DCC phosphorylation on serine and threonine residues. Treatment of neurons with specific Src family kinase inhibitors abrogated netrin-1 induced commissural axon outgrowth and turning of retinal growth cones. Moreover, we have shown that phosphorylation of DCC on Y1418 appears to be critical for DCC induced neurite outgrowth of N1E-115 neuroblastoma cells and activation of Rac-1 in COS-7 cells. These findings have provided tremendous insight on the biochemical regulation of DCC and have raised the possibility that other neuronal receptors which lack intrinsic kinase activity could also be regulated by non-receptor tyrosine kinases.

5.2. CDGAP and RSK

In this thesis, we have focused most of our attention on understanding the role of ERK1/2 in CdGAP phosphorylation and regulation. Although we have provided convincing evidence that threonine phosphorylation of CdGAP is important in regulating its activity, it has raised some inevitable technical issues. Given that CdGAP is mainly phosphorylated on serine residues *in vivo*, mutating threonine residues has not provided significant changes in total CdGAP phosphorylation *in vivo* and has required the use of phosphopeptide mapping in order to detect changes in CdGAP phosphorylation. In fact, phosphopeptide mapping of CdGAP phosphorylated inside cells demonstrated that mutation of threonine residues does not induce changes in the major phosphopeptides. Therefore, mutation of serine residues will almost certainly enable us to detect significant changes in total CdGAP phosphorylation. An alternative approach in studying serine and threonine phosphorylation is the use of commercially available antibodies that detect these residues. Unfortunately, we have found that most of these antibodies give poor results and do not offer an interesting alternative to metabolic P^{32} labeling.

So far, RSK-1 is the only kinase that phosphorylates CdGAP mainly on serine residues and is likely responsible for some of CdGAP's phosphorylation on serines. In fact, the protein sequence of CdGAP contains at least three consensus RSK phosphorylation sites, which are all serine residues (Fig. 2.6). Interestingly, mass spectrometry analysis has revealed that the tryptic peptide ENSLPEIVPPPFHTVLELPDNKRKLSSK which contains one of the RSK consensus sites is a phosphopeptide with at least one phosphorylation site (Table 5.1). In the event this serine residue is phosphorylated, it would create a potential binding site for the 14-3-3 protein. These proteins belong to a family consisting of highly conserved acidic proteins, with molecular weights of 25-30 KDa. There are at least seven mammalian isoforms, which are found in all eukaryotic cells. 14-3-3 acts as an adaptor which is able to move freely from cytoplasm to nucleus and vice-versa (Muslin and Lau, 2005). They are phosphoserine-binding proteins that bind to the consensus motifs RSXpSXP and RXY/FXpSXP. These consensus motifs are present in almost all of the 14-3-3 binding partners (Yaffe and Elia, 2001). Numerous families of proteins interact with 14-3-3 in a phosphorylation-dependent manner, including proteins implicated in actin dynamics such

Table 5.1. Mass spectrometry analysis of CdGAP phosphopeptides

Overexpressed CdGAP was immunoprecipitated from COS-7 cells and digested with trypsin for direct MALDI and IMAC (phosphopeptide enrichment). Results show that among the four phosphopeptides identified, three are located in the proline-rich domain (PRD) of CdGAP and one is from the central domain. Similar to the phosphopeptide mapping, these results suggest that most of the phosphorylation sites are located within the PRD. The first phosphopeptide contains the RSK consensus site (RENS) and the third harbors the ERK site identified in CdGAP (PPT(776)-P).

Masses m/z	Position AA	# de site (P)	Sequence AA
3264.7034	270-297	1 P	(R)ENSLPEIVPPPFHTVLELPDNK RKLSSK(S)
4823.2422	649-693	2 P	(K)IIESEEEFSSLPPAAQKTSPIPE SSPAPFPFPEAPGSLPSSSAPR(E)
2922.4195	763-788	2 P	(R)NLSPPLTPAPPPPTPLEEEPEV LLSK(Æ)
2216.9406	802-820	2 P	(R)TDVYTEQPTPKESPGIPTP(-)

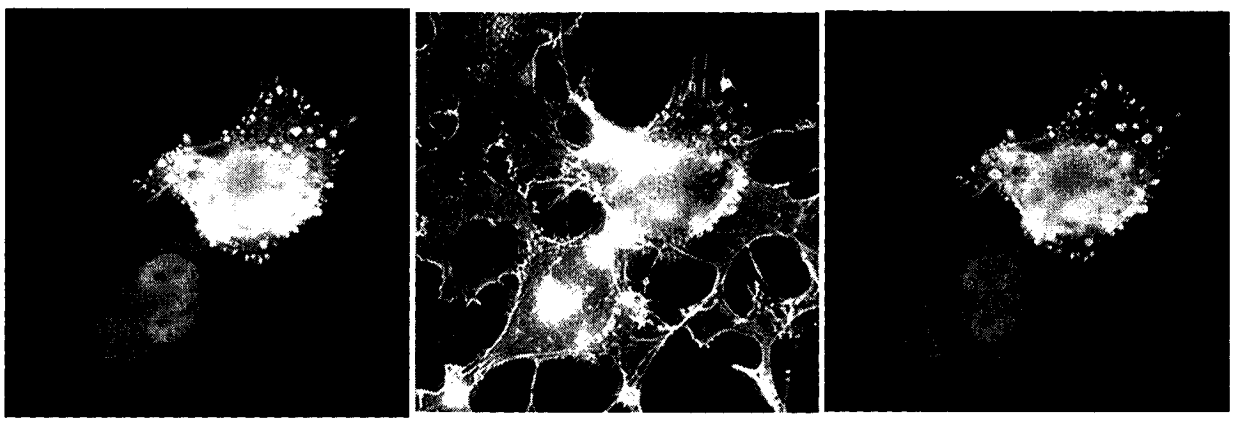
as RhoGEF and RhoGAP proteins (Jin et al., 2004). RSK has been shown to create 14-3-3 binding sites in a number of proteins involved in a wide range of biological processes (Chun et al., 2005; Fujita et al., 2003; Kinuya et al., 2000; Lehoux et al., 2001; Lizcano et al., 2000; Tan et al., 1999). 14-3-3 proteins regulate protein function by a number of mechanisms including nucleocytoplasmic shuttling, acting as a scaffold in promoting protein-protein interactions, and sequestering cytotoxic proteins, to mention only a few. 14-3-3 protects cells against apoptosis by binding and sequestering the proapoptotic protein BAD in the cytosol where it cannot induce apoptosis by interacting with BCL-X_L and BCL2 in the mitochondria. Interestingly, overexpression of CdGAP in cell lines can cause plasma membrane blebbing which is a hallmark of apoptosis (Fig.5.1A). This phenotype is not simply caused by the overexpression of a RhoGAP protein since p50RhoGAP does not cause blebbing (Fig 5.1B). It will be interesting to investigate whether 14-3-3 interacts with CdGAP and protects cells against plasma membrane blebbing or potentially apoptosis, by sequestering CdGAP in the nucleus or the cytoplasm. In fact, immunocytochemistry reveals that overexpressed CdGAP localizes both in the nucleus and the cytoplasm, when compared to activated ERK which is mainly present in the nucleus (Fig. 5.2).

As mentioned previously, the RSK family consists of four members which seem to have overlapping and specific functions. We have found that RSK-1 -2 and -3 can phosphorylate CdGAP *in vitro* with equal efficiency (Fig. 5.3A). However, only RSK-1 protein seems to be highly expressed in the cell lines used to study CdGAP phosphorylation (Fig. 5.3B). By using the same amount of recombinant proteins, we have found that all three kinases are specifically recognized by their respective antibodies (Fig 5.3C). However, it has been reported that over 95 % of endogenous RSK3 is lost to the insoluble fraction during clearing of crude cell lysates (Zhao et al., 1996). Therefore, the absence of RSK-3 in the cell lines could be caused by technical issues. These results do not rule out the possibility that isoforms other than RSK-1 could interact with CdGAP and it will be interesting to investigate their role in CdGAP function.

Figure 5.1. CdGAP causes plasma membrane blebbing

Serum growing COS-7 cells were transfected with pRK5Myc-CdGAP (A) or pRK5Myc-p50RhoGAP (B) for 48 h with the DEAE-Dextran method. CdGAP and p50RhoGAP were visualized by indirect immunofluorescence using monoclonal anti-Myc antibodies and secondary antibodies coupled to FITC. Picture merge was done using Northern Eclipse software. Actin was visualized by staining with TRITC-coupled to phalloidin. (A) In the left panel, we can see that CdGAP causes plasma membrane blebbing and seems to be localized in these bubble-like structures. In the middle and the right panel (merge), it seems that the blebs are surrounded by an actin ring, a phenomenon that has been reported in the literature. (B) We can see that in the three cells, p50RhoGAP causes what is known as pseudopodia (long extension) but does not seem to cause plasma membrane blebbing.

A

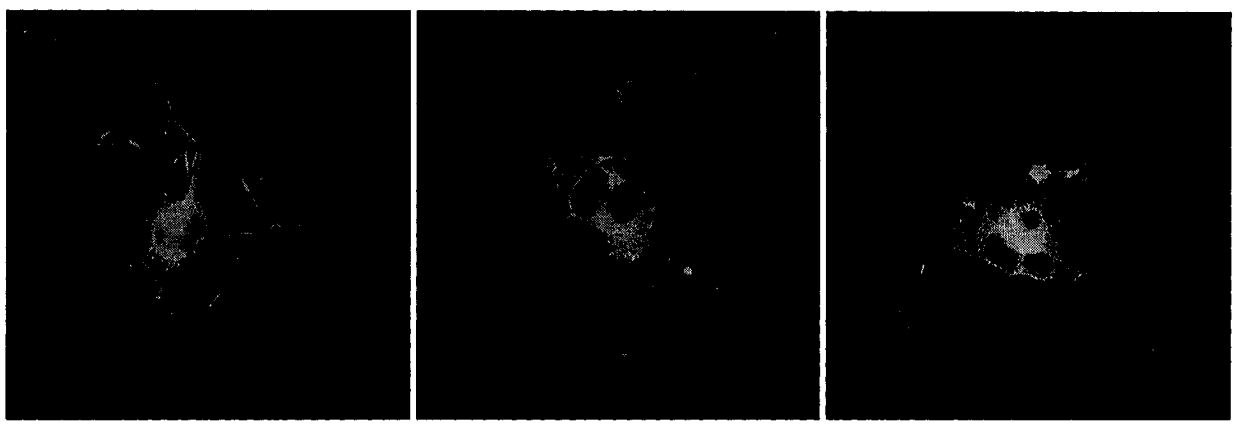


MycCdGAP
FITC

F-actin
Phalloidin

Merge

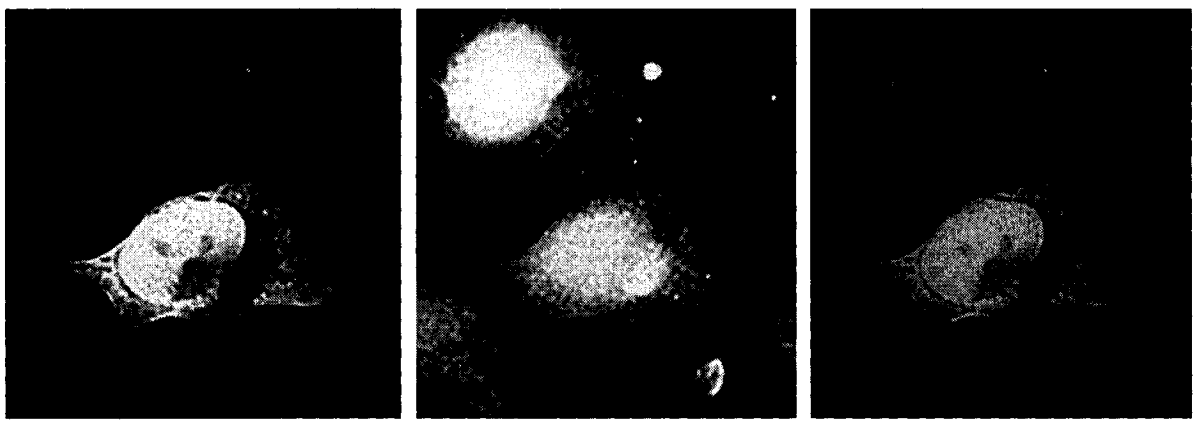
B



Myc-p50RhoGAP
FITC

Figure 5.2. Localization of overexpressed CdGAP

Serum growing COS-7 cells were transfected with pRK5Myc-CdGAP for 48 h with the DEAE-Dextran method. CdGAP and endogenous activated ERK1/2 were visualized by indirect immunofluorescence using monoclonal anti-Myc antibodies and polyclonal anti-phospho-ERK1/2 antibodies and secondary antibodies coupled to FITC and TRITC, respectively. Picture merge was done using Northern Eclipse software. In the left panel, we can see that CdGAP is localized in the nucleus and the cytoplasm, whereas the middle panel shows that activated ERK is mainly in the nucleus.



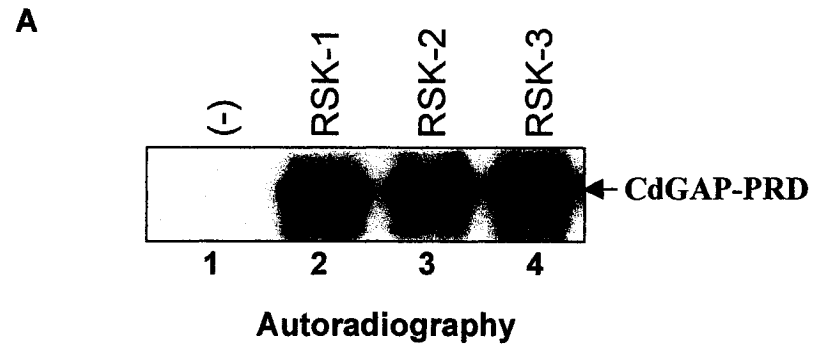
CdGAP
FITC

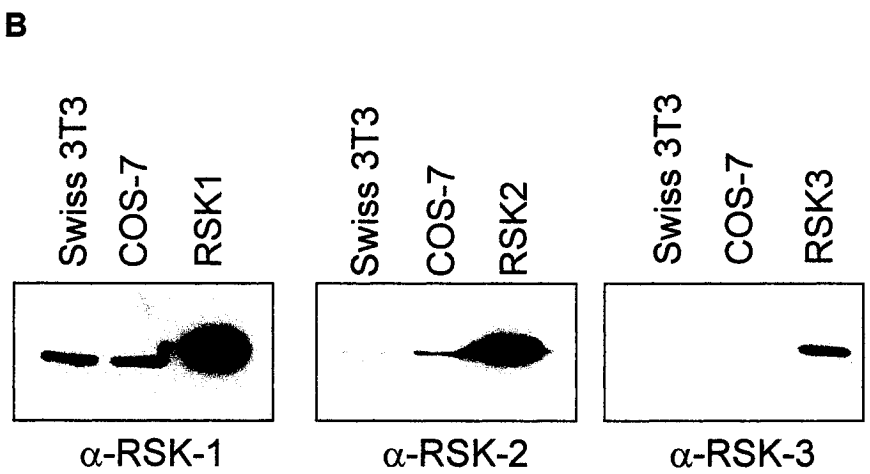
Endogenous
P-ERK1/2
TRITC

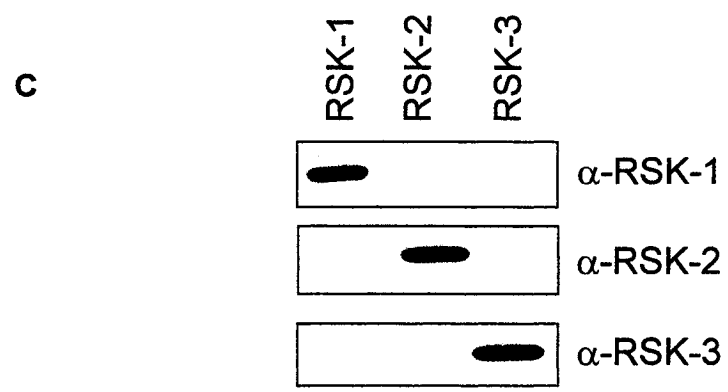
Merge

Figure 5.3. Expression of RSK isoforms and phosphorylation of CdGAP

(A) All four RSK isoforms phosphorylate CdGAP with similar efficiency. An *in vitro* kinase assay was performed by incubating recombinant His-tagged CdGAP-PRD without (lane 1) or with equal amounts of activated RSK-1 (lane 2), RSK-2 (lane 3), RSK-3 (lane 4) in the presence of 10 μCi/ml [γ-^{32}P]-ATP. The products were resolved by SDS-PAGE and phosphorylated substrates were detected by autoradiography. (B) Expression of RSK isoforms in cell lines. Cell lysates from Swiss 3T3 (lane 1) and COS-7 (lane 2) fibroblasts as well as equal amounts of recombinant RSK1 to -3 (lanes 3) as positive controls were resolved by SDS-PAGE and proteins were transferred on nitrocellulose membrane for immunoblotting (IB) using polyclonal anti-RSK-1, -2, -3 antibodies. (C) Specificity of RSK antibodies. Equal amounts of recombinant RSK1 to -3 were resolved by SDS-PAGE and proteins were transferred onto nitrocellulose membrane for immunoblotting (IB) using polyclonal anti-RSK-1, -2, -3 antibodies


A
(-)
RSK-1
RSK-2
RSK-3
CdGAP-PRD
1
2
3
4
Autoradiography


B
Swiss 3T3
COS-7
RSK1
Swiss 3T3
COS-7
RSK2
Swiss 3T3
COS-7
RSK3
α-RSK-1
α-RSK-2
α-RSK-3


C
RSK-1
RSK-2
RSK-3
α-RSK-1
α-RSK-2
α-RSK-3

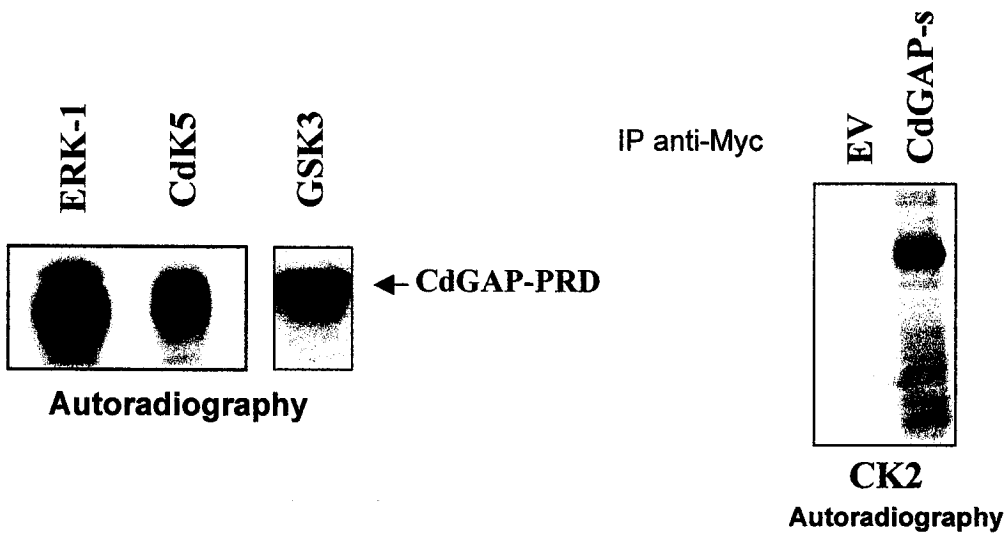
5.3. OTHER KINASES THAT PHOSPHORYLATE CDGAP

The CdGAP protein sequence contains a number of consensus phosphorylation sites for a variety of Ser/Thr kinases including ERK, RSK, PKC, CK2, Cdk5 and GSK3, to name only a few. We have found that in addition to ERK and RSK, CdGAP is also phosphorylated *in vitro* by GSK3, CKII and CdK5 (Fig.5.4). We have identified GSK3 from a yeast two hybrid assay by using the proline-rich domain of CdGAP as a bait. GSK3 interacts with CdGAP inside cells and like ERK, it phosphorylates CdGAP on T776 (Danek et al., manuscript in preparation). Unlike ERK, GSK3 enzymes are mainly active in the absence of extracellular signals. This provides an attractive mechanism by which CdGAP's activity could be regulated both in the presence or absence of external factors.

The biological relevance of CdGAP phosphorylation by Cdk5 and CKII has not been determined yet, but it is tempting to speculate. Cyclin-dependent kinase 5 (Cdk5), is a proline-directed kinase that was originally identified as a member of the cyclin-dependent kinase family based on sequence homology (Lew et al., 1992). Whereas Cdks are traditionally involved in regulating cell cycle progression, Cdk5 does not participate in cell cycle regulation. Despite the demonstrated association of Cdk5 with cyclin D, activation of Cdk5 is not dependent on cyclin D (Xiong et al., 1992). Instead, two regulatory proteins that share little sequence homology with cyclins, p35 and p39, have been identified as activators of Cdk5 (Humbert et al., 2000; Tang et al., 1995). Despite the ubiquitous expression of Cdk5, kinase activity of Cdk5 is largely detected only in the nervous system, since p35 and p39 expression is found almost exclusively in the nervous system. Similar to Rho GTPases, p35/Cdk5 has an important role in neuronal morphogenesis and survival (Nikolic, 2002; Nikolic, 2004). It was shown that p35/Cdk5 forms a complex with Rac and Pak-1 and activated p35/Cdk5 causes Pak1 hyperphosphorylation in a Rac-dependent manner, which appears to be important in promoting neuronal migration and neurite outgrowth (Nikolic et al., 1998). Furthermore, α2-chimaerin associates with Cdk5/p35, and together participate in Sema 3A signaling in which α2-chimaerin GAP activity is required (Brown et al., 2004). Given that CdGAP is also expressed in neuronal cells (Fig 2.1C), it will be interesting to investigate whether

Figure 5.4. Phosphorylation of CdGAP by GSK3, CKII and CdK5

An *in vitro* kinase assay was performed by incubating recombinant His-tagged CdGAP-PRD or immunoprecipitated CdGAP from COS-7 cells with activated-ERK-1, -GSK3, -Cdk5 and -CK2 in the presence of 10 μCi/ml [γ-^{32}P]-ATP. The products were resolved by SDS-PAGE and phosphorylated substrates were detected by autoradiography. EV: empty vector.


ERK-1
CdK5
GSK3
CdGAP-PRD
Autoradiography
IP anti-Myc
EV
CdGAP-s
CK2
Autoradiography

phosphorylation of CdGAP by Cdk5 could inhibit its GAP activity thereby promoting neurite outgrowth.

CK2, formerly known as casein kinase 2, is a ubiquitous, highly pleiotropic and constitutively active kinase with specificity for serine/threonine residues in the vicinity of acidic amino acids. Ironically, although well over 300 potential physiological targets of CK2 have been identified, it seems very unlikely that CK2 has any role in the *in vivo* phosphorylation of casein, the protein from which it originally derived its name. As the list of likely physiological targets for CK2 continues to grow, it becomes increasingly evident that CK2 has the potential to participate in the regulation of a diverse selection of cellular processes including transcription, signaling, proliferation and development (Meggio and Pinna, 2003). CK2 has been shown to regulate cell morphology by regulating the activities of a number of proteins involved in actin dynamics (Canton and Litchfield, 2005). However, CK2 has not been shown to interact with RhoGEF or RhoGAP proteins, therefore, if CdGAP is an actual physiological substrate of CK2, it could provide an attractive link between CK2 signaling and the actin cytoskeleton.

5.4. DCC AND MAPK

Given that growth cones are often located thousands of cell diameters away from the cell body, they need to be able to synthesize proteins locally in order to produce a rapid response to guidance cues. It has been reported that growth cones contain all the necessary machinery for local protein translation (Brittis et al., 2002). It was demonstrated that guidance molecules like netrin-1 steer axon growth by activating translation initiation factors and triggering rapid local changes in protein levels in growth cones (Campbell and Holt, 2001).

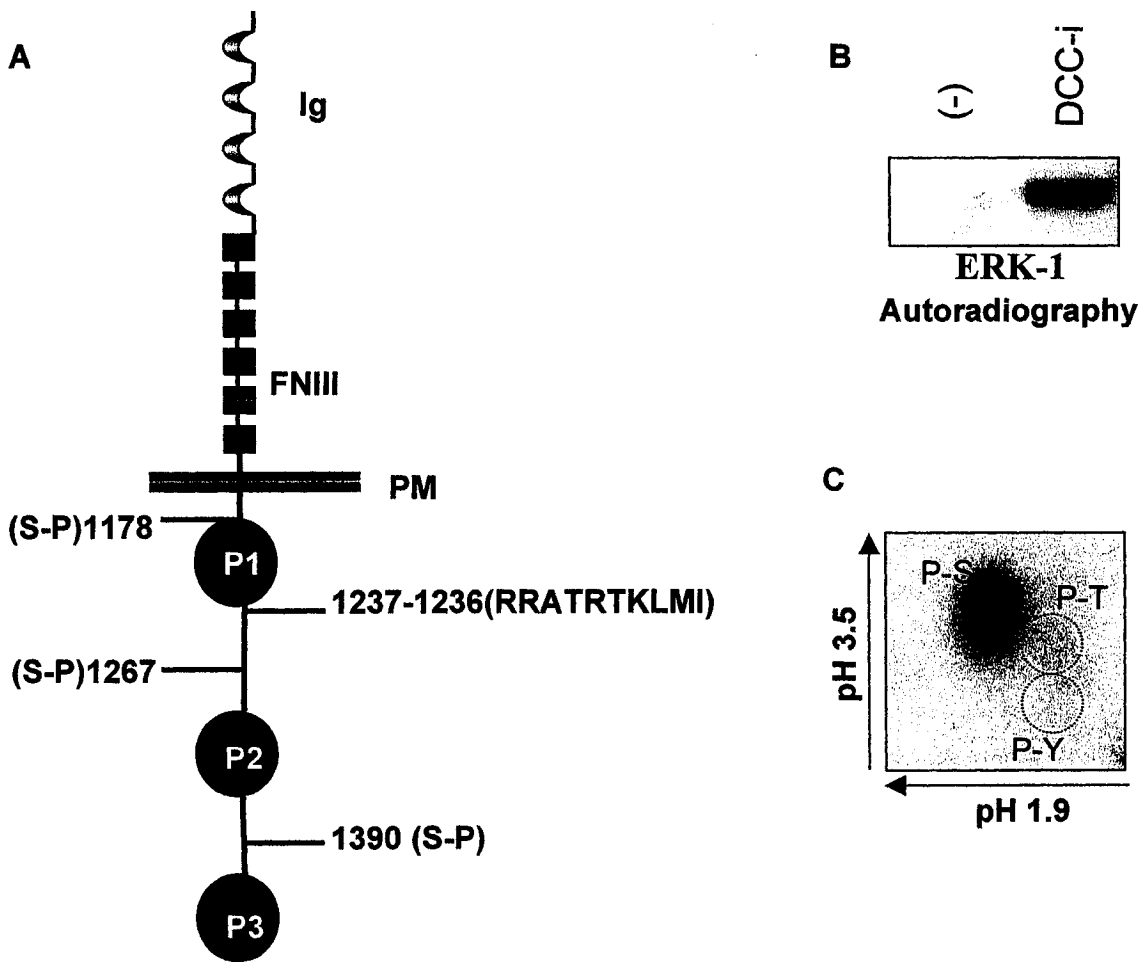
An important step in translation involves the release of a sequestered initiation factor, eIF-4E, from its binding protein, eIF-4EBP1. In its non-phosphorylated state, eIF-4EBP1 binds eIF-4E and represses translation. Phosphorylation of eIF-4EBP1 disrupts binding, releasing eIF-4E enabling it to bind the mRNA cap and activate translation (Gingras et al., 1999). It was shown that the Erk pathway, through mechanisms requiring the mammalian target of rapamycin (mTOR) protein, regulates the phosphorylation of multiple sites in 4E-BP1 *in vivo* and this is sufficient for the release of 4E-BP1 from eIF4E (Gingras et al., 1999).

Recently, it was reported that netrin-1 promotes axon outgrowth and protein synthesis through DCC-mediated MAPK activation (Forcet et al., 2002). It was shown that DCC binds and activates both ERK-1 and ERK-2 in HEK 293 cells and dissociated E13 commissural neurons. Inhibition of ERK activation by pharmalogical inhibitors against MEK blocked netrin-1-induced axon outgrowth of commissural neurons as well as turning of Xenopus retinal growth cones. These results shed light on the molecular mechanisms linking netrin-1 to protein synthesis for axon growth and guidance.

Given that DCC is highly phosphorylated on serine and threonine residues (Fig 4.1E and F), and that it contains at least three minimal ERK consensus phosphorylation sites (S/T-P) and a putative ERK docking site known as the D domain (Fig. 5.5A), and that ERK1/2 interact with DCC *in vivo*, we have investigated whether ERK-1 can phosphorylate DCC *in vitro*. We have found that ERK-1 can phosphorylate DCC *in vitro* mainly on serine residues as predicted by the consensus phosphorylation sites. It will be interesting to mutate these sites in order to determine if they are phosphorylated *in vivo*

Figure 5.5. Consensus ERK phosphorylation sites in DCC and phosphorylation of DCC by activated ERK-1 *in vitro*.

(A) The intracellular domain of rat DCC contains three minimal consensus phosphorylation sites for ERK (Ser-1178, Ser-1267, and Ser-1390) and a putative ERK docking domain (RRATRTK[redacted]M[redacted]) which fits the consensus D domain R/K-R/K-$X_{(1-5)}$-[redacted]-X-[redacted] (Jacobs et al., 1999; Tanoue et al., 2000). (B) An *in vitro* kinase assay was performed with or without (-) recombinant GST-tagged intracellular domain of DCC incubated with activated ERK-1 in the presence of 10 μCi/ml [γ-^{32}P]-ATP. The products were resolved by SDS-PAGE and phosphorylated substrates were detected by autoradiography. (C) The phosphorylated protein band corresponding to DCC-i was cut and subjected to phosphoaminoacid analysis. 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).


A
Ig
FNIII
PM
(S-P)1178
P1
1237-1236(RRATRTKLMI)
(S-P)1267
P2
1390 (S-P)
P3
B
(-)
DCC-i
ERK-1
Autoradiography
C
P-S
P-T
P-Y
pH 3.5
pH 1.9

and whether they can affect DCC function, which would suggest a negative or positive feedback loop.

5.5. SIGNALING PATHWAYS UNDERLYING NETRIN-1 AND DCC

We and others have shed light on some of the molecular mechanisms involved in netrin-1 and DCC signaling. We have shown the importance of the Src family tyrosine kinases in DCC function (Meriane et al., 2004). In agreement with our findings, other laboratories have also reported an important role for Src family members in netrin-1 signaling (Ren et al., 2004; Li et al., 2004; Liu et al., 2004). However, there is a debate on which of Fyn or Src is most important for DCC phosphorylation and function. These studies have also identified focal adhesion kinase (FAK) as a major contributor of netrin-1-induced axon outgrowth and guidance. It was reported that the P3 domain of DCC directly interacts with FAK (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Moreover, netrin-1 induces tyrosine phosphorylation of FAK mainly through DCC (Liu et al., 2004; Ren et al., 2004). Reciprocally, FAK seems to be required for netrin-1-mediated DCC tyrosine phosphorylation (Li et al., 2004; Ren et al., 2004). The kinase activity of FAK does not seem to be required for DCC tyrosine phosphorylation (Ren et al., 2004). However mutation of the Src binding site in FAK abrogates DCC phosphorylation (Ren et al., 2004), suggesting a possible role for FAK in bridging DCC and Src. In addition, netrin-1 appears to cause Fyn and Src tyrosine phosphorylation and activation (Li et al., 2004; Liu et al., 2004). Both kinases seem to interact with DCC and appear to be important for FAK and DCC tyrosine phosphorylation. Pharmacological inhibitors against the Src family tyrosine kinases block netrin-1-induced phosphorylation events and also neurite outgrowth and guidance.

It was previously shown that Rac1 and Cdc42 but not RhoA activities are required for neurite outgrowth induced by the netrin-1 signaling and that the adaptor protein Nck-1 couples DCC to the activation of Rac1 (Li et al., 2002a; Li et al., 2002b; Shekarabi and Kennedy, 2002). Also, findings in this thesis have shown that phosphorylation of DCC is important for netrin-1 induced Rac1 activation (Meriane et al., 2004). However, an important piece of the puzzle is missing: what is the link between the receptor and activation of the Rho GTPases? As mentioned all through this thesis, membrane receptors

couple extracellular factors to changes in the actin cytoskeleton mainly by modulating the activities of RhoGAP or RhoGEF proteins. Therefore, in order to obtain a more complete story we will have to determine whether such a Rho regulatory protein is located downstream of the DCC receptor. One potential candidate is the RhoGEF Trio which has been shown to have an important role in neuronal morphogenesis (Bateman et al., 2000; Estrach et al., 2002; Newsome et al., 2000). Most importantly, Trio has been found to interact with frazzled, the ortholog of DCC in the fruit fly (Forsthoefel et al., 2005). Also, Trio interacts with Dock, the drosophila ortholog of Nck (Newsome et al., 2000). Therefore it will be interesting to investigate whether Trio can be recruited to the DCC receptor via the Nck adaptor molecule.

5.6.CONCLUSIONS

Our understanding of protein phosphorylation has now reached the stage where its importance in almost every fundamental cellular event is recognized. The findings reported in this thesis have further solidified this notion. We have shown that both cytoplasmic and plasma membrane proteins involved in actin dynamics are regulated by phosphorylation.

More specifically, in the first part of this thesis we have shown that CdGAP phosphorylation inside cells is enhanced by external agents that activate components of the Ras-MEK-ERK pathway. We have found that ERK phosphorylates CdGAP on Thr-776 which seems to negatively regulate its GAP activity both *in vitro* and *in vivo*. Given that oncogenic Ras induces changes in the actin cytoskeleton mainly through activation of Rac, and that Rac is required for Ras-mediated transformation, it is not suprising that signaling components downstream of Ras inhibit a negative regulator of Rac. It will be interesting to investigate whether CdGAP can act as a tumor suppressor and prevent Ras from causing cellular transformation. In the second part of this thesis we have shown that the human ortholog of CdGAP behaves in a similar fashion. Interestingly, human CdGAP is located on chromosome 3.q.21.2, a region which is often deleted in breast cancer and leukemias. Moreover, since PAK and Raf synergize to activate MEK, it is possible that ERK inhibits CdGAP in order to enable PAK activation of MEK and consequently its own activation.

For over a decade, the axon guidance community has been intrigued as to how the receptor DCC is regulated at the biochemical level. Only by combining the dissection and isolation of primary neurons with classical biochemical approaches can one provide a satisfying answer to this question. Therefore we have been fortunate to possess both expertises in our laboratory. We have shown that in dissociated commissural neurons netrin-1 mediates phosphorylation of DCC mainly through the Src tyrosine kinase family. The major challenge now is to determine the Ser/Thr kinases that phosphorylate DCC, the phosphorylation sites and the role of these sites in regulating DCC functions.

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APPENDIX

4. Dissection of embryonic rat spinal cord.

ii) the procedures involving biohazards 1.To study the signaling mechanisms regulated by netrin-1, various techniques are used involving: transfection of plasmids, microinjection of plasmids or proteins, cell lines described above as well as primary commissural neurons dissected from the rat spinal cord. 2. Production of transgenic mice expressing dominant negative Nck proteins in brain tissues

3. Various proteins are expressed as GST fusion proteins in E.coli

4. The yeast two-hybrid system will be used to identify binding partners for the netrin-1 receptors DCC and UNC-5H.

iii) the protocol for decontaminating spills Cover the spills with absorbent paper towels. Pour 10% sodium hypochlorite starting at the perimeter of the spills and working towards the center. Allow 20 minutes contact time before clean up. Wipe down any walls or equipment that may have been splashed. All

materials used to clean the contaminated area will be transferred to biohazards waste bags. The McGill safety office will be notified of any spills.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

yes, experiments involving expresssion of proteins in E.coli or in yeast will require the manipulation of large volumes of bacterial cultures.

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

yes

). What precautions are being taken to reduce production of infectious droplets and aerosols?

1. homogenizers: will be used in a safety cabinet and all surfaces desinfected after use
2. pipettes: all pipetting procedures will be done with a mechanical pipetting. Pipettes will be plugged with cotton and all liquids gently expelled.
3. Animal housing: cage taps will be used and all animals handling will be carried out in a biological safety cabinet. All animal surgery will be done in a biological safety cabinet and all instruments used will be soaked in desinfected liquids and autoclaved after use. Tissue culture will be carried out in an isolated room 1/37A in the Strathcona Anatomy Building.

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Stratcona Anatomy Building	1/37A	Microzone	BK-2-4	801-2108	25-04-2002