Characterization of the signaling pathways underlying netrin-1 receptor deleted in colorectal cancer

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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 " Rac1 and Cdc42 but not RhoA or Rho kinase activities are required for neurite outgrowth induced by the netrin-1 receptor DCC (deleted in colorectal cancer) in N1E-115 neuroblastoma cells" involved performing all experiments except for the explant assay, which was performed by Dr. Tim Kennedy, and reverse transcriptase (RT)-PCR experiment performed by Dr. Masoud Shekarabi. Dr. Klaus Aktories provided Toxin B, Dr. Masoud Shekarabi provided pEGFP-DCC construct, and Etienne Saint-Cyr-Proulx performed one of the experiment in N1E-115 cells with GFP-DCC construct. Both Dr. Nathalie Lamarche-Vane and I contributed to the writing of the manuscript.

Chapter 3

My contribution to the manuscript entitled "The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism" involved performing all experiments, with the exception of the experiments in Figure 1A, B, D, and Figure 2 performed by Dr. Mayya Meriane. The experiments in Figure 1C, Figure 3A, D were performed by Ms. Ibtissem Triki, the dissection of the spinal cord and dissociation of commissural neurons were performed by Drs. Tim Kennedy and Masoud Shekarabi. Dr. Louise Larose provided all the Nck-1 constructs. Both Dr. Nathalie Lamarche-Vane and I contributed to the writing of the manuscript.

Chapter 4

I performed all the experiments for the manuscript entitled "Disruption of each of the PxxP motifs in DCC does not block the interaction of DCC with Nck-1". Both Dr. Nathalie Lamarche-Vane and I contributed to the writing of the manuscript.

ABSTRACT

Netrins are a small family of secreted proteins that function as chemotropic cues directing cell and axon migration during neural development. They are bifunctional molecules attracting and repelling different classes of axons. DCC (deleted in colorectal cancer) is a transmembrane receptor for netrin-1 implicated in mediating both responses. The intracellular mechanisms mediating the response of an axon to netrin-1 are currently unclear. Previous studies indicated that extracellular guidance cues induce the neuronal growth cone to advance, retract, or turn by regulating the actin cytoskeleton within the growth cone. The Rho family GTPases, in particular, RhoA, Rac1 and Cdc42, are well-described regulators of actin reorganization in non-neuronal cells, and there is now compelling evidence implicating a role for them as signaling components within the neuronal growth cone.

In the first part of this thesis, we have demonstrated that the Rho GTPases are required for embryonic spinal commissural axon outgrowth induced by netrin-1. Using N1E-115 neuroblastoma cells we found that both Rac1 and Cdc42 activities are required for DCC-induced neurite outgrowth. In Swiss 3T3 fibroblasts, DCC was found to trigger actin reorganization through activation of Rac1. These results implicate the small GTPases as important signaling components in the molecular mechanisms underlying DCC.

In the second part, we found that DCC interacts constitutively with the adaptor protein Nck in commissural neurons. Moreover, dominant negative Nck-1 inhibits the ability of DCC to induce neurite outgrowth in N1E-115 cells and to activate Rac1 in fibroblasts in response to netrin-1. These studies provide evidence for an important role of Nck-1 in a novel signaling pathway from an extracellular guidance cue to changes in the actin-based cytoskeleton responsible for axonal guidance.

In the last part, we found that disruption of each of the PxxP motifs in the cytoplasmic domain of DCC is not able to block the interaction of DCC with Nck-1, suggesting that more than one PxxP motifs or non-PxxP sequences may mediate the interaction of DCC with Nck-1.

Taken together, the data in this thesis contribute to our understanding of the intracellular mechanisms mediating the response of an axon to netrin-1 during neural development.

RÉSUMÉ

Les nétrines représentent une famille de protéines sécrétées qui fonctionnent comme des facteurs d'attraction ou de répulsion des axones au cours du développement du système nerveux central. Chez les vertébrés, l'attraction du cône de croissance axonal induite par nétrine-1 implique le récepteur transmembranaire DCC (deleted in colorectal cancer) tandis que les propriétés de répulsion des nétrines impliquent les protéines homologues à UNC-5. Les mécanismes intracellulaires induits lors de la réponse des axones à nétrine-1 sont encore très peu connus. Cependant, plusieurs études démontrent l'importance du cytosquelette d'actine lorsque le cône de croissance neuronal doit avancer, se rétracter, ou tourner en présence de facteurs extracellulaires. Les protéines de la famille Rho GTPases, soient RhoA, Rac1 et Cdc42, jouent un rôle primordial dans la réorganisation du cytosquelette d'actine dans les cellules non-neuronales. De plus, plusieurs études récentes tendent à démontrer l'importance des Rho GTPases dans la signalisation neuronale.

Dans la première partie de ma thèse, nous avons démontré que les Rho GTPases sont nécessaires durant la croissance des axones commissuraux induite par nétrine-1 au cours du développement de la moëlle épinière embryonnaire de rat. Nous avons aussi montré que Rac1 et Cdc42 sont requis pour la croissance neuritique induite par DCC dans les neuroblastes de souris N1E-115. De même, l'expression de DCC dans des fibroblastes de souris Swiss 3T3 induit la formation de lamellipodia via l'activation de Rac1, et ce, de façon dépendante de nétrine-1.

Dans la deuxième partie de ma thèse, nous avons trouvé que la protéine adaptatrice Nck-1 interagit constitutivement via les domaines SH3 avec DCC dans les neurones commissuraux. La protéine dominante négative Nck-1 dépourvue de son domaine SH2 inhibe la capacité de DCC à induire des croissances neuritiques dans les cellules N1E-115 et empêche l'activation de Rac1 dans les fibroblastes. Ces études démontrent que Nck-1 joue un rôle important dans les voies de signalisation induites par nétrine-1 en couplant le récepteur DCC à l'activation de Rac1.

Finalement, nous avons trouvé que l'abolition par mutagénèse dirigée de chaque domaine PXXP dans l'extrémité cytoplasmique de DCC n'est pas suffisante pour inhiber l'interaction de DCC avec Nck-1. Ceci suggère que plus d'un domaine PXXP est requis

pour médier l'interaction DCC/Nck-1. Cependant, nous ne pouvons exclure la possibilité qu'un motif autre que PXXP soit impliqué dans l'interaction DCC/Nck-1.

En conclusion, ces résultats contribuent significativement à améliorer nos connaissances sur les mécanismes intracellulaires qui régissent la réponse cellulaire d'un axone à nétrine-1 au cours du développement neuronal.

LIST OF ABBREVIATIONS

ARF	ADP-ribosylation factor
Abl	abelson tyrosine kinase
Arp2/3	actin-related protein 2 and 3 complexes
bFGF	basic fibroblast growth factor
Bcr	breakpoint cluster region
BSA	bovine serum albumin
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
Cdc42	cell division cycle
CdGAP	Cdc42 GTPase-activating protein
Cdk	cyclin-dependent kinase
cGMP	cyclic guanosine monophosphate
CRIB	Cdc42/Rac interactive binding
CRMP-2	collapsin response mediator protein-2
CST	corticospinal tract
DB	DCC-binding
DCC	deleted in colorectal cancer
DH	dbl homology
DInR	*Drosophila* insulin receptor
Dock	dreadlocks
Dok	downstream of tyrosine kinase
DRG	dorsal root ganglion
Dscam	from human protein 'Down syndrome cell adhesion molecule'
E13	embryonic day 13
ECM	extracellular matrix molecules
EGF	epidermal growth factor
Ena	enabled
Eph	erythropoietin-producing hepatocellular receptor
Ephexin	Eph-interacting exchange factor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
GAP	GTPase activating protein
GCK	germinal center kinase
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein

GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
GTP	guanine triphosphate
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
LIMK	LIM kinase
LOH	loss of heterozygosity
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MBS	myosin-binding subunits
Mena	mammalian Enabled
Mig-2	migration 2
MLC	myosin light chain
MRCK	myotonic dystrophy kinase-related Cdc42-binding kinase
Msn	misshapen
Nap1	Nck-associated protein 1
Nap125	Nck-associated protein of 125Kd
NCAM	neuron cell adhesion molecule
NGF	nerve growth factor
NIK	Nck-interacting kinase
$p75^{NTR}$	p75 neurotrophin receptor
PAK	p21 activated kinase
PBS	phosphate-buffered saline
PC12	pheochromocytoma cell line
PDGF	platelet-derived growth factor
PH	pleckstrin homology
PI3-K	phosphatidylinositol 3-kinase
PIR121	p53-inducible messenger RNA with a relative molecule mass of 140Kd
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfony
PTB	phosphotyrosine-binding
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
Rac	Ras-related C3 botulinum toxin substrate
RBD	Rho-binding domain

RGC	retinal ganglion cell
Rho	Ras homologous
Robo	roundabout
ROCK	Rho kinase
RPTP	receptor protein tyrosine phosphatase
RT	reverse transcriptase
RTK	receptor tyrosine kinase
SAP	SLAM-associated protein
Scar	suppressor of cAMP receptor
SDF-1a	stromal cell-derived factor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser/Thr	serine/threonine
SH2	Src-homology 2
SH3	Src-homology 3
Shh	sonic hedgehog
SLAM	signaling lymphocyte activation molecule
SLAP	SLP-76 associated protein
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
Sos	son of sevenless
SRE	serum response element
srGAP	Slit-Robo GAP
TGFβ	transforming growth factor β
Tiam1	mouse T-lymphoma invasion and metastasis-1 gene
TOR	target of rapamycin
TRITC	tetramythylrhodamine isothiocyanate
UNC	uncoordinated
VASP	vasodilator-stimulated phosphoprotein
VHD	villin headpiece domain
WAVE	WASP family Verprolin-homologous protein
WH1	WASP-homology domain1
WIP	WASP interacting protein

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 GENERAL INTRODUCTION

The formation of appropriate connections between neurons and their target cells is a fundamental step during development of invertebrate and vertebrate nervous systems. As the nervous system develops, newborn neurons extend axons towards their appropriate targets. The neuronal growth cones are highly mobile, hand-like structures at the tip of developing neurites (Fig. 1.1). They have two types of cytoskeletal structures. Microtubules are found in abundance in the organelle-rich central domain. In the peripheral domain, actin filaments predominate, forming tight bundles in filopodia and a dense interwoven meshwork at the leading edge and in lamellipodia. Filopodia act as sensor to detect the guidance cues in the environment and lead the growth cone to the right target (Mueller, 1999; Tessier-Lavigne and Goodman, 1996). Therefore, extracellular guidance cues instruct the growth cone to advance, retract, or turn by regulating the actin cytoskeleton within the growth cone. Small GTPases of the Rho family- RhoA, Rac1, and Cdc42- are regulators of actin organization in non-neuronal cells (Hall, 1998). There is now growing evidence showing that Rho GTPases regulate cytoskeletal dynamics in neuronal growth cones as well (Luo, 2000).

It has been proposed that activation of Rac1 or Cdc42 by attractive guidance cues induce filopodia and lamellipodia formation leading to growth cone extension, whereas activation of RhoA by repulsive cues induce filopodia and lamellipodia retraction leading to growth cone collapse (Mueller, 1999). Recent studies have provided compelling evidence that Rho GTPases play important roles in mediating guidance cues signaling (Hu et al., 2001; Jin and Strittmatter, 1997; Newsome et al., 2000; Wahl et al., 2000; Wong et al., 2001). At the same time, much effort has been made in defining the pathways linking Rho GTPases to the actin cytoskeleton during axon outgrowth and guidance (Banzai et al., 2000; Bashaw et al., 2000; Bito et al., 2000; Hing, 1999; Newsome et al., 2000). These findings provide insight into understanding how guidance cues and their receptors orchestrate the establishment and maintenance of neuronal connectivity.

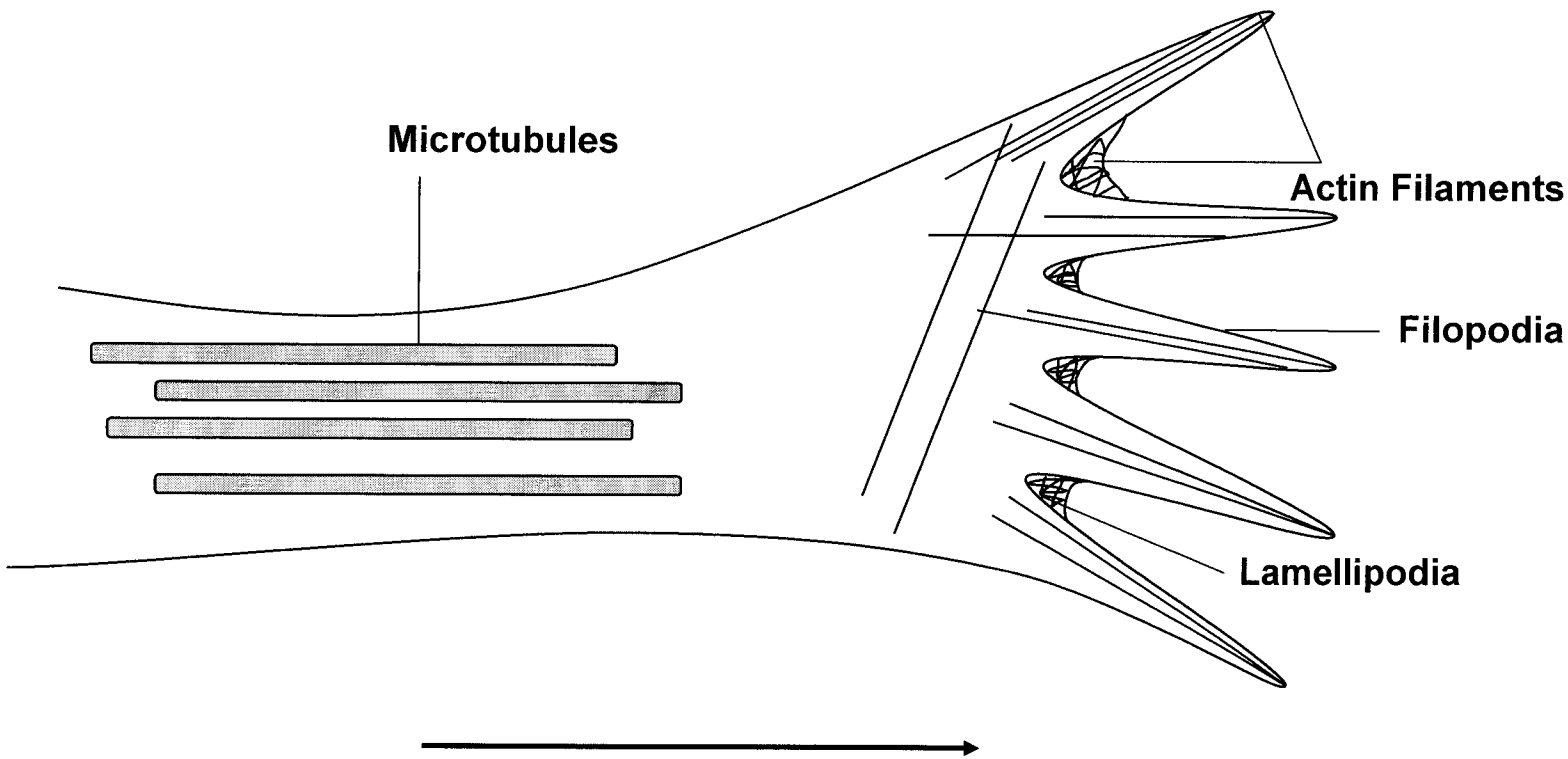
Microtubules
Actin Filaments
Filopodia
Lamellipodia

Figure 1.1. Schema of the neuronal growth cone.

The neuronal growth cone is a hand-like structure at the tip of a developing neurite. It has two types of cytoskeletal filaments. Microtubules are in the central domain, whereas filamentous actin containing filopodia and lamellipodia actin structures are at the leading edge of the growth cone. Arrow indicates direction of growth cone extension.

1.1 AXON GUIDANCE CUES AND THEIR RECEPTORS

The complexity of neural connectivity depends upon the precise navigation of axons to their targets in the developing nervous system. How is this achieved? Signals from the environment are assumed to steer axons in the appropriate direction through their receptors on the growth cone, but the molecular details of this process have been largely unknown. Recently, impressive progress has been made into identifying families of guidance cues and their corresponding receptors that may underlie this process (Fig. 1.2). Members of different families of guidance cues are either membrane-bound proteins such as semaphorins and ephrins or secreted proteins such as netrins and Slits. In the case of netrins and Slits, small number of ligands interact with a small number of receptors; in the case of semaphorins and ephrins, large families of related ligands interact with corresponding families of receptor proteins. In addition to these four conserved families of guidance cues, other cues also guide axons, including neurotrophins, hepatocyte growth factor (HGF)/scatter factor, transforming growth factorβ (TGFβ) family members, receptor protein tyrosine phosphatases (RPTP), and immunoglobulin family cell adhesion molecules (Ig-CAMs) (Fig. 1.2).

1.1.1 Ig-CAMs

Ig-CAMs have tandem immunoglobulin domains in their extracellular regions. Family members contain neural CAM (NCAM), L1/NgCAM, NrCAM, TAG-1/axonin-1. Many members of this family mediate homophilic adhesion, functioning as both a ligand on one cell and a receptor on the other cell. Isoforms of NCAM have been implicated in a number of events in axon growth and guidance (Walsh and Doherty, 1997). Some members also bind heterophilically to other family members, for example L1 and axonin-1 associate with each other. Commissural axons express both L1 and axonin-1 during their trajectory to the floor plate and crossing it, whereas the floor plate itself expresses NrCAM. *In vivo* injections into chick embryos of anti-axonin antibodies or of soluble axonin-1 as a blocking agent resulted in defasciculation of commissural axons and pathfinding errors such that some axons failed to cross and instead grew along the ipsilateral floor plate border (Stoeckli and Landmesser, 1995). Similar injections of antibodies to L1 resulted in axon defasciculation but axons still crossed the floor plate

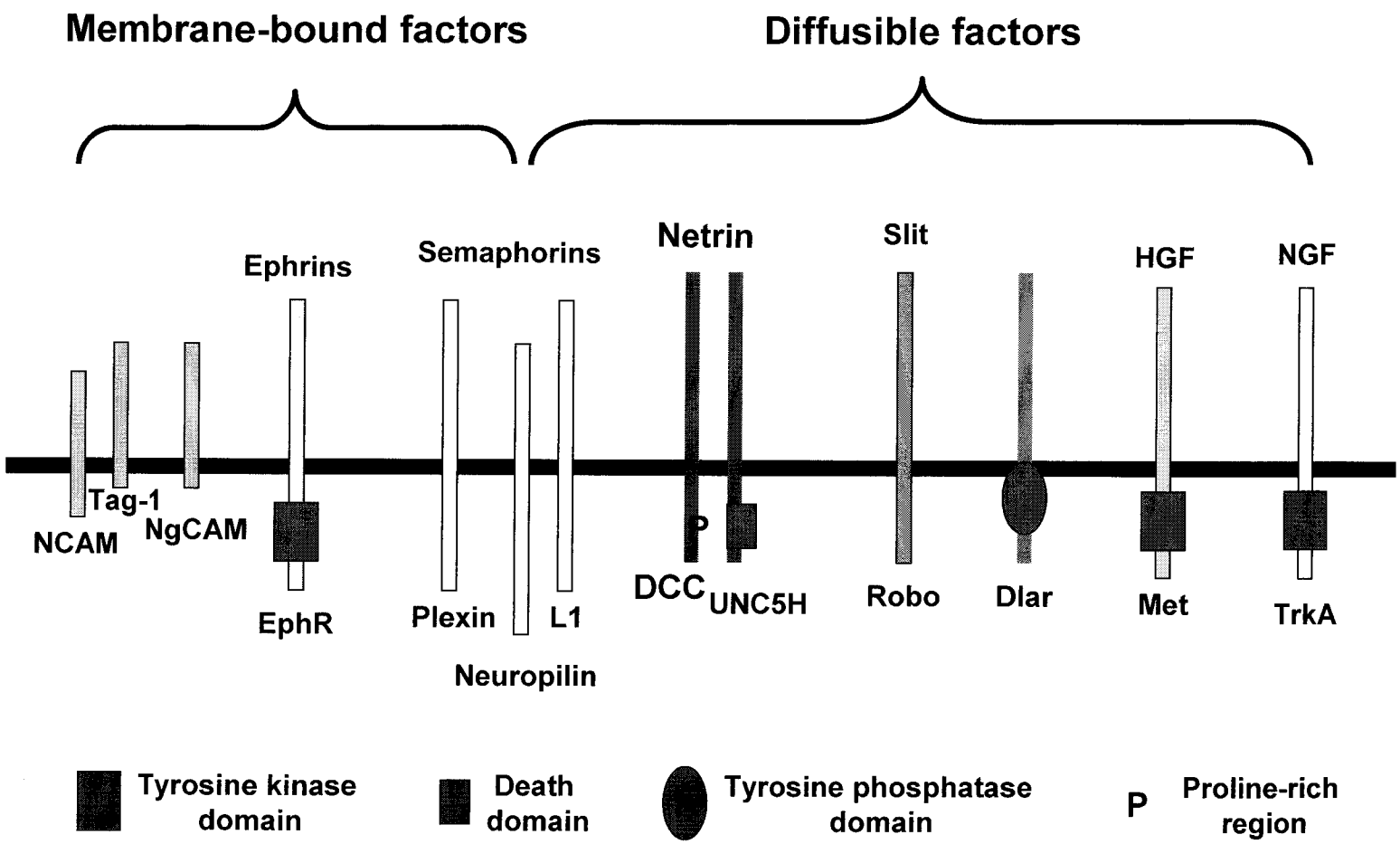
Membrane-bound factors
Diffusible factors
Ephrins
Semaphorins
Netrin
Slit
HGF
NGF
Tag-1
NCAM
NgCAM
EphR
Plexin
L1
Neuropilin
P
DCC
UNC5H
Robo
Dlar
Met
TrkA
Tyrosine kinase domain
Death domain
Tyrosine phosphatase domain
P
Proline-rich region

Figure 1.2. Some of the major families of axon guidance cues and their receptors. Members of the different families of guidance cues are either membrane-bound proteins or diffusible factors. However, some family of guidance cues such as semaphorins include both secreted and membrane proteins. Guidance cues can be categorized as either attractive or repulsive cues. Some families of guidance cues such as netrins and ephrins are bifunctional molecules, they function as both attractive and repulsive molecules.

correctly. Injections of antibodies to NrCAM led to pathfinding defects similar to those seen following perturbations of axonin-1. This implicates that interactions between axonin-1 and NrCAM are involved in the entry of commissural axons into the floor plate, but also raise the possibility that interfering with axon-axon interactions mediated by axonin alter growth cone responses to guidance cues. Polysialic acid (PSA) associated with NCAM modulates homophilic interaction between NCAM molecules and heterophilic interaction mediated by NCAM and other molecules. The effect of removing PSA was tested in the plexus region where motor axons rearrange before entering the limb bud of the chick embryo. PSA was found to be present at low levels in regions where axons were tightly fasciculated before reaching the plexus, but to be abundant in regions of defasciculation. Specific removal of PSA by application of a bacterial endoneuraminidase resulted in axons with straight and less complex trajectories (Tang et al., 1992). The effect of endoneuraminidase on axon pathfinding was reversed by treatment with antibodies against L1 but not by those against NCAM, implicating L1-mediated interactions in the normal fasciculation process at the plexus (Tang et al., 1994).

1.1.2 Extracellular matrix molecules (ECM)

Many ECM molecules including laminin, collagen, fibronectin, and a variety of proteoglycans, act as promoters or inhibitors of neurite outgrowth and extension *in vitro* (Hynes, 1992). Receptors for ECM molecules are predominantly integrins, Ig superfamily members, and proteoglycans (Reichardt and Tomaselli, 1991). On the basis of their *in vitro* activities and *in vivo* expression patterns, many ECM molecules are expected to play roles in axon guidance, but not much is known about their actual guidance functions *in vivo*. In *Drosophila melanogaster*, loss of lamininA function results in the stalling of a subset of sensory axons, implicating laminin as a permissive substrate for these axons (Garcia-Alonso et al., 1996). Similarly, interfering with integrin function in *Xenopus* retinal axons *in vivo* causes a shortening of the axons (Lilienbaum et al., 1995). In addition to promoting neurite outgrowth, laminin also modulates the navigation behaviour of growth cones in response to guidance cues. It has been shown that growth cone attraction is converted to repulsion by the presence of laminin-1 (Hopker et al., 1999).

1.1.3 Receptor-like protein tyrosine phosphatases (RPTPs)

Phosphotyrosine signaling is regulated by the action of multiple protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Many PTPs are transmembrane receptor PTPs and most of these are highly expressed in the developing nervous system (Van Vactor, 1998). RPTPs reside in axons as well as in the membranes of growth cone lamellipodia and filopodia (Ledig et al., 1999), strongly suggesting roles in nervous system development. *D. melanogaster* photoreceptor development is an excellent system to study neuronal differentiation and axon guidance. Photoreceptors are arrayed in ommatidial clusters of eight neurons (R1-R8). Individual ommatidia project their axons as a single fascicle through the optic nerve to the primary visual center in the brain. The pioneer R8 axon terminates in the medulla. R1-R6 axons fasciculate with R8 until they reach the lamina where they defasciculate and terminate. Lastly, R7 grows to the end of R8 where it defasciculates and terminates more distally in the medulla. Two studies show that *D. melanogaster* RPTP-DPTP69D is critical in the guidance decisions of R1-R6 axons and R7 (Garrity et al., 1999; Newsome et al., 2000). In loss of function mutant larvae, growth cones of R1-R6 often fail to stop in the lamina and continue on to the R8 termination zone. Using an eye-specific mosaic system to screen for the retinal axon guidance defects, DPTP69D was implicated in the role of axon guidance of R1-R6, as well as R7. Again, R1-R6 axons failed to stop in the lamina, instead extending into the R7 and R8 termination zones. R7 growth cones also stopped short on R8 in the medulla, failing to reach their distal targets. RPTP also regulates axon guidance across the midline of the *D. melanogaster* embryo. Both DPTP10D and DPTP69D are required for repulsion of growth cones from the midline of embryonic central nervous system (CNS) (Sun et al., 2000a). In vertebrate, mammalian RPTPδ, RPTPκ, RPTPμ, (Burden-Gulley et al., 2002; Drosopoulos et al., 1999; Sun et al., 2000b) and chick PTPδ (Rashid-Doubell et al., 2002) have been implicated in the growth and guidance of several populations of developing vertebrate neurons.

1.1.4 Semaphorins and their receptors

Semaphorins are a large family of secreted and membrane proteins, defined by the presence of a conserved ~420 amino acid Sema domain at their NH2-terminus. The first

semaphorins were identified by searching for molecules expressed on specific axon fascicles in the grasshopper CNS (Kolodkin et al., 1992) and by purifying a potent inducer of vertebrate sensory growth cone collapse *in vitro* (Luo et al., 1993). Semaphorins are grouped in eight classes, on the basis of their structure. Classes 1 and 2 are found in invertebrates, classes 3 to 7 are found in vertebrates, and class V semaphorins are encoded by alcelaphine herpesvirus type 1 and poxvirus (Comeau et al., 1998; Ensser and Fleckenstein, 1995). Semaphorins signal through multimeric receptor complexes. The composition of these receptor complexes is not fully understood. Semaphorin receptor complexes include plexins, neuropilins, neural cell adhesion molecule L1 (for Sema3A), the receptor tyrosine kinase Met (for Sema4D), and the catalytically inactive receptor tyrosine kinase OTK (for *D. melanogaster* Sema1a). Plexins comprise a large family of transmembrane proteins divided into four groups (A to D), on the basis of sequence similarity (Tamagnone et al., 1999). *D. melanogaster* plexinA is a functional receptor for the transmembrane Sema1a (Winberg et al., 1998b), vertebrate plexin-As are functional receptors for secreted class 3 semaphorins (Cheng et al., 2001; Tamagnone et al., 1999), and other plexins bind directly to semaphorins of different classes (Swiercz et al., 2002; Tamagnone et al., 1999). Neuropilins bind directly to both class 3 semaphorins and plexins (Raper, 2000). Neuropilins do not appear to have a signaling function, but rather contribute to ligand specificity. Genetic analysis of semaphorins function in flies and in mice suggests that they primarily act as short-range inhibitory cues that direct axons away from inappropriate regions (Raper, 2000). Evidence suggests that semaphorins may also act as attractive cues (Raper, 2000).

1.1.5 Netrins and their receptors

Netrins are a small family of secreted proteins that function as tropic cues directing cell and axon migration during neuronal development. The first netrin cloned, UNC-6, was identified using a genetic screen for mutations affecting axon guidance in *Caenorhabditis elegans* (Ishii et al., 1992). Netrin family members have now been identified in multiple vertebrate and invertebrate species and shown to have a highly conserved function as axon guidance cues (Culotti and Merz, 1998). Netrins are bifunctional molecules attracting and repelling different classes of axons. Growth cone

attraction mediated by netrin-1 involves the transmembrane netrin receptor deleted in colorecter cancer (DCC) (Keino-Masu et al., 1996). In *C. elegans*, the identification of UNC-5 first implicated it as a receptor required for the repellent response to UNC-6 (Hedgecock et al., 1990). Three UNC-5 homologues have now been identified in mammals (Ackerman, 1997; Leonardo, 1997). Current evidence suggests that short-range repulsion mediated by netrin requires UNC-5 family members, whereas long-range repulsion requires both UNC-5 and DCC family members (Keleman and Dickson, 2001).

1.1.6 Ephrins and Eph receptors

Ephrins were identified in the search for the graded cues that guide retinal axons to their appropriate topographic locations in the optic tectum (Cheng et al., 1995). They are membrane-bound ligands for the Eph (erythropoietin-producing hepatocellular receptor) family of receptor tyrosine kinase (Drescher et al., 1995). Mammals have 13 Eph receptors and 8 ephrins. Both ephrins and Eph receptors are divided into two classes: ephrin-As (epheinA1-ephrinA5), which are anchored to the membrane by a glycosylphosphatidylinositol (GPI) linkage and bind EphA receptors (EphA1-EphA8); ephrin-Bs (ephrinB1-ephrinB3), which have a transmembrane domain and a short cytoplasmic tail and bind EphB receptors (EphB1-EphB4, EphB6) (Wilkinson, 2001). EphA4 can bind both ephrinAs and ephrinBs. In the visual system, topographic mapping of retinal axons along the anterior-posterior axis depends on repulsion mediated by ephrin-A ligands and their EphA receptors (Wilkinson, 2001). Ephrin-A ligands are expressed in a gradient in the tectum, and Eph receptors are expressed in a complementary gradient in the retina. Mapping along the dorsal-ventral axis, in contrast, involves attractive signaling mediated by ephrinB ligands and EphB receptors (Hindges et al., 2002; Mann et al., 2002). Correct mapping of retina axons along this axis evidently requires both 'forward' signaling, in which ephrin-B ligands activate EphB receptors, and 'reverse' signaling, in which EphBs serve as ligands to signal back through the transmembrane ephrin-Bs. The GPI-linked ephrin-As are also able to signal in the reverse direction (Davy et al., 1999) and may act in this way to mediate attraction or adhesion during mapping of vomeronasal axons to the accessory olfactory bulb (Knoll et al., 2001).

1.1.7 Slits and Robos

Slits are large secreted proteins that signal through Roundabout (Robo) family receptors. Robo was first identified in a genetic screen for midline guidance defects in *D. melanogaster* (Seeger et al., 1993). Genetic studies suggest that Robo is a receptor for a midline repulsive cue (Kidd et al., 1998) that was subsequently identified as Slit (Battye et al., 1999; Kidd et al., 1999). This repulsive action of Slit was found to be conserved in vertebrates (Brose et al., 1999; Li et al., 1999). However, in a parallel approach, Slit was also purified as a factor that stimulates sensory axon branching and elongation (Wang et al., 1999b). Thus, Slits, like netrins, are multifunctional. The best-understood functions of Slit proteins are in midline guidance in *D. melanogaster* and in the formation of the optic chiasm in vertebrates. In *D. melanogaster*, Slit is expressed at the ventral midline, where it acts as short-range repellent signaling through Robo to prevent ipsilateral axons from crossing the midline and commissural axons from recrossing (Battye et al., 1999; Kidd et al., 1999). Two other Slit receptors, Robo2 and Robo3, specify the lateral positions of axons that run parallel to the midline, presumably in response to a long-range gradient of Slit activity diffusing away from the midline (Rajagopalan et al., 2000; Simpson et al., 2000). Vertebrate Slit proteins are also expressed by ventral midline cells (Brose et al., 1999), and commissural axons are repelled by Slit after they have crossed the midline (Zou et al., 2000). Mice deficient for both Slit1 and Slit2 lack any obvious defects in midline guidance in the spinal cord (Plump et al., 2002), but Slit3 is still expressed at the midline in these mice. Slits1/2-deficient mice do have striking defects in the formation of the optic chiasm, where Slit3 is not expressed (Plump et al., 2002).

1.2 NETRINS AND RECEPTORS

1.2.1 Netrins

Netrins are a family of secreted proteins that guide growing axons during neural development. The first netrins found in vertebrates were purified from embryonic chick brain (Serafini et al., 1994). Four members of the netrin gene family have been identified in mammals: *netrin-1*, *netrin-3*, *netrin-G1*, and *netrin-4*, also called *β-netrin* (Koch, 2000; Nakashiba et al., 2000; Serafini, 1996; Wang et al., 1999a; Yin et al., 2000). All encode ~75kDa proteins made up of three domains (V, VI, and C). Domains V and VI are homologous to domains V and VI of laminins. No domain homologous to the netrin C domain is present in the laminin family. The netrin C domain binds heparin with high affinity (Kappler et al., 2000). Unlike other netrins, netrin-G1 binds to the plasma membrane via a GPI lipid anchor linked to the C domain.

Netrins are bifunctional molecules. They attract some axons and repel other axons. The first member of the netrin family UNC-6 was identified using a genetic screen for defects in neural development by examining *C. elegans* mutants with uncoordinated (unc) phenotypes. Loss of UNC-6 function mutants have defects in the trajectories of axons that normally extend circumferentially toward or away from the ventral midline of the developing nematode. In vertebrates, netrin-1 is expressed by floor plate cells as commissural axons extend toward the ventral midline (Kennedy et al., 1994). The production of a hypomorphic allele in mice that almost completely abolishes *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. Anterior commissure is also defective. These indicate that netrin-1 is required for the development of multiple commissural projections. Netrin-1-deficient mice have severe defects in commissural axons. Many commissural axons are shortened and fail to invade the ventral spinal cord, and many are misrouted, only a few axons reach the floor plate. The neurons of the trochlear nucleus exhibit chemorepulsion by netrin-1 *in vitro*, seem normal in the netrin-deficient animals, extending dorsally to form a normal projection. The lack of trochlear phenotype indicates the existence of netrin-1-independent guidance mechanism. The fact that some commissural axons do reach the

floor plate in the netrin mutant mice suggests that additional attractive guidance cues, distinct from netrin-1, are expressed by the floor plate. Consistent with this, netrin-1 mutant floor plate explants remain capable of reorienting commissural axons in coexplanted dorsal spinal cord, and the DCC antibody fails to block reorientation induced *in vitro* by normal floor plate or netrin-1-transfected cells. What might these cues be? Sonic hedgehog (Shh) is expressed in the floor plate of the vertebrate neural tube, and act as a long-range cue from its source, so it is a potential candidate. Indeed, Charron et al. (Charron et al., 2003) showed that COS cells expressing Shh cause reorientation of commissural axons within the spinal cord explant, like netrin-1-expresssing cells do. Shh also induce turning of growth cones of isolated *Xenopus* spinal axons. Therefore, morphogen Shh mimic the additional chemoattractant activity of the floor plate *in vitro* and act directly as a chemoattractant on isolated axons.

In the visual system, several lines of evidence have shown that netrin-1 plays a critical role in retinal ganglion cell (RGC) axons exit from the retina into the developing optic nerve (Deiner et al., 1997). First, netrin-1 protein is expressed by neuroepithelial cells at the developing optic nerve head that are in close contact with RGC axons. Secondly, RGC axons *in vitro* respond to netrin-1 as an attractive guidance molecule. Lastly, mice with a targeted deletion of netrin-1 gene exhibit pathfinding errors at the optic disc where RGC axons fail to exit from the retina into the optic nerve and instead grow inappropriately into the other side of the retina. As a result of this aberrant pathfinding, these mice exhibit optic nerve hypoplasia (Deiner et al., 1997).

Netrin-1 acts as a long-range cue or short-range cue in different circumstances. Several lines of evidence indicated that netrin-1 functions as a long-range cue. Netrin-1 protein diffuses away from its source through a collagen gel to attract or repel growing axons (Kennedy et al., 1994). The growth cones of dissociated neurons from the embryonic *Xenopus* laevis turn when challenged *in vitro* with a gradient of netrin-1 delivered from a micropipette (de la Torre et al., 1997; Ming et al., 1997). These results support the hypothesis that following secretion, netrin-1 diffuses several hundred microns and act as a long-range cue to direct axon extension. Netrins have also been suggested to function as a short-range cue. A target derived short-range function for netrin has been proposed to contribute to the development of nerve-muscle synapses in *D. melanogaster*

(Winberg et al., 1998a). Netrin-1 expressed by cells at the optic disc also appears to function as a short-range cue for RGC axons as they exit the retina during mouse embryogenesis (Deiner et al., 1997). Analysis of the subcellular distribution of netrin-1 protein in the adult rat spinal cord demonstrated that the majority of netrin-1 protein is not freely soluble but is associated with ECM or cell membranes (Manitt et al., 2001). When netrin-1 was initially purified from embryonic day 10 (E10) chick brains, the axon outgrowth promoting activity corresponding to netrin-1 fractionated as membrane-associated proteins (Serafini et al., 1994), while only very limited activity was present in the fraction containing soluble proteins. Western blot analysis following subcellular fractionation of homogenates of E10 chick brains confirmed this distribution. The majority of netrin-1 protein was found to be membrane-associated with only a small amount of netrin-1 detected in the fraction containing soluble protein. These findings suggest that following secretion netrin-1 may not diffuse a long distance from the surface of cells producing it, and a major function of netrin-1 in the embryonic and adult CNS may be short-range.

In addition to its role in the nervous system, Srinivasan et al. (Srinivasan et al., 2003) extend the netrin activities to non-neuronal tissues, and show that it also acts to keep cells stick together during the development of mammary glands. They found that netrin-1 prevents cap-cell movement. Loss of either netrin-1 or neogenin (DCC family member) disrupted adhesion between the cap-cell layer and adjacent cells, and resulted in cap-cells moving into regions they would not normally go. Furthermore, addition of netrin-1 to isolated neogenin-producing cells caused them to aggregate. So it seems that netrin-1 may be required in the developing mammary gland to make sure that cells stick together.

1.2.2 Netrin receptors

Candidate netrin receptors were first identified in *C. elegans* based on the similarity of *unc-5*, *unc-40*, and netrin *unc-6* mutant phenotypes (Hedgecock et al., 1990). Mutation of *unc-5* caused defects in ventral to dorsal migration, away from *unc-6* expressing cells. Mutation of *unc-40* caused defects in dorsal to ventral migration, toward *unc-6* expressing cells. Mutation of *unc-6* caused defects in both trajectories. Cloning of

unc-40 and *unc-5* indicated that they encode transmembrane members of the Ig superfamily, and both are expressed by neurons as they extend axons during development in *C. elegans* (Chan, 1996; Leung-Hagesteijn et al., 1992). Orthologs of *unc-40* and *unc-5* have been identified in different species and are represented by the DCC and UNC-5 families of netrin receptors, respectively.

The DCC family includes DCC and neogenin in vertebrates, frazzled in *D. melanogaster*, and UNC-40 in *C. elegans* (Chan, 1996; Fearon et al., 1990; Keino-Masu et al., 1996; Kolodziej, 1996; Vielmetter et al., 1994). DCC is a transmembrane receptor which has an extracellular domain composed of four immunoglobulin (Ig) domains followed by six fibronectin type III repeats, a transmembrane domain, and an intracellular domain (Fig 1.3). In the cytoplasmic domain of DCC, there are three conserved motifs: P1, P2, and P3. Strong evidence indicates that DCC is a receptor mediating the chemoattractant response to netrin-1. Neogenin is expressed in neurons and non-neuronal cells in the CNS during embryogenesis (Vielmetter et al., 1994). Embryonic spinal commissural neurons express *DCC* but not *neogenin* as they extend axons toward the floor plate (Keino-Masu et al., 1996). Neogenin binds netrin-1 but its function in the CNS remains unclear.

The UNC-5 family includes UNC-5 in *C. elegans* and *D. melanogaster*, UNC5H1, UNC5H2, and UNC5H3 in vertebrates. They are transmembrane proteins with two Ig domains, two thrombospondin type I domains, a transmembrane domain, and an intracellular domain containing a ZU-5 domain and a death domain (Fig. 1.3). In *C. elegans*, the trajectories of axons extending away from a source of UNC-6 are disrupted in *unc-5* mutants, suggesting that UNC-5 is required for the chemorepellent response (Hedgecock et al., 1990). In *D. melanogaster*, *unc-5* is expressed in motoneurons whose axons exit the CNS without crossing the midline and then avoid netrin-expressing muscles in the periphery (Keleman and Dickson, 2001). In both *C. elegans* and *D. melanogaster*, ectopic expression of UNC-5 in neurons that normally do not respond to netrin or are attracted toward a netrin source caused these axons to be repelled by netrin *in vivo* (Hamelin et al., 1993; Keleman and Dickson, 2001). These findings provide further evidence that UNC-5 mediates the repulsive response to netrin. In mammals, UNC5Hs all bind to netrin-1, suggesting that they act as netrin receptors

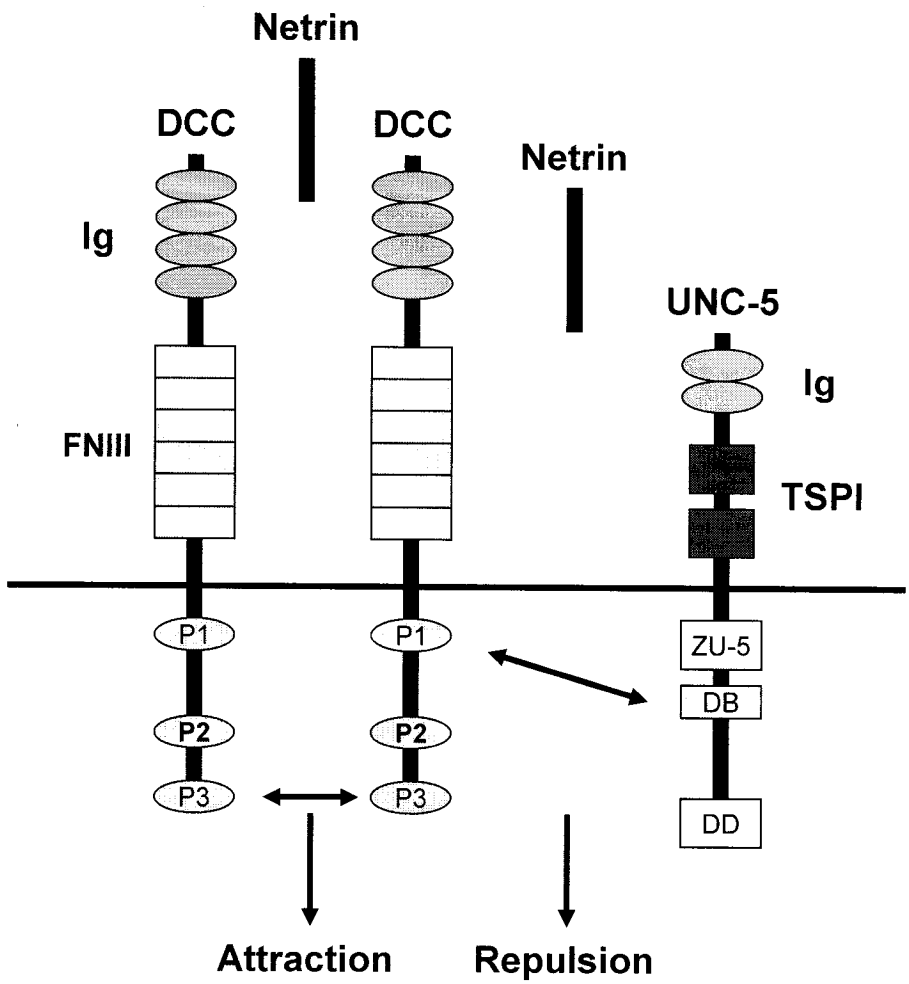
Netrin
DCC
DCC
Netrin
Ig
UNC-5
Ig
FNIII
TSPI
P1
P1
ZU-5
DB
P2
P2
P3
P3
DD
Attraction
Repulsion

Figure 1.3. Signaling by netrin receptors.

DCC is a transmembrane receptor which has four immunoglobulin (Ig) domains followed by six fibronectin type III (FNIII) repeats in the extracellular domain. In the cytoplasmic domain, there are three conserved motifs: P1, P2, and P3. UNC-5 is a transmembrane protein with two Ig domains, two thrombospondin type I (TSPI) domains in the extracellular domain. The cytoplasmic domain contains a ZU-5 domain, a DCC-binding domain (DB), and a death domain (DD). Netrin binding to DCC causes DCC clustering via its P3 domain and promotes attraction. DCC also interacts with UNC-5 via its P1 domain and the DB domain of UNC-5. The DCC-UNC-5 complex signals repulsion.

(Ackerman, 1997; Leonardo, 1997). The first *in vivo* evidence of vertebrate UNC5 homologs in axon guidance comes from the UNC5H3 mutant mice (Finger et al., 2002). In the mutant mice, the corticospinal tract (CST), the major tract responsible for the coordination of limb movements, appeared shorter and thinner compared with that of wild-type mice, suggesting CST defects in UNC5H3 mutant mice.

In addition to the DCC and UNC5 receptor family members, netrin-1 also binds to the membrane-associated adenosine A2B receptor, a G-protein-coupled receptor that induces cAMP accumulation upon adenosine binding (Corset et al., 2000). Corset et al. show that A2B is a netrin-1 receptor and induces cAMP accumulation upon binding to netrin-1, and netrin-1-dependent outgrowth of dorsal spinal cord axons directly involves A2B receptor. However, this result is challenged by Tessier-Lavigne and colleagues. They show that netrin-1 binds DCC directly, and activation of the adenosine A2B receptor is not required for rat commissural axon outgrowth or *Xenopus* spinal axon attraction to netrin-1 (Stein, 2001). Thus, they conclude that DCC plays a central role in netrin signaling of axon growth and guidance independently of A2B receptor activation.

1.2.3 Netrin-1 receptor DCC

1.2.3.1 Expression of DCC

The *DCC* gene was originally identified as a candidate tumor suppressor gene on human chromosome 18q that is frequently lost in human colorectal carcinomas (Fearon et al., 1990). It is the largest human tumor suppressor gene identified to date and encodes a transmembrane protein with sequence similarity to cell adhesion molecules such as NCAM and belongs to the immunoglobulin family. *DCC* gene is expressed at low levels in almost all normal adult tissues, including colonic mucosa, with highest levels in neural tissues (Cooper et al., 1995; Reale et al., 1994). During vertebrate development, *DCC* gene expression is predominately restricted to the developing CNS. Within the CNS, initiation of DCC expression correlates with the onset of neurogenesis and is maintained at high levels in all regions of the developing CNS actively undergoing neurogenesis. DCC expression is not restricted to a particular neuronal subset. Instead, DCC is ubiquitously expressed in the majority of postmitotic neurons within the early CNS (Gad et al., 1997). As the neural structures matured, DCC expression is detected only in a small

number of structures such as the olfactory bulb, the hippocampus, and the cerebellum that are known to sustain active neurogenesis well into postnatal life (Gad et al., 1997; Shu et al., 2000). In the developing mouse forebrain, DCC protein is expressed in specific axonal populations projecting from the developing olfactory bulb, neocortex, hippocampus, and epithalamus/habenular complex. In the developing olfactory bulb and neocortex, DCC expression is particularly evident during the targeting phase of axon outgrowth and is then rapidly downregulated (Shu et al., 2000). In the developing rat spinal cord, Keino-Masu et al. (Keino-Masu et al., 1996) found that *DCC* transcripts are strongly expressed in the commissural neuron and motor neuron cell bodies, but not in undifferentiated neuroepithelial cells. DCC protein is expressed on commissural axons as they project toward the floor plate, and on their growth cones.

1.2.3.2 Function of DCC

1.2.3.2a Role in commissural axon outgrowth

In embryonic vertebrate nervous system, spinal commissural neurons express DCC as their axons extend toward the floor plate. Netrin-1 binds to DCC-transfected cells with high affinity (Keino-Masu et al., 1996). These findings suggest that DCC may be involved in axon outgrowth. Direct evidence is provided by using a DCC blocking antibody, a monoclonal antibody raised against the extracellular domain of DCC, which inhibits netrin-1 and floor plate-evoked outgrowth of commissural axons in collagen gel explants; however, the antibody does not interfere with the binding of netrin-1 to DCC. It has been reported that DCC binds to heparin through a polybasic sequence in the fifth fibronectin repeat, and that the antibody blocking netrin responses interferes with heparin binding to DCC (Bennett et al., 1997). Geisbrecht et al. (Geisbrecht et al., 2003) mapped the interactions between netrin and its receptors. They found that netrin binds exclusively to the fifth fibronectin III repeat of DCC and to each Ig domain of UNC5, which is consistent with the previous finding that DCC binds directly to netrin-1 to mediate axon guidance (Stein, 2001). However, they found no evidence for an interaction between DCC and heparin and instead demonstrated that a loop on the fifth fibronectin III repeat of DCC previously implicated in mediating interactions with heparin is important for

netrin binding. Since netrin binds heparin, the interactions between DCC and heparin are likely mediated by netrin.

1.2.3.2b Is DCC a tumor suppressor protein?

Although *DCC* was first identified as a candidate tumor suppressor gene, evidence that DCC is in fact a tumor suppressor protein has been difficult to obtain. *DCC* expression is either lost or reduced in ~70% of colon tumors (Fearon et al., 1990), but few tumors seem to contain deletions at chromosome 18q that are likely to be restricted to DCC (Fearon and Vogelstein, 1990; Thiagalingam et al., 1996). There may be multiple tumor suppressors in this region. Inactivating mutations in two *Smad* genes and /or loss of *DCC* are LOH (loss of heterozygosity) events identified in tumors. No single gene accounts for all of the LOH; most 18q events alter more than one gene and 18q are associated with, but not shown to promote, tumorigenesis. DCC expression appears to be reduced during tumor progression (Reyes-Mugica et al., 1997). However, late stage tumors may contain multiple mutations, further complicating the analysis of tumor suppressors identified in LOH studies. In *DCC* knockout mice, an increase in tumor frequency was not found. However, the significance of this finding is difficult to interpret because the mice die shortly after birth and tumors may not have time to develop (Fazeli, 1997). Re-expression of *DCC* in tumor cells has been shown to suppress tumorigenicity (Tanaka et al., 1991). Correlations made between DCC expression and the phenotypes of primary tumor cells suggest that DCC does not influence tumor formation but inhibits a later step in tumor development such as tumor cell migration and metastasis (Kong et al., 1997a; Kong et al., 1997b; Reale et al., 1996; Reyes-Mugica et al., 1998). These findings suggest that DCC may function as a tumor suppressor by inhibiting cell motility, which is different from its role in the CNS.

Recent studies of *DCC* overexpression in 293T cells suggest that DCC functions as a tumor suppressor by acting as a pro-apoptotic dependent receptor (Mehlen et al., 1998). In the absence of netrin-1, DCC promotes apoptosis through activation of a novel pro-apoptotic caspase-dependent pathway (Forcet et al., 2001). Overexpression of *DCC* induces apoptosis in several carcinoma cell lines, but netrin-1 cannot rescue DCC-induced cell death (Chen et al., 1999).

1.2.3.2c Phenotype of DCC knockout mice

To elucidate the functions of the *DCC* gene, Frazeli et al. (Fazeli, 1997) inactivated the *DCC* gene in the mouse genome through the use of homologous recombination and examined the effects of this inactivation on both the intestine and the developing nervous system. All neonatal mice homozygous for the *DCC* allele appear to be grossly normal at birth but die within 24h. The DCC knockout neonatal mice exhibit striking behavioural phenotypes, including the inability to suckle, laboured respiration, abnormal body posture and abnormal limb flexion in response to pinch stimuli. However, the absence of DCC function does not affect either the normal or the neoplastic mouse gastrointestinal tissue. *DCC* knockout mice show several defects in commissural axon extension toward the floor plate throughout the developing spinal cord that are similar to those seen in netrin-1-deficient mice but are more severe. Within the dorsal spinal cord of E9.5-E11.5 *DCC* knockout mice, there is a reduction in the number of commissural axons, although those that do extend appear to adopt a normal dorsal-to-ventral trajectory. Few axons extend into the ventral spinal cord and reach the floor plate. In addition, it appears to be a misrouting of many of the axons that project into the ventral spinal cord, with some projecting more medially and others more laterally. In addition to defects in spinal cord development, the corpus commissures and the hippocampal commissure are completely absent. The axons that normally form these commissures are present but failed to cross the midline and remained ipsilateral, projecting to aberrant locations and forming tangles. The anterior commissure in *DCC* knockout mice is also severely reduced. Thus, the defects seen in the brain of *DCC* knockout mice are similar to those observed in netrin-1-deficient mice but are more severe. 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. Some commissural axons do reach the floor plate in the *DCC* knockout mice suggesting the existence of a DCC-independent mechanism for guidance of commissural axons to the floor plate. Evidence has been provided that Shh is

the cue distinct from netrin-1 and guides commissural axons toward the floor plate through a DCC-independent mechanism (Charron et al., 2003).

1.2.3.3 Interactions between DCC and other guidance receptors

Netrin-1 causes DCC to multimerize, which appears to be required for the axon guidance function of DCC (Stein, 2001). The cytoplasmic domain of DCC interacts homophilically through the P3 region and this interaction is repressed in the full-length receptors in the absence of netrin-1 (Fig. 1.3). The presence of netrin-1 stimulates DCC multimerization by association of both extracellular domains and intracellular domains. Similarly, DCC and UNC5 cytoplasmic domain interactions are repressed in the full-length receptors, and netrin-1 stimulates association of the two receptors by promoting the association of both extracellular and intracellular domains. The interaction of DCC and UNC5 is mediated by P1 region in DCC and DB (DCC-binding) domain in UNC5, and this interaction can switch DCC response to netrin-1 from attraction to repulsion (Fig. 1.3). In this case, UNC5 uses DCC as a coreceptor and only low concentration of netrin is needed (long-range repulsion). UNC5 can act on its own to repel axons at higher netrin concentrations (short-range repulsion) (Keleman and Dickson, 2001).

In *D. melanogaster*, where the activity of Slit in axon guidance was first described (Kidd et al., 1999), commissural axons increase their expression of the Slit receptor Robo as soon as they cross the midline (Kidd et al., 1998). This explains how commissural axons acquire sensitivity to Slit. But how do they lose their sensitivity to netrin? Previous work demonstrated that dissociated *Xenopus* embryonic spinal axons in culture steer toward a gradient of netrin, and that this response requires endogenously expressed DCC (Ming et al., 1997). Stein and Tessier-Lavigne found that netrin-mediated attraction in dissociated stage 22 *Xenopus* spinal neurons is abolished following bath application of Slit2. Though stage 22 spinal neurons are not themselves repelled by a source of Slit2, spinal neurons cultured from older stage 28 embryos are repelled by Slit2 but are not attracted to netrin. These younger and older populations of neurons appear to mimic the behaviour of pre- and post-crossing spinal commissural neurons with respect to their changing responses to netrin and Slit2 over time. Therefore, Slit specifically silences the ability of netrin to attract axons. They also showed that this silencing effect depends on a

direct interaction between the cytoplasmic domains of Robo and DCC. Full-length DCC and Robo expressed in tissue culture cells form a complex in the presence of Slit2, but not netrin alone. It appears that Robo and DCC ectodomains suppress, in the absence of the Slit2 ligand, the association between their cytoplasmic domains. This interaction is mediated by short conserved domains in each receptor-CC1 in Robo and P3 in DCC. CC1 domain is essential for silencing, however, Slit2-mediated repulsion does not require this domain, indicating that Robo-mediated silencing is distinct from its function in repulsion. Although netrin attraction is silenced by Slit2, netrin outgrowth promoting activity is not affected, providing evidence that these two different netrin activities are distinct. This study provides new insight into how axons respond to multiple guidance cues. *In vivo*, axons are simultaneously exposed to a number of different attractive and repulsive cues. It has been widely thought that the axon integrates all of these signals in order to reach its target. However, multiple guidance signals can also be combined in a way with one signal silencing the response to another.

1.2.3.4 Interaction of netrin family members with identified netrin receptors

Although five netrins have now been identified in vertebrates, it seems that not all of them interact with known netrin receptors. It has been shown that netrin-1 and netrin-3 bind to DCC, neogenin, UNC5H1, UNC5H2, and UNC5H3 (Keino-Masu et al., 1996; Leonardo, 1997; Wang et al., 1999a). Netrin-1, netrin-2, and netrin-3 promote DCC-dependent axon outgrowth from multiple neuronal cell types (de la Torre et al., 1997; Deiner et al., 1997; Hong et al., 1999; Keino-Masu et al., 1996; Wang et al., 1999a). Netrin-1 and netrin-3 repel the axons of embryonic trochlear motoneurons (Colamarino and Tessier Lavigne, 1995; Wang et al., 1999a). Recombinant netrin-4/β-netrin evokes the outgrowth of axons from explants of embryonic rat olfactory bulb, but it is not known if this response requires DCC or if these neurons express *DCC* (Koch, 2000). There is no evidence showing that any known netrin receptors bind to netrin-G1. Recombinant netrin-G1 did not promote the outgrowth of cerebellofugal axons from explants of embryonic cerebella plate (Nakashiba et al., 2000), axons that extend toward a source of netrin-1 (Shirasaki et al., 1996). All these findings indicate that DCC and UNC-5 homologs

function as receptors for netrin-1, netrin-2, netrin-3, and possibly netrin-4/β-netrin, and suggest that an unidentified receptor mediates the response to netrin-G1.

1.2.3.5 Modulation of guidance receptors

How does a growth cone respond to a bifunctional guidance cue? Are responses mediated by different combination of the receptors on the growth cone surface, or are there other properties intrinsic to the growth cone that dictate the response? To address this question, Bashaw et al. (Bashaw and Goodman, 1999) generated chimeric receptors, having the ectodomain of Frazzled combined with the cytoplasmic domain of Robo (Fra-Robo) and ectodomain of Robo with the cytoplasmic domain of Frazzled (Robo-Fra), and then expressed these chimeric receptors in all neurons of the *D. melanogaster* embryonic CNS. They observed that these neurons expressing high levels of the Fra-Robo chimeric receptor were repelled by netrin-expressing midline cells leading to a lack of axons crossing the CNS midline. On the other hand, neurons expressing the Robo-Fra chimeric receptor were attracted to the Slit-expressing midline cells, with many axons inappropriately crossing the CNS midline. They conclude that the ectodomains of these receptors determine ligand recognition, while the cytoplasmic domains specify the response. So, the cytoplasmic domains of guidance receptors are key effectors, directing either attractive or repulsive responses. In independent studies, Hong et al. (Hong et al., 1999) focused on attractive and repulsive responses to a single cue, netrin-1, and also found that the cytoplasmic domain of a receptor dictates the growth cone response. When a chimeric receptor consisting of a DCC ectodomain and an UNC5 cytoplasmic domain was tested in the axon turning assay, it mediated a repulsive response to the netrin-1 gradient.

1.2.3.6 Modulation of netrin signaling

The levels of DCC receptors at the cell surface may also be regulated by proteolytic cleavage. It was believed that the main function of metalloproteases is to create penetrable paths for axon extension and to modulate the activities of receptors and ligands through proteolytic processing (Adams et al., 1997; Cabrera et al., 1996; Fambrough et al., 1996; Schlondorff and Blobel, 1999). However, Galko and Tessier-

Lavigne (Galko and Tessier-Lavigne, 2000) showed that chemical inhibitors of metalloproteases do indeed potentiate axon outgrowth mediated by netrin-1. They also found that DCC is a substrate for metalloprotease-dependent ectodomain shedding, and that inhibitors block proteolytic processing of DCC and cause an increase in DCC protein levels in axons within the spinal cord. These findings indicate that dorsal spinal cord explants display a metalloprotease activity that mediates the proteolytic degradation of the netrin receptor DCC to a presumably non-functional form, and that the inhibition of metalloprotease activity leads to enhanced responsiveness to netrin-1. This functional effect may result from inhibition of DCC cleavage, although the possibility that it may also result partly or entirely from inhibition of cleavage of other substrates cannot be excluded. These results imply that the balance of metalloprotease activity within the dorsal spinal cord or along the trajectory of commissural neurons may be an important regulator of commissural axon guidance. For instance, it appears that the C-terminal domains of netrins are homologous to the N-terminal domains of tissue inhibitors of metalloproteases (Banyai and Patthy, 1999). It will be interesting to determine whether netrin-1, in addition to activating DCC, also stabilizes its receptor by inhibiting metalloproteases.

UNC-71 is a metalloprotease protein with an inactive metalloprotease domain in *C. elegans*. Double mutants in unc-71 and netrin signaling molecules all showed an enhancement of netrin-mediated commissural axon outgrowth, suggesting that UNC-71 functions in parallel to netrin-mediated commissural axon outgrowth and guidance. Although ectodomain shedding of DCC by metalloproteases has been implicated in axon outgrowth (Fambrough et al., 1996; Galko and Tessier-Lavigne, 2000), UNC-71 is unlikely the metalloprotease that cleaves DCC because of its inactive metalloprotease domain.

The neuronal growth cone integrates various guidance cues and responds by adjusting the overall direction in which the axon extends. Some guidance cues are produced near the advancing growth cone, tethered to the surface of the nearby cells or to the extracellular matrix, and locally steer growth cones. Other secreted and diffusible guidance cues are thought to act over long distances by forming gradients that either attract or repel extending axons. But little is known about how these cues are presented to

axons. Using *D. melanogaster* as a model system, Hiramoto et al. (Hiramoto et al., 2000) show that netrin is captured far from its site of synthesis and presented to approaching axons by Frazzled, *D. melanogaster* homolog of DCC. Netrin thereby instructs growing axons to follow a precise trajectory within the CNS. In *D. melanogaster*, in addition to the expression in the CNS midline, a substantial fraction of netrin also localizes to axons in a dorsolateral CNS region, far from where netrin is made. This dorsolateral region also contains abundant Frazzled; indeed, both Frazzled and netrin are found on the same dorsolateral axons. In the absence of Frazzled or following the forced expression of Frazzled in the wrong places, netrin distribution changes dramatically. So Frazzled plays a key role in capturing and localizing netrin at specific sites in the fly CNS. To determine if this netrin localization is able to guide axons, they looked at the dMP2 neurons, which are known to require netrin and Frazzled for guidance. In embryos lacking netrin or Frazzled, the turning behaviour of dMP2 neurons is altered. In embryos lacking Frazzled, this altered behaviour is corrected by selectively expressing Frazzled in lateral neurons, but not in dMP2 neurons themselves, consistent with the observation that dMP2 neurons do not express Frazzled. The ability of Frazzled to guide dMP2 axons and localize netrin does not require its cytoplasmic domain. The extracellular domain of Frazzled captures and immobilizes netrin in regions encountered by extending axons. So rather than serving only as a netrin-binding protein on the surface of growing neurons, Frazzled, and possibly other DCC family members, also helps netrin to steer axons that do not themselves express Frazzled.

1.2.3.7 Protein synthesis, degradation, and growth cone guidance

Axonal growth cones turn in response to little concentration differences in extracellular guidance cues (Baier and Bonhoeffer, 1992). What exactly happens inside the growth cone to make it turn in response to these extracellular gradients? Campell and Holt (Campbell and Holt, 2001) have demonstrated that these turning responses are dependent upon localized protein synthesis and degradation within the growth cone. In earlier work, they showed that *Xenopus* retinal axons are responsive to both the netrin-1 and Sema3A guidance cues (Campbell and Holt, 2001; de la Torre et al., 1997). Netrin-1 appears to guide these axons at an early stage (stage 24), attracting them to the head of the optic

nerve on the first leg of their journey towards the tectum (de la Torre et al., 1997). Only later (after about stage 32) do retinal axons respond to Sema3A, which provides a repulsive signal to help keep them on course as they approach the tectum (Campbell and Holt, 2001). In this assay, an isolated *Xenopus* axon growing on a coverslip is confronted with a gradient of netrin-1 or Sema3A, delivered from a micropipette positioned just ahead and to the side of the advancing growth cone. Retinal axons turn towards the pipette if it delivers netrin-1 and away from it if it provides Sema3A.

Several years ago, Harris et al. (Harris et al., 1987) found that when growth cones are cut from their soma, they stay alive for up to 3 hours *in vivo* and continue to navigate correctly towards the tectum. Later, Campell and Holt (Campbell and Holt, 2001) found that the isolated growth cones also stay alive in culture for 2 hours, long enough to perform the turning assay. In these assays, the growth cones of severed axons respond to both netrin-1 and Sema3A as the intact neurons. These findings suggest that turning relies only on those molecules, including any mRNAs, already available in the growth cone. They found both mRNA and ribosomes in the growth cones of *Xenopus* retinal axons. Exposure to netrin-1 or Sema3A triggers a burst of protein synthesis within the growth cone, as evidenced by a rapid phosphorylation of eIF4E, and increased incorporation of labelled amino acids. Both of these responses are rapid, occurring within a few minutes of stimulation. Rapamycin, an inhibitor of the TOR (target of rapamycin) pathway, blocked protein synthesis induced by either netrin-1 or Sema3A, but inhibitors of phosphatidylinositol 3-kinase (PI3-K) only blocked protein synthesis induced by Sema3A. This suggests that netrin-1 and Sema3A might act at least in part through different pathways to regulate translation within the growth cone. The machinery for proteasome-mediated protein degradation is also present in the growth cones of *Xenopus* retinal axons, and netrin-1 (but not Sema3A) induces a rapid rise in the levels of proteins tagged with ubiquitin and thus destined for degradation in the proteasome.

Netrin-1 thus induces rapid protein synthesis and degradation within the growth cone, while Sema3A appears to induce only protein synthesis. To test if local protein turnover is involved in growth cone turning, they applied inhibitors of translation or degradation to both intact and isolated retinal axon growth cones in the *in vitro* turning assay. Inhibitors of either protein synthesis or degradation block attraction by netrin-1,

while only protein synthesis inhibitors block repulsion by Sema3A. However, none of these inhibitors blocks axon extension itself, implicating axon turning and outgrowth through different mechanisms. How does local protein synthesis and degradation contribute to growth cone turning? One possibility might be that the asymmetric synthesis or degradation of proteins within the growth cone directly mediates the turning response. For example, in the case of a netrin-1 gradient, cytoskeleton proteins might be synthesized on the side of the growth cone facing up the gradient, and degraded on the side facing down. This could lead to the turning response towards the netrin-1 source.

Other studies have revealed that netrin-1 triggers a rapid phosphorylation and activation of extracellular signal-regulated kinase (ERK)-1/2, which is mediated through the netrin-1 receptor DCC (Forcet et al., 2002). Based on these observations, and the fact that translation initiation factors are also regulated by an ERK-1/2-dependent pathway, Forcet et al. (Forcet et al., 2002) proposed that netrin-1, signaling through DCC, activates mitogen-activated protein kinase (MAPK) in a way that could regulate local protein synthesis within the growth cone. This hypothesis was tested recently by Campbell et al. (Campbell and Holt, 2003). They confirmed that p42/p44 MAPK mediates netrin-1 responses in retinal growth cones, and inhibition of p42/p44 blocks netrin-1-induced protein synthesis. Unexpectedly, p38 MAPK is also activated by netrin-1 in retinal growth cones and is required for chemotropic responses and translation. These findings suggest that the differential activation of MAPK pathways may underlie guidance cue directed migration.

1.2.3.8 Signal transduction underlying netrin and DCC

Although much progress has been made into identifying axon guidance cues and their receptors, the signal transduction mechanisms underlying them are just beginning to be elucidated. In the case of netrin and DCC, the signaling events downstream of DCC that mediate axon growth and guidance are still poorly understood. Recently, Forcet et al. (Forcet et al., 2002) demonstrated that netrin-1-mediated axon outgrowth requires DCC-dependent MAPK activation. They show that DCC activates ERK-1/2 in both transfected cells and commissural neurons in a netrin-1-dependent manner. This activation can cause transcriptional activation, as evidenced by examining transcription factor Elk-1 activation

and serum response element (SRE)-dependent gene expression. ERK-1/2 activation is associated with recruitment of ERK-1/2 to a DCC receptor complex. They then examined whether ERK-1/2 contributes to the effects of DCC on axon growth and guidance. Inhibition of ERK-1/2 antagonizes netrin-1-dependent axon outgrowth and guidance. In netrin-1 and DCC knockout animals they observed diminished phospho-ERK-1/2 staining in the central spinal cord, which is consistent with activation of ERK-1/2 mediated by netrin-1. These results support a role for the MAPK pathway in response to the chemoattractant netrin-1. DCC-stimulated MAPK activation leads to activation of transcription factor Elk-1 and SRE-regulated gene expression, providing a mechanism for transcriptional control by netrin-1. Elk-1 is present not just in neuronal cell bodies but also in axon terminals (Sgambato et al., 1998), but whether axonal Elk-1 participates in axon guidance is unknown. Other targets of ERK-1/2 involved in axon guidance may be translational regulators. New protein translation is stimulated by netrin-1 and required for netrin-mediated attraction of *Xenopus* retinal growth cones *in vitro* (Campbell and Holt, 2001). Principal factors for translation initiation like eIF4E and eIF4E-BP1 are phosphorylated by an ERK-1/2-dependent pathway (Herbert et al., 2002), providing a potential mechanism linking netrin-1 to protein synthesis for axon growth and guidance.

Several studies indicate that protein kinase A (PKA) plays a key role in determining if a neuronal growth cone is attracted or repelled by netrin-1. Elevating the intracellular concentration of cAMP causes PKA activation (Nairn et al., 1985). Utilizing dissociated neurons from embryonic *Xenopus* spinal cord, Ming et al. (Ming et al., 1997) demonstrated that PKA activation is correlated with chemoattraction to netrin-1, while PKA inhibition switches the response to repulsion. It is known that PKA can phosphorylate RhoA, leading to the translocation of membrane-associated RhoA to the cytoplasm (Lang et al., 1996), thus PKA activation may influence growth cone guidance by inhibiting RhoA activity and having a chemoattractant response to netrin-1. Mena, a profilin-binding protein required for axon guidance in mice, is also an *in vivo* substrate of PKA, thus PKA may regulate actin dynamics through Mena (Lanier et al., 1999). These findings also suggest that if a growth cone encountered an extracellular cue that altered the intracellular concentration of cAMP, the response of the extending axon to netrin-1 might be reversed. In agreement with this, Hopker et al. (Hopker et al., 1999) found that

the combined expression of netrin-1 and laminin-1 at the entry of the optic nerve triggers a specific decrease in cAMP levels that drives axons away from the eye toward an attractive, netrin-rich, laminin-poor optic nerve. In this situation, the response of a growth cone to netrin is switched by the presence or absence of laminin.

Cytosolic calcium is known to play an important regulatory role in growth cone motility and to regulate the dynamics of actin polymerization and depolymerization, processes necessary for local changes of the cytoskeleton associated with growth cone turning (Gu and Spitzer, 1995; Lankford and Letourneau, 1989). The netrin-1-induced turning response depends on Ca^{2+} influx through plasma membrane Ca^{2+} channels, as well as Ca^{2+}-induced Ca^{2+} release from cytoplasmic stores. Reduction of Ca^{2+} signals by blocking either of these two Ca^{2+} sources converts the netrin-1-induced response from attraction to repulsion (Hong et al., 2000). Calcium signaling can be transduced by calmodulin (CaM) and CaM-dependent kinases. Deviations of axons in growth, fasciculation and pathfinding have been observed in *D. melanogaster* embryos in which Ca^{2+}/CaM function was selectively disrupted (VanBerkum and Goodman, 1995). Another potential downstream target of Ca^{2+} is adenylyl cyclase (Xia and Storm, 1997). Type I and type III adenylyl cyclases are stimulated by Ca^{2+} and CaM *in vivo* to produce cAMP that can activate PKA. The ratio of cAMP to cGMP activities sets the polarity of the netrin-1-induced axon guidance: high ratios favour attraction, whereas low ratios favour repulsion (Nishiyama et al., 2003). cAMP signaling directly modulates the activity of Ca^{2+} channels in axonal growth cones, and regulation of Ca^{2+} channels is a pivotal early event in the transduction of netrin-1 signals.

Brief electrical activity in the neuron also modulates netrin-1-induced responses. When cultured *Xenopus* spinal neurons are exposed to pulsatile electrical stimuli, which are able to trigger action potentials in the neuron, the growth cone exhibits marked attractive turning in the same gradient produced by the low dose of netrin-1, which otherwise does not induce turning response. Thus, electrical stimulation can enhance the sensitivity of the growth cone to netrin-1. Electrical activity can also convert the repulsive effect of netrin-1 to attraction. These effects require Ca^{2+} influx through the opening of membrane depolarization-induced voltage-dependent Ca^{2+} channels. The effects of

electrical stimulations are mediated through elevation of cAMP, presumably through the activation of Ca^{2+}-dependent adenylate cyclase (Xia and Storm, 1997).

Tyrosine phosphorylation plays important roles in signal transduction. Evidence has been shown that both UNC-40 and UNC-5 homologs are phosphorylated on tyrosine residues (Tong et al., 2001). Furthermore, tyrosine phosphorylation of UNC5 family members is enhanced by netrin in a mammalian cell line and in *C. elegans in vivo*. Although it is not yet clear how tyrosine phosphorylation may contribute to netrin-dependent attraction or repulsion, this finding provides the first indication that tyrosine phosphorylation may be involved in netrin signals.

Ena/VASP has been implicated in growth cone repulsion by netrin/UNC-6 and its receptor UNC-5 in *C. elegans* (Colavita and Culotti, 1998), and by Slit and its receptor Robo in *D. melanogaster* (Bashaw et al., 2000). Gitai et al. (Gitai et al., 2003) provide evidence that Ena is also an effector of an attractive axon guidance pathway. They generate a gain-of-function UNC-40 molecule that produces strong axon guidance and outgrowth defects in *C. elegans*. These defects are suppressed by loss-of-function mutations in CED-10 (a Rac GTPase), UNC-34 (an Ena homolog), and UNC-115 (a putative actin-binding protein similar to human actin-binding LIM protein, ABLim). Thus, Ena functions in axonal attraction as well as axonal repulsion. UNC-40 has two conserved cytoplasmic motifs that mediate distinct downstream pathways: CED-10, UNC-115, and the UNC-40 P2 motif act in one pathway, and UNC-34 and the UNC-40 P1 motif act in the other. Thus, UNC-40 might act as a scaffold to deliver several independent signals to the actin cytoskeleton.

1.2.3.9 The intrinsic regulation of growth cone response to netrin-1

RGC axons follow a complex path and are confronted with multiple choices as they exit the eye and project to the brain. One of the first steering decisions that growing RGC axons make is to turn away from the retina surface and extend into the optic nerve head to exit the eye. Then they continue along the optic nerve, cross the chiasm, extend through the optic tract, and enter the optic tectum, where they stop and branch within the optic tectum to form a functional terminal arbour. Previous work has shown that netrin-1 guides retinal axons as they exit the eye by serving as a short-range attractive signal (de la

Torre et al., 1997). Retinal axons show chemoattractive turning toward netrin-1 gradients and are guided out of the eye by localized netrin-1 expression in the optic nerve head. Thus, netrin-1 initially attracts and later repels retinal axons. Shewan et al. (Shewan et al., 2002) show that both intrinsic and extrinsic cues are involved in regulating this developmental switch in responsiveness. They demonstrate that a correlated, developmental downregulation of cAMP and A2B receptor expression is responsible for the differential response of retinal axons to netrin-1 guidance. Using growth cones from explants prepared from whole retinas at distinct times in development, as well as growth cones from young retinas aged in culture (to the equivalent of the stage when axons reach the tectum), they assayed the response to netrin-1. They found that growth cones from young retinal explants have attractive response and growth cones from old retinal explants have repulsive response to netrin-1. These results support the idea that a developmentally regulated decrease in cAMP levels is involved in the regulation of netrin-1 response within the visual pathway. cAMP levels were significantly lower in growth cones of old retinal explant cultures, which were repelled by netrin-1, than in their younger counterparts, which were attracted by netrin-1. The contribution of cAMP downregulation to the change in growth cone response to netrin-1 was further demonstrated by manipulating cAMP levels. Treatment of old growth cones with Sp-cAMPs, a cAMP analog, resulted in a switch in response to netrin-1, from repulsion to attraction. The downregulation of cAMP was paralleled by a similar and significant downregulation in the adenosine A2B receptor levels at the growth cone. Similar to increasing cAMP levels, bath application with an A2B receptor agonist converted the repulsion of old growth cones to attraction, where bath application of specific A2B receptor antagonists converted the attraction response by young growth cones to repulsion. DCC receptor levels were also downregulated in the retinal growth cones as they matured, but to a lesser degree than the A2B receptor. The results presented by Shewan et al. imply that age-related intrinsic regulation of responsiveness can occur to a certain degree without the influence of extrinsic signals.

1.3 RHO GTPASES: REGULATION AND FUNCTION

1.3.1 Ras superfamily of small GTPases

The Ras family of small GTPases are monomeric guanine nucleotide-binding proteins. Since the first small GTPase Ha-Ras was discovered (Capon et al., 1983), over 60 members in mammals have been identified in the Ras superfamily, which are grouped into five subfamilies including Ras, Rho, Rab, ARF, and Ran (Fig. 1.4). Small GTPases function as molecular switches to control a wide range of essential biological process in all eukaryotic cells. They exist in either an inactive, GDP-bound form or an active, GTP-bound form and this GDP/GTP cycle is regulated by three families of proteins, guanine nucleotide exchange factors (GEFs) (Table 1.1; Whitehead et al., 2003), guanine nucleotide dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs) (Table 1.2; Jenna et al., 2003) (Fig 1.5). GEFs activate the GTPase through GDP/GTP exchange and GAPs inactivate GTPase by stimulating the intrinsic GTPase activity, which returns the protein to the GDP-bound form to complete the cycle and terminate signal transduction. GDIs appear to stabilize the inactive, GDP-bound form of the protein. Activated GTPases interact with downstream effectors to trigger a variety of cellular responses.

GTPases play major roles in the regulation of cell growth, morphogenesis, cell motility, axon guidance, cytokinesis, and membrane trafficking. Ras GTPases are key regulators of cell growth and differentiation. They have been found in mutated oncogenic forms in a large number of human tumours. Mutations in codon 12, 13, or 61 of one of the three *ras* genes, Ha-*ras*, K-*ras*, and N-*ras*, convert these genes into active oncogenes (Bos, 1989). Rho proteins control cell growth and morphology in all eukaryotic cells. Rab GTPases represent a large family of Ras-like enzymes that play key roles in the secretory and endocytic pathways and are located in distinct cellular compartments. ADP-ribosylation factors (ARFs) were initially recognized and purified because of their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A subunit. They are critical components of several vesicular trafficking pathways. Ran GTPases play a central role on protein and RNA trafficking in and out of the nucleus. They are one of the most abundant GTPases. One important feature of the GTPases is the cross-talk between the subfamilies. The cross-talk occurs at different levels including GTPases, regulators, and

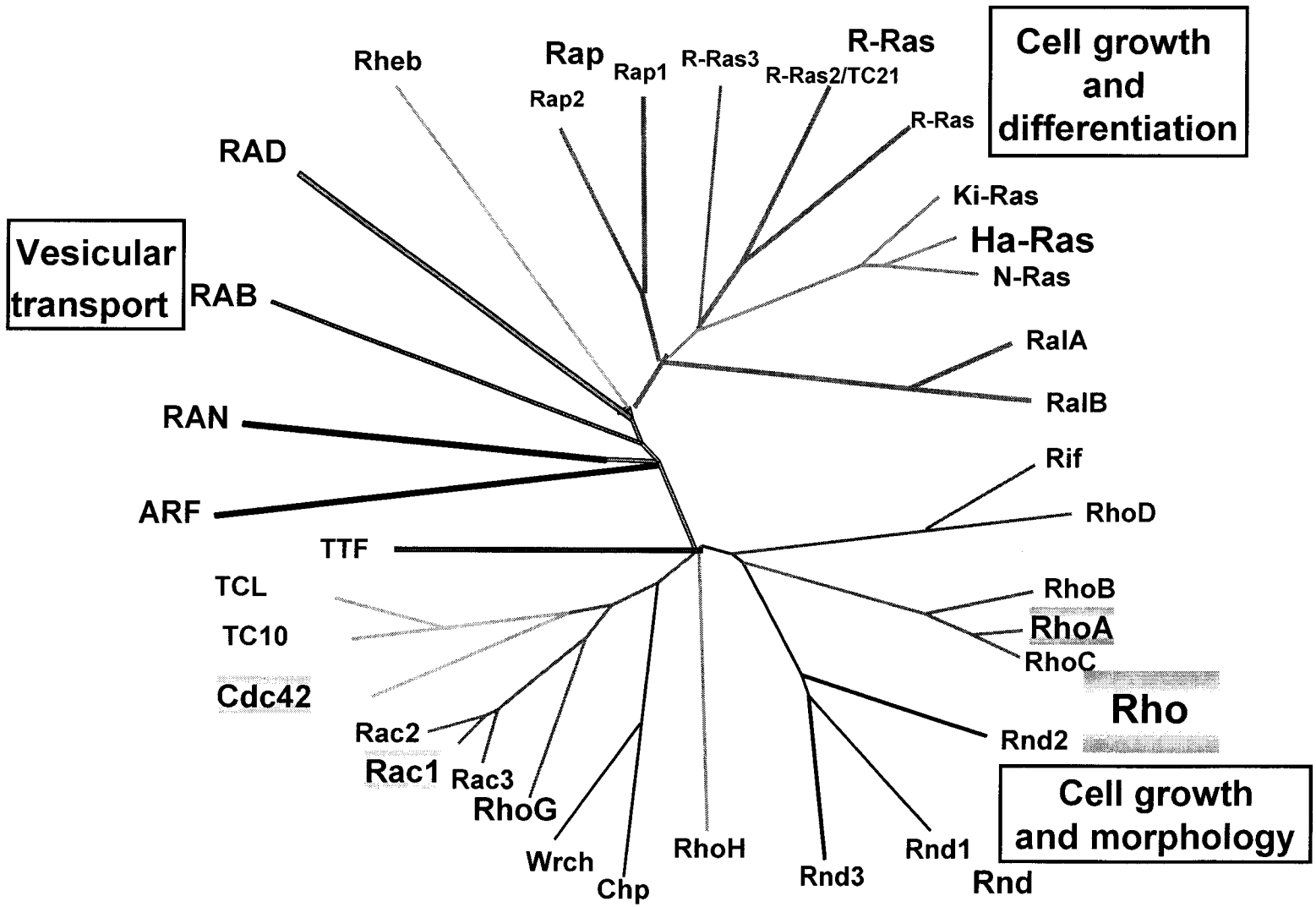
Cell growth and differentiation
Rheb
Rap
Rap1
Rap2
R-Ras3
R-Ras
R-Ras2/TC21
R-Ras
Ki-Ras
Ha-Ras
N-Ras
RalA
RalB
Vesicular transport
RAD
RAB
RAN
ARF
TTF
Rif
RhoD
RhoB
RhoA
RhoC
Rho
TCL
TC10
Cdc42
Rac2
Rac1
Rac3
RhoG
Wrch
Chp
RhoH
Rnd3
Rnd1
Rnd2
Rnd
Cell growth and morphology

Figure 1.4. Ras superfamily of small GTPases.

The Ras superfamily of small GTPases has over 60 members in mammals, which are grouped into five subfamilies including Ras, Rho, Arf, Ran, and Rab. They play major roles in the regulation of cell growth, morphogenesis, and vesicular transport. In the Rho subfamily, the best-characterized members are RhoA, Rac1, and Cdc42. They have been shown to play important roles in the regulation of a wide range of biological processes affecting both cell morphology and cell growth control.

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	Has 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 estrigen 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 domian
Pix/Cool/ARFGEF6	Rac1	Muted in X-linked mental retardation
Kalirin	Rac1	Regulates dendritic morphogenesis
Duet	NR	Contains serine/threonine kinase activity
STEF	Rac1	Closely related to Drosophila Sif

Table 1.1. Properties of mammalian RhoGEFs (Whitehead et al., 2003).

Exchange specificities are for DH domain related activities only. NR = not reported.

Subfamily	Name	GAP activity	Role in...
Bcr	Bcr	**Rac1**, Rac2, Cdc42	Cell cycle progression
	Abr	Rac1, Cdc42	Mediation of glucocorticoid effects
p190-RhoGAP	p190-A	Rac1, Cdc42, **RhoA***	Ras-RhoA cross-talk
	p190-B	Rac1, Cdc42, **RhoA***	Fibroblast migration Melanoma and breast cancer cell invasion; Neurogenesis
Chimaerin	α1	**Rac1**, Cdc42	Rac1, Cdc42 effector?
	α2	**Rac1**, Cdc42	
	β1	**Rac1**, Cdc42	
	β2	**Rac1**, Cdc42	Malignant glioma? Mitosis,cytokinesis
	MgcRacGAP	Rac1, Cdc42	Hematopoiesis; Spermatogenesis? Neurogenesis
	Rotund	ND	Spermatogenesis
	CYK-4	Rac1, Cdc42	Cytokinesis
	PARG1	Rac1, Cdc42, RhoA*	
GRAF	GRAF	**RhoA,** Cdc42	Hematopoietic disorders?
	Oligophrenin1	RhoA, Cdc42, Rac1	X-linked mental retardation
	PSGAP	**RhoA, Cdc42**	
Myosin-IX	Myosin-IXB	RhoA	
	Myosin-IXA	ND	Bardet-Bield sydrome?
	Myr5	**RhoA,** RhoB, RhoC	
	Myr7	RhoA	
RLIP	RLIP76	Cdc42, Rac1	Ral-Rho crosstalk
	RIP1	Cdc42, Rac1	Ral-Rho crosstalk
	RalBP1	Cdc42	Ral-Rho crosstalk
	Cytocentrin	ND	Ral-Rho crosstalk; Mitosis
p85	p85α p85β	(-)	actin reorganization
p50-RhoGAP	p50-RhoGAP (Cdc42Hs-GAP)	Rac1, Cdc42,* **RhoA**	
OCRL	OCRL1	(-)	Lowe syndrome
p122-RhoGAP	p122-RhoGAP	**RhoA**	RhoA effector? Activates PLC-δ1
C1	Hs C1	**RhoA**	
CeGAP	CeGAP	Rac1, Cdc42, RhoA Ras, let60, Rab3A	
3BP1	3BP1	**Rac1,** Cdc42	Abl signaling
	Nadrin	**Rac1,** Cdc42, **RhoA**	Exocytosis in PC12 cells
CdGAP	CdGAP	**Rac1, Cdc42**	endocytosis? mitogenic signaling
ARHGAP6	ARHGAP6	**RhoA**	Microphthalmia with Linear Skin defects?
srGAP	srGAP	Cdc42	axon growth and guidance
Grit	Grit	Rac1, Cdc42, RhoA	neurite outgrowth

Table 1.2. Specific GAP activity and putative cellular and pathological functions of characterized mammalian RhoGAPs (Jenna et al., 2003).

Rho GTPases experimentally identified as *in vivo* RhoGAP substrates are indicated in bold. The proposed cellular and/or pathological roles are indicated. Asterisks indicate the preferred substrates. ND indicates non-characterized activity. (-) indicates that the protein does not present any GAP activity *in vitro*.

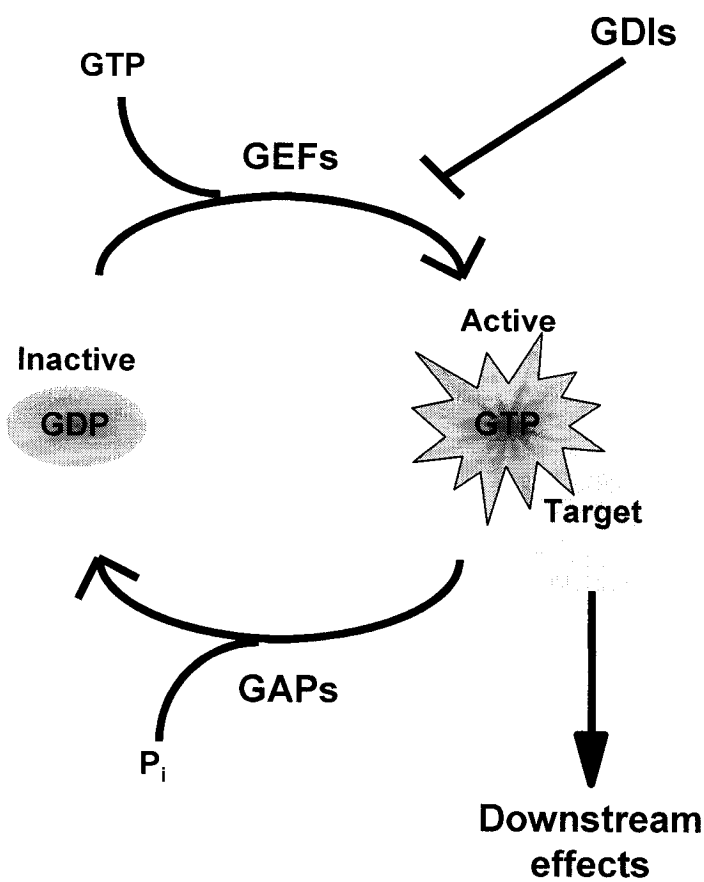
GDIs
GTP
GEFs
Active
Inactive
GDP
GTP
Target
GAPs
P_i
Downstream
effects

Figure 1.5. GDP/GTP cycle of the small GTPases.

Small GTPases exist in either an inactive, GDP-bound form or an active, GTP-bound form. This GDP/GTP cycle is regulated by three families of proteins, GEFs, GAPs, and GDIs. GEFs activate GTPases through GDP/GTP exchange and GAPs inactivate GTPases by stimulating the intrinsic GTPase activity. GDIs inactivate GTPases by stabilizing the proteins in the GDP-bound form. Activated GTPases interact with downstream effectors to trigger a variety of cellular responses.

effectors. For example, constitutively active Ras is a potent activator of Rac1 (Ridley et al., 1992), whereas both Ras and Rac1/Cdc42 activate MAPK pathway.

1.3.2 Rho GTPases

Rho GTPases are about 30% identical at the amino acid level to Ras GTPases. The core consensus motifs that have been defined as being essential for GTP binding and hydrolysis in Ras are also maintained in the Rho GTPases, and these proteins exhibit similar biochemical properties (Lamarche and Hall, 1994; Nobes and Hall, 1994). However, the putative effector domain of the Rho proteins is significantly different from that of Ras, therefore, the cellular targets of the activated Rho GTPases are different from those of activated Ras. The Rho family comprises 21 genes in humans, encoding 23 signaling proteins: Rho (A, B, and C), Rac (1a, 1b, 2, and 3), Cdc42 (Cdc42Hs and G25K, TC10, and TCL), RhoD, RhoG, Rnd (1, 2, 3, and RhoE), TTF, Rif, Wrch, Chp, and RhoBTB (1 and 2) (Wherlock and Mellor, 2002) (Fig. 1.4). Related genes have been identified in yeast, worms, and flies, indicating a high degree of evolutionary conservation of the Rho GTPases.

In the Rho family of the small GTPases, the best-characterized members are RhoA (Ras homologous), Rac1 (Ras-related C3 botulinum toxin substrate), and Cdc42 (Cell division cycle). They have been shown to play important roles in the regulation of a wide range of biological processes affecting both cell morphology and cell growth control. Their biological function was initially described in Swiss 3T3 fibroblasts. Activation of RhoA by lysophosphatidic acid (LPA) leads to the assembly of stress fibers which are actin-myosin bundles across the cells (Ridley and Hall, 1992). Therefore, RhoA acts as a molecular switch to control a signal transduction pathway that links membrane receptors to the actin cytoskeleton. The next member of the Rho family analyzed was Rac1. Activation of Rac1 by growth factors such as platelet-derived growth factor (PDGF) induces lamellipodia formation which are sheet-like extensions at the edge of the cells containing cross-linked F-actin meshwork (Ridley et al., 1992). On the other hand, activation of Cdc42 by bradykinin, IL-1 or TNFα, was shown to induce filopodia formation which are long, thin protrusions at the periphery of the cells composed of F-actin bundles (Kozma et al., 1995; Puls, 1999). In some cell types such as fibroblasts,

there is cross-talk between the Rho GTPases. Cdc42 can activate Rac1 and Rac1 activation leads to RhoA activation (Nobes and Hall, 1995). These observations suggest that members of the Rho GTPase family are key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton.

Now, there is evidence showing that Rho GTPases affect microtubules as well. RhoA promotes the accumulation of detyrosinated microtubules in the vicinity of the leading edge of the cell (Cook et al., 1998). Cdc42 regulates the orientation of the microtubule organizing centre either in front of or behind the nucleus (Wittmann and Waterman-Storer, 2001). Rac1 causes phosphorylation and thus inactivation of the microtubule destabilizing protein, stathmin, which could enhance polymerization or stabilization of microtubules (Daub et al., 2001). Also, Rac1 and Cdc42 increase the interaction of IQGAP with CLIP-170, which binds to the polymerizing ends of microtubules, this might capture microtubule ends at the plasma membrane (Fukata et al., 2002). More recently, Rac1 has been shown to mediate the effect of laminin on microtubules in the growth cone (Grabham et al., 2003).

1.3.2.1 Rho GTPases and axon guidance

The Rho GTPases have emerged as common regulators of actin dynamics that drive growth cone motility. Most of the studies on Rho function in neuronal cells have been obtained by expressing constitutively active or dominant negative mutant proteins in neuronal cells. Studies in N1E-115 neuroblastoma cells have shown that Rac1 and Cdc42 promote neurite outgrowth by regulating the formation of lamellipodia and filopodia in the growth cone, whereas RhoA activity inhibits neurite outgrowth (Kozma, 1997). Expression of dominant negative mutants of Rac1 and Cdc42 blocks neurite outgrowth induced by serum starvation and inactivation of RhoA by C3 transferase (bacterial toxin which inactivates RhoA activity) stimulates neurite outgrowth. Although studies *in vitro* have implicated the role of the GTPases in neurite ourgrowth, they do not address whether GTPases play important role in axonal outgrowth *in vivo*. Previous studies in *D. melanogaster* provided insight into the role of the GTPases in axonal growth *in vivo*. In *D. melanogaster* embryonic sensory neurons, expression of either constitutively active or dominant negative Rac1 results in selective defects in axonal outgrowth without notably

affecting dendrite growth. Cdc42 mutations affected both axons and dendrites (Luo et al., 1994). Rho GTPases are also involved in guiding growing axons. Expression of dominant negative Rac1 causes motor axon guidance errors at specific choice points (Kaufmann, 1998). Moreover, either downregulation of Rac1 and Cdc42 or activation of RhoA is required for midline repulsion in *D. melanogaster* (Fritz and VanBerkum, 2002). In *C. elegans*, mutations in a Rho-like GTPase mig-2 (migration 2) caused both outgrowth defects and occasional guidance defects (Zipkin et al., 1997).

As axons grow, their growth cones encounter many cues that guide them towards or away from specific cells or pathways. Guidance cues act by selectively stabilizing or destabilizing actin-based filopodia and lamellipodia. Accumulating evidence indicates that Rho GTPases participate in mediating these actions. Rac1 may directly mediate F-actin reorganization downstream of Sema3D (Jin and Strittmatter, 1997). In support of this finding, Fournier et al. (Fournier et al., 2000) showed that ligand-aggregated semaphorin receptors colocalize with Rac1 in growth cones and during growth cone collapse. Furthermore, the cytoplasmic domain of semaphorin receptor plexinB1 has been found to bind to Rac1 directly (Driessens et al., 2001; Vikis et al., 2000), suggesting that Rac1 plays a role in mediating semaphorin signaling. PlexinB interacts directly with Rac1 *in vitro* and, at high molar excess, inhibits the ability of Rac1 to associate with and activate its downstream effector PAK1. PAK1 activation leads to LIM kinase (LIMK) activation and myosin light chain kinase (MLCK) inhibition, which limits depolymerization and the retrograde flow of actin filaments (Edwards et al., 1999; Sanders et al., 1999). Genetic studies in *D. melanogaster* showed that plexinB mediates motor axon guidance by inhibiting active Rac1 and enhancing RhoA signaling (Hu et al., 2001). In contrast to vertebrate plexinB, which does not directly interact with RhoA, plexinB in *D. melanogaster* binds directly to and activates RhoA (Hu et al., 2001; Vikis et al., 2002). RhoA recruitment to plexins may activate ROCK, which phosphorylates and thereby activates LIMK, which in its activated state suppresses cofilin-mediated actin depolymerization. Although the biochemical interactions demonstrated *in vitro* have not been confirmed *in vivo*, dosage-sensitive genetic interactions between plexinB and the GTPases suggest that plexinB mediates repulsion by inactivating Rac1 and activating RhoA. A more recent report by Vikis et al. (Vikis et al., 2002) confirmed these

observations in the mammalian system downstream of Sema4D-activated plexinB1. Using overexpression experiments in COS and human embryonic kidney 293 (HEK293) cells, these authors demonstrate that plexinB1 competes with PAK for Rac1-GTP binding, and the binding of activated Rac1 to plexinB1 additionally increases the cell surface expression of plexinB1 and leads to a slightly enhanced binding affinity for the Sema4D ligand. Thus, Rac1 could be involved in modulating plexin responsiveness to different semaphorins during axon guidance *in vivo*.

The low affinity neurotrophin receptor p75 has been shown to bind directly to RhoA and to activate its activity. This binding and activation is abolished when neurotrophin binds to the receptor, inactivating RhoA and so permitting axonal outgrowth (Yamashita and Barde, 1999). There is also some evidence implicating that RTK TrkA may be linked to axon outgrowth. TrkA activates RhoG through PI3-K therefore activating Rac1 and Cdc42 whereas inactivating RhoA upon nerve growth factor (NGF) stimulation (Nusser et al., 2002). The GEF protein Trio has been shown to activate RhoG which leads to neurite outgrowth (Estrach et al., 2002); however, a direct link between TrkA signaling and Trio has yet to be demonstrated.

In addition to TrkA receptors, another family of RTKs-Eph receptors also involve the Rho GTPases. The first strong evidence that extracellular guidance cues can directly modulate the activities of Rho GTPases is from Wahl et al. (Wahl et al., 2000). The growth cones of cultured RGCs collapse when treated with soluble ephrin-A5, a response that is thought to mimic their repulsion by ephrin-A5 during development of the retinotectal projection. Using glutathione S-transferase (GST)-effector domain fusion proteins to affinity pull-down Rho GTPases in their active GTP-bound form, they showed that ephrin-A5 activates RhoA and inhibits Rac1. Moreover, specific inhibitors of RhoA and ROCK block the collapse of RGC growth cones. Therefore, coordinated activation and inhibition of Rho GTPases by guidance cues result in a response of the growth cone to extend or retract. However, the same guidance cue may also activate two distinct RhoA signaling pathways. Recently, Arakawa et al. (Arakawa et al., 2003) showed that stromal cell-derived factor SDF-1α, a neural chemokine, is a physiological ligand that can activate two distinct RhoA-dependent pathways with opposite consequences. At low concentration, SDF-1α induces a RhoA/mDia pathway that mediates axon outgrowth in

concert with Rac1. In contrast, at higher concentration, SDF-1α inhibits axon formation through RhoA/ROCK pathway. These findings also shed light on the new possibility that RhoA- and Rac1-dependent pathways may interact not just in an antagonistic but also in a cooperative way in the regulation of the timing and the extent of axon elongation in cultured cerebellar granule cells.

In addition to the best-studied members of the Rho family, there is also evidence that other members are involved in neuronal signaling. Among them, TC10 and RhoT have been shown to induce neurite outgrowth in both PC12 and N1E-115 cells (Abe et al., 2002). TC10 and RhoT bind to N-WASP and activate N-WASP to induce Arp2/3 complex-mediated actin polymerization leading to neurite outgrowth. An affinity screen using GST-fusion proteins identified RhoD and Rnd1 as interactors of plexinA1 (Zanata et al., 2002). The interaction of plexinA1 with Rnd1 induces growth cone collapse; the interaction with RhoD, in contrast, blocks this Rnd1-triggered effect. It is possible that Rnd1 versus RhoD recruitment fine-tunes the balance of RhoA versus Rac1 activity to induce growth cone collapse.

1.3.3 RhoGEFs

Extracellular factors are believed to transmit their signals to the Rho family GTPases through the regulatory protein GEFs that promote the exchange of GDP for GTP. To date, GEFs have attracted more attention, partially because many were originally identified as potent oncogenes capable of transforming NIH 3T3 cells (Eva and Aaronson, 1985). The first characterized GEF was Dbl, an oncoprotein isolated from diffuse B lymphomas (Hart et al., 1991; Ron et al., 1991). Dbl was shown to share a conserved 180 amino acid domain, now known as Dbl homology (DH) domain with Cdc24, a GEF for yeast Cdc42 (Zheng et al., 1994). Since the initial characterization of Dbl and Cdc24, additional members of the Dbl family have been characterized such as Vav (Katzav et al., 1989), Tiam-1 (Habets et al., 1994), FGD1 (Olson, 1996), and Trio (Debant et al., 1996). To date, ~ 60 GEFs have been identified in humans that share two conserved domains (Venter, 2001), a DH domain followed by a pleckstrin homology (PH) domain. The DH domain has been shown to be responsible for the activation of the GTPases, whereas the PH domain is thought to play a crucial role in membrane

localization by interacting with specific lipids. In addition to the DH and PH domains, most of them contain other protein-protein interaction motifs suggesting multifunctional roles for GEFs. The large family of RhoGEF suggests that the function of individual GEF is more specialized in mammals. Indeed, many mammalian GEFs are tissue- and cell-type specific. Several GEFs appear to be highly specific toward a single GTPase such as Tiam-1, a Rac1-specific GEF, and FGD1, a GEF specific for Cdc42; However, other GEFs may activate several GTPases such as Vav which can activate all three members: RhoA, Rac1, and Cdc42 (Hart et al., 1994). Trio contains two DH domains, the first DH domain activates RhoG and Rac1 and the second DH domain activates RhoA (Blangy et al., 2000; Debant et al., 1996). The mechanisms by which GEFs are activated by membrane receptors are still unclear.

1.3.3.1 GEFs and axon guidance

The first RhoGEF that has been extensively studied in the context of growth cone guidance is Trio. Trio was originally identified in mammals as a binding partner of the receptor tyrosine phosphatase LAR (Debant et al., 1996). Trio contains two GEF domains. Genetic and biochemical data suggest that GEF1 activates RhoG thereby activating Rac1 and Cdc42, and GEF2 activates RhoA (Bellanger, 1998; Blangy et al., 2000; Debant et al., 1996; Newsome et al., 2000; Steven, 1998). Unc73 and *D. melanogaster* Trio are orthologs of mammalian Trio. The analysis of *C. elegans unc73* mutants and *D. melanogaster trio* mutants has indicated that Trio functions to regulate axon outgrowth and guidance (Awasaki et al., 2000; Newsome et al., 2000; Steven, 1998). In *D. melanogaster*, Trio was identified in a search for novel *D. melanogaster* RhoGEFs. Trio mutations disrupt axonal projections in both the embryonic and adult CNS (Awasaki et al., 2000). Trio interacts with non-receptor tyrosine kinase Abl. Trio and Abl mutations mutually enhance each other, increasing the severity of the CNS defects and the rate of lethality (Liebl et al., 2000). Trio, dreadlock (Dock), and Pak (effector of Rac1 and Cdc42) mutations result in similar projection errors. Mutations in the three genes also show dosage-sensitive genetic interactions (Newsome et al., 2000). These data suggest that Trio, Dock, and Pak function in a common pathway to control axon guidance, whether this occurs as well in vertebrates remains to be elucidated.

The axon guidance receptors acting upstream of Trio are still not known. One candidate is the receptor tyrosine phosphatase LAR (Bateman et al., 2000; Debant et al., 1996). Human Trio interacts with the cytoplasmic domain of LAR (Debant et al., 1996), and mutations in the gene encoding the *D. melanogaster* LAR orthologue, DLAR, disrupt the projections of many axons that are also affected in Trio mutants (Krueger et al., 1996). However, the carboxyl-terminal region of human Trio that mediates the association with human LAR is absent in *D. melanogaster* Trio (Debant et al., 1996). Thus, although the fly Trio and DLAR act in the same growth cones, they are unlikely to function in a common pathway.

Rac1-specific GEF Tiam-1 has been shown to cause N1E-115 cells to elaborate neurites and prevent LPA-induced neurite retraction (Leeuwen et al., 1997). Tiam-1 is expressed in migrating cortical neurons during neural development. In mice lacking Tiam-1 expression, cerebellar granule neurons failed to migrate (Ehler et al., 1997). Another Rac1-GEF, STEF, is also expressed in the developing CNS and causes Rac1-dependent neurite outgrowth in N1E-115 cells (Matsuo et al., 2002). The mechanisms of these GEFs in axon guidance remain to be discovered. They are likely to bind directly or indirectly to guidance receptors leading to Rho GTPase regulation.

There is some evidence that guidance receptors bind directly to GEFs. Ephexin (Eph-interacting exchange factor) is a novel GEF that interacts directly with the cytoplasmic domain of EphA4 receptor and acts as a direct link between Eph receptors and Rho GTPases (Shamah et al., 2000). In the absence of stimulation, ephexin is constitutively bound to the cytoplasmic domain of EphA4 receptor and in a position to activate Cdc42 and Rac1. In this state, growth cone extension is promoted. When EphA4 receptors are stimulated with ephrinA1, ephexin activates RhoA while reducing the activation of Cdc42 and Rac1. The net effect is to induce growth cone collapse. Ligand binding is associated with the inhibition of PAK1. As PAK1 and its downstream effectors are involved in the reorganization of the actin cytoskeleton, a major function of ephexin may be to regulate cytoskeleton dynamics by modulating PAK1 activity. RhoA-dependent pathways are essential for plexinB1 function (Driessens et al., 2001; Hu et al., 2001). Recent work has showed that B-type plexins can bind to two RhoGEF proteins, PDZ-RhoGEF and Leukemia-associated Rho GEF (LARG) (Driessens et al., 2002; Perrot

et al., 2002; Swiercz et al., 2002), and that plexinB clustering or ligand-induced dimerization of plexinB is sufficient to stimulate endogenous RhoA activity (Driessens et al., 2002; Perrot et al., 2002), suggesting that plexinB may stimulate RhoA through the recruitment of PDZ-RhoGEF. In a functional assay, they showed that Sema4D, a ligand for plexinB, induced the retraction of neurites and cell rounding in PC12 cells that have endogenous plexinB2 expression, and this effect is blocked by the PDZ domain of PDZ-RhoGEF (Perrot et al., 2002). Furthermore, Sema4D-induced growth cone collapse could be completely blocked by the C3 transferase and the ROCK inhibitor Y-27632 in primary hippocampal neurons which have endogenous plexinB1 expression, suggesting RhoA activity is required for growth cone collapse induced by Sema4D. Most importantly, dominant negative RhoGEF could also inhibit Sema4D-induced growth cone collapse. Therefore, PDZ-RhoGEF functions as a crucial link between plexinB1 and RhoA activation, thereby regulating the actin cytoskeleton during growth cone collapse.

1.3.4 RhoGAPs

RhoGAPs enhance the low intrinsic GTPase activity of Rho GTPases, which subsequently leads to inactivation. Following biochemical identification of the large cytosolic Bcr (breakpoint cluster region) phosphoprotein, the first GAP for Rho proteins, a large family of proteins with GAP activity for Rho GTPases has been discovered in the past decade (Diekmann et al., 1991; Garrett et al., 1991; Lancaster et al., 1994). More than 40 members have been described from yeast to human. Recent human genome analysis has identified around 90 RhoGAPs in human. The overabundance of RhoGAPs suggests that each RhoGAP might play a specialized role in regulating individual Rho GTPase activity. All these RhoGAPs share a conserved RhoGAP domain, the 'GAP catalytic domain' which mediates binding and catalytic activity with Rho proteins. Rho GAP domain consists of about 180 amino acids. In addition to their RhoGAP catalytic domain, most of these Rho GAPs have other motifs involved in signaling, including Src-homology 2 or 3 (SH2 or SH3), PH, DH and Serine/Threonine (Ser/Thr) kinase domains, as well as SH3-binding sites. The activity spectrum of RhoGAPs with Rho GTPases varies widely *in vitro*, but it can be more specific *in vivo*. Some RhoGAPs recognize a single Rho protein and catalyze its GTPase activity such as Slit-Robo GAPs (srGAPs)

(Wong et al., 2001), whereas others can interact with all three members: RhoA, Rac1 and Cdc42 such as p190-B (Sordella et al., 2002).

1.3.4.1 GAPs and axon guidance

The first RhoGAP studied extensively in the context of neuronal morphogenesis is n-chimaerin. It was shown to cooperate with Rac1 and Cdc42 to induce the formation of lamellipodia and filopodia in neuronal cell lines (Kozma, 1996). Recent studies of p190-A RhoGAP (originally named p190), one of the first RhoA-specific GAPs identified, in gene targeted mice show that the p190-A is required for axon outgrowth, guidance and fasciculation, as well as in neuronal morphogenesis (Brouns et al., 2000; Brouns et al., 2001). In p190-A knockout mice, excessive accumulations of polymerized actin were found in the neural tube floor plate cells, suggesting a negative role of p190-A in the regulation of Rho-mediated actin assembly within neuroepithelium. P190-A was co-localized with F-actin at the distal end of the axon and its overexpression induced neurite formation in N2A neuroblastoma cells, indicating that p190-A could be an important regulator of RhoA-mediated actin reorganization in neuronal growth cones. In addition, p190-A was found to be the principal Src kinase substrate in brain and p190-A-mediated neurite outgrowth is promoted by extracellular matrix protein laminin. In *D. melanogaster*, RNA interference blocking of p190-A expression leads to the retraction of axonal branches by upregulating RhoA activity and affecting a signaling pathway from RhoA to the effector Drok to the actin/myosin contractility component myosin regulatory light chain (Billuart et al., 2001). Given the observation from both mammalian and non-mammalian systems, an attractive model would be that activation of Src kinases by neural adhesion molecules results in p190-A phosphorylation, which downregulate RhoA activity and promote axon outgrowth and guidance by ROCK-mediated actin changes. In addition, mice lacking the Eph family receptor, EphB2 (Nuk), exhibit an anterior commissural guidance defect very similar to that seen in p190-A mutant mice, suggesting that Eph ligands might signal through p190-A (Henkemeyer et al., 1996).

There is some evidence showing the direct interaction of the guidance receptors and RhoGAPs, which mediates axon growth and guidance. For example, mammalian Robo1 transduces repulsive signal by directly interacting with a novel family of GAPs,

the srGAPs (Wong et al., 2001). In Robo1-transfected cells, binding of Slit to Robo1 was found to increase the interaction of srGAP1 with the cytoplasmic domain of Robo1 and was accompanied by the activation of RhoA and inhibition of Cdc42. I*n vitro*, srGAP1 associates with Cdc42, suggesting that srGAP1 directly modulates Cdc42 activity. Furthermore, dominant negative srGAP1 blocked Slit-mediated inactivation of Cdc42 and repulsion in cell culture, whereas a constitutively active Cdc42 blocked the repulsive effect of Slit. Therefore, these results implicate the importance of Cdc42 in Robo signaling and indicate that srGAP1 mediates the repulsion by inhibiting Cdc42 activity.

Another RhoGAP identified recently is Grit, which has GAP activity for RhoA/Rac1/Cdc42 (Nakamura et al., 2002). Grit was found abundant in neuronal cells and directly interacted with TrkA, a high-affinity receptor for NGF. Overexpression of the TrkA-binding region of Grit inhibited NGF-induced neurite elongation. These results suggest that Grit regulates neurite outgrowth by modulating the Rho family of small GTPases. $P75^{NTR}$ has been shown to interact constitutively with RhoA and mediate down-regulation of RhoA upon neurotrophin stimulation (Yamashita and Barde, 1999). The association between Trk and $P75^{NTR}$ was demonstrated previously, and neurotrophin was suggested to reinforce the Trk- $P75^{NTR}$ interaction (Bibel et al., 1999). Based on this finding, it is possible that TrkA/Grit could be prompted to associate with p75/RhoA complex upon NGF stimulation; Grit could thereby down-regulate RhoA activity and positively contribute to NGF-induced neurite outgrowth.

1.3.5 Effectors

The GTP-bound form of the Rho GTPases is able to interact with effector or target molecules to trigger a variety of cellular responses. Over 30 potential effector proteins have been identified that interact with members of the Rho family. The most common mechanism of effector activation by Rho GTPases appears to be the disruption of intramolecular autoinhibitory interactions, to expose functional domains within the effector protein. For example, the Rac1/Cdc42 targets PAK1-3 Ser/Thr kinases have an intramolecular regulatory domain that inhibits kinase activity. Upon GTPase binding, the inhibitory sequence is displaced, leaving the kinase domain free to bind substrates (Bagrodia et al., 1999; Tu and Wigler, 1999). A more distantly related PAK family

member, PAK4, does not contain an autoinhibitory domain, and this PAK is not significantly stimulated by GTPase binding (Abo et al., 1998). WASP and N-WASP, two related Cdc42 targets, also appear to be regulated by an intramolecular interaction. Cdc42-GTP competes with WASP C-terminus for binding to the N-terminus and induces a conformational change in the WASP N-terminus quite different from its conformation when autoinhibited. Thus, Cdc42-GTP activates N-WASP by releasing intramolecular interactions (Machesky and Insall, 1998; Rohatgi et al., 1999). However, the activation mechanism of WAVE1, a member of the WASP family of proteins, is quite different from WASP and N-WASP. N-WASP is autoinhibited, whereas WAVE1 is trans-inhibited. The action of Rac1 and Nck is to disassemble the trans-inhibited WAVE1 complex, which releases the active WAVE1 protein in association with HSPC300 (Eden et al., 2002). Another example is the Cdc42 effector IQGAP. Calmodulin-Ca^{2+} binds to IQGAP and inhibits its binding to actin and Cdc42 (Joyal et al., 1997). This might provide a calcium-sensitive regulatory mechanism for controlling the activation of IQGAP.

Almost all Rho GTPase effectors have multiple domains, and some of these might regulate their activity. For example, the PH domains present in WASP and N-WASP are thought to promote association with the membrane through lipid binding, where they may encounter activated Rho GTPases (Miki, 1996). Protein-protein and protein-lipid interactions can regulate the subcellular localization of GTPase effectors. For example, WASP and PAK contain the SH3-binding motif, PxxP, which has been reported to bind to the adaptor protein Nck (Galisteo et al., 1996; Rivero-Lezcano et al., 1995). Since Nck also has an SH2 domain, it could recruit these effectors to activated receptor tyrosine kinases.

To date, significant progress has been made in identifying effectors involved in actin reorganization. At least two effectors, ROK and Dia1, appear to be required for RhoA-induced assembly of stress fibers and focal adhesions. ROKα (also known as ROCK, Rho kinase) and ROKβ are Ser/Thr kinases, which have a kinase domain, a coiled-coil region, a Rho-binding domain (RBD), a PH domain and a Cysteine-rich region. ROKs are activated by binding to RhoA-GTP (Ishizaki et al., 1996; Matsui et al., 1996). Watanabe et al. (Watanabe et al., 1999) showed that expression of activated

ROCK and Dia1 simultaneously induces the assembly of stress fibers that are indistinguishable in appearance from those induced by RhoA. RhoA-activated Dia1 may act through profilin to promote extension of actin filaments by recruiting actin monomers. Two substrates of ROK which are likely to be key players in actin-myosin filament assembly are myosin light chain (MLC) and the myosin-binding subunits (MBS) of MLC phosphatase (Amano et al., 1996; Kawano et al., 1999). Phosphorylation of MLC occurs at Ser^{19}, which stimulates the actin-activated ATPase activity of myosin II and promotes the assembly of actomyosin filaments. On the other hand, MLC phosphatase is inhibited by phosphorylation, indirectly leading to an increase in MLC phosphorylation. Another ROK target is LIM kinase (LIMK). When phosphorylated, LIMK is able to inhibit (by phosphorylation) cofilin, leading to stabilization of filamentous actin structures.

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). Mutations in WASP leads to reduced mobility of lymphoid immune cells, reflecting the fact that WASP and its relatives play a critical role in organization of the actin cytoskeleton (Machesky and Insall, 1999). The WASP family proteins consist of WASP, N-WASP and WAVE (Mullins, 2000). N-WASP, first discovered in brain, is a ubiquitous protein (Miki, 1996). Both WASP and N-WASP contain several modular domains, including an N-terminal PH domain and WASP-homology domain1 (WH1), a CRIB (Cdc42/Rac1 interactive binding) domain that binds the Rho family GTPase Cdc42, a proline-rich sequence, a WH2 motif that interacts with monomeric actin and a C-terminal A motif that binds to the Arp2/3 complex (Machesky and Insall, 1999; Mayer, 2001; Mullins, 2000). It was observed that overexpression of N-WASP plus Cdc42 induces very long microspikes (Miki, 1998), suggesting that N-WASP may be involved in the formation of filopodia downstream of Cdc42. In contrast to WASP and N-WASP, WAVE does not possess a CRIB domain and is not capable of interacting with Cdc42. However, GTP-bound Rac1 can activate WAVE and is involved in WAVE translocation from the cytosol to membrane ruffles (Eden et al., 2002; Miki, 1998). Accumulating data suggest that the Arp2/3 complex, a stable complex of seven proteins including the actin related proteins Arp2 and Arp3, regulate the assembly of new

actin filaments. Proteins of the WASP family bind directly, via their C-terminal A motif, to the Arp2/3 complex and stimulate its ability to nucleate de novo actin filaments and induce filament branching (Machesky and Insall, 1999; Mullins, 2000).

Cdc42 also interacts with two Ser/Thr kinases that are thought to be involved in filopodia formation, MRCKα and β (myotonic dystrophy kinase-related Cdc42-binding kinase). MRCKs are Cdc42-specific effector proteins that contain a PH domain and a ROK-like kinase domain which phosphorylates MLC. Kinase dead MRCKα inhibits Cdc42-induced filopodia, and overexpression of MRCKα synergizes with Cdc42 to induce large filopodia in Hela cells (Leung et al., 1998). Another protein that is able to synergize with Cdc42 to produce large filopodia-like structures is the new PAK4 (Abo et al., 1998). PAK4 has a CRIB motif and a kinase domain similar to PAKs 1-3, but its sequence is divergent outside of these regions. PAK4 acts quite differently to PAKs 1-3, in that it binds to Cdc42 and not to Rac1, and it is not activated by GTPase binding.

To date, there are few example of unique Rac1 effectors that have been implicated in actin reorganization. Some common target proteins appear to be utilized by both Rac1 and Cdc42 in the induction of lamellipodia and filopodia, respectively. PAKs1,2,3 are Ser/Thr kinases, related to yeast Ste20, which have been better described. PAK1 and PAK3 are highly enriched in neurons. *In vitro*, PAK1 and 3 bind equally well to Cdc42 and Rac1, though Cdc42 stimulates PAK1 activity more strongly than Rac1 (Manser et al., 1995). PAK2 only inhibits the intrinsic GTPase activity of Rac1, and not Cdc42, suggesting that it may be a Rac1 target (Zhang et al., 1998). However, it is difficult to decide whether PAK1,2,3 are targets for Rac1, Cdc42 or both *in vivo*. There have been conflicting reports linking PAKs to actin changes. Activated mutants of PAK1 have been reported to induce both filopodia and membrane ruffles in Swiss 3T3 cells and to cause neurite outgrowth in PC12 cells, similar to the effects of constitutively active Cdc42 and Rac1 (Daniels et al., 1998; Sells et al., 1997). Interestingly, PAK1-induced cytoskeleton changes are partly independent of its kinase activity, but require membrane targeting. Other groups, however, have failed to find any effects of a role of PAK1 as a direct Rac1/Cdc42 effector on the actin cytoskeleton (Joneson, 1996; Lamarche, 1996). Taken together, these results suggest that PAKs may affect the actin cytoskeleton in cooperation with other signals present in the cells tested. A variety of substrates for PAKs have been

identified that could affect the actin cytoskeleton. Rac1, like RhoA, induces phosphorylation of LIMK, and PAK has been shown to phosphorylate LIMK *in vitro* (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. PAK has been shown to phosphorylate and inactivate MLC kinase, decreasing MLC phosphorylation and reducing actomyosin assembly (Sanders et al., 1999).

IQGAP1 and 2 are effectors for Rac1 and Cdc42 and may be involved in actin polymerization (Brill et al., 1996; Hart 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-Cdc42 (Fukata et al., 1997). It has been suggested that IQGAP oligomers may form upon binding to GTPase 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).

Recently, WAVE1, has been shown to mediate signals from Nck and Rac1 leading to actin nucleation (Eden et al., 2002). WAVE1 exists in a heterotetrameric complex that includes PIR121 (p53-inducible messenger RNA with a relative molecule mass of 140KD), Nap125 and HSPC300. The WAVE1 complex is inactive. Rac1 and Nck cause dissociation of the WAVE1 complex, which releases active WAVE1-HSPC300 and leads to actin nucleation, suggesting that WAVE1 is a Rac1 effector.

1.3.5.1 Effectors and axon guidance

Cdk5 is a member of the cyclin dependent kinase (Cdk) family, which are Ser/Thr kinases that require the association of a regulatory protein for activity (Meyerson et al., 1992). Cdk5 is activated in postmitotic neurons by two highly related proteins, p35 and p39 (Kwon and Tsai, 2000). In neurons the p35/Cdk5 kinase is a Rac1 effector. It was

found to associate with Rac1 via p35 in a GTP-dependent manner both in transfected cells and brain tissue (Nikolic et al., 1998). In cultured rat cortical neurons, loss of p35/Cdk5 kinase by overexpression of a kinase inactive Cdk5 mutant or antisense p35 cDNA resulted in reduced neurite outgrowth (Nikolic et al., 1996). In contrast, overexpression of both p35 and p39 induced cortical neurons to extend longer neurites. This was the first report to suggest that the kinase regulates neurite outgrowth. Subsequent experiments on primary neurons and neuronal cell lines have confirmed the involvement of the p35/Cdk5 and p39/Cdk5 kinases in neurite outgrowth (Pigino et al., 1997; Xiong et al., 1997). Axonal outgrowth defects seen in *Xenopus* RGCs overexpressing a constitutively active Rac1 mutant were partially rescued by co-expression of the kinase inactive Cdk5 mutant, suggesting that the effects of active Rac1 on the neuronal cytoskeleton are mediated in part by p35/Cdk5 kinase activity (Ruchhoeft et al., 1999). In $p35^{-/-}$ mice, the loss of the p35/Cdk5 kinase did not appear to cause defects in neurite outgrowth. However, abnormal neurite morphology was observed in pyramidal neurons. These neurons were completely disorganized with no recognizable pattern of neurite arrangement (Chae et al., 1997). In addition to a general disorganization of neurites, animals that lack the p35/Cdk5 kinase also showed axonal pathfinding and fasciculation defects. Thus, the afferent and efferent tracts that link the cortex and thalamus appeared altered and the corpus callosum thinner and defasciculated (Chae et al., 1997; Gilmore et al., 1998; Kwon et al., 1999). In *D. melanogaster*, studies have shown that loss or gain of Dp35/Cdk5 kinase is responsible for a wide range of regulatory defects during motor axon outgrowth (Connell-Crowley et al., 2000). The observed defects include overextended and stalled nerves, loss of target recognition and pathfinding errors.

Evidence that PAK functions in growth cone guidance comes from the analysis of PAK orthologs in *D. melanogaster* (Hing, 1999). Loss-of-function mutations in PAK result in photoreceptor axon guidance errors similar to those that occur in Trio mutants, and the two genes show strong genetic interactions (Hing, 1999; Newsome et al., 2000). Activation of PAK depends not only on signals transduced by Trio and Rac1, but also on signaling through the SH2-SH3 adaptor protein Dock, the *D. melanogaster* orthologue of mammalian Nck. Dock binds directly to PAK through the second SH3 domain, and loss-of-function Dock mutations lead to photoreceptor projection errors identical to those of

PAK mutants (Garrity et al., 1996; Hing, 1999). A membrane-tethered form of PAK substantially rescues the projection errors in a Dock mutant, suggesting that the primary function of Dock is to recruit PAK to the plasma membrane, where it is then activated by the GTP-bound forms of Rac1 or Cdc42. The regulation of PAK kinase activity by Trio, Rac1 and Dock shows how guidance cues acting both via and independently of Rho GTPases might be combined to create spatial asymmetries in the activity of a cytoskeletal regulator. To date, there is no reported studies on the role of PAK in neurite outgrowth in cultured primary neurons. However, overexpression of membrane targeted PAK1 in PC12 cells mimicked NGF-induced neurite outgrowth (Daniels et al., 1998). Membrane localization of PAK1 was found to be important to induce dramatic cytoskeletal changes. The effects of cytoplasmic PAK1 on PC12 cells were either not evident or much weaker (Daniels et al., 1998; Obermeier et al., 1998). Interestingly, PAK1 kinase activity was dispensable for neurite outgrowth in PC12 cells in both studies. Constitutive active and kinase dead PAK1 mutants had similar effects, suggesting that in these experimental systems, membrane association rather than kinase activity was the predominant cause of cytoskeletal modifications. It has been shown that ephrinA1-induced activation of EphA4 downregulates PAK1 kinase activity via the exchange factor ephexin, while plexinB inhibits the Rac1-PAK complex formation therefore inhibits PAK kinase activity (Hu et al., 2001; Shamah et al., 2001). These data suggest that inhibition of PAK kinase activity is a cause of growth cone collapse. A novel PAK isoform, PAK5, has been identified in neurons and is highly expressed in mammalian brain, it promotes neurite outgrowth in N1E-115 cells (Dan et al., 2002).

The expression level of ROKβ is hardly detectable in brain, whereas ROCK (ROKα) is highly enriched in pyramidal neurons of the cortex and hippocampus and cerebellar Purkinje neurons (Hashimoto et al., 1999). Genetic studies of ROCK function in growth cone guidance have not yet been reported, but *in vitro* experiments using a specific ROCK inhibitor support the idea that ROCK is a negative regulator of growth cone motility (Bito et al., 2000; Hirose et al., 1998; Wahl et al., 2000). Inhibition of ROCK was found to block growth cone collapse in RGCs and neurite retraction in N1E-115 cells (Hirose et al., 1998; Wahl et al., 2000). Inhibition of ROCK also results in excessive and precocious axonogenesis in cultured cerebellar granule neurons (Bito et al.,

2000). Growth cones are larger and more motile when ROCK is inhibited. ROCK has also been suggested to function during RhoA-mediated growth cone collapse of chick dorsal root ganglion (DRG) neurons, by phosphorylating the neuron-enriched collapsin response mediator protein-2 (CRMP-2) on threonine 555 (Arimura et al., 2000). CRMP-2 proteins are conserved from *C. elegans* to mammals and have been demonstrated to mediate growth cone collapse following exposure to repulsive axon guidance molecules of the semaphorin family (Nakamura et al., 2000; Quinn et al., 1999; Wang and Strittmatter, 1996). Interestingly, in chicken DRG neurons, ROCK-catalyzed phosphorylation of CRMP-2 rapidly increased as a consequence of exposure to LPA, a well-known activator of RhoA also shown to induce growth cone collapse. Inhibition of ROCK specifically abolished LPA-induced CRMP-2 phosphorylation and growth cone collapse (Arimura et al., 2000). Together, these data demonstrated that ROCK and CRMP-2 are essential downstream components of the LPA-induced growth cone collapse in chicken DRG neurons.

The WASP/Arp2/3 complex also appears to be involved in neurite outgrowth. N-WASP is found throughout the brain and is concentrated at nerve terminals (Fukuoka et al., 1997). Arp2/3 are concentrated in the actin filaments of NGF-stimulated growth cones from rat sympathetic neurons and PC12 cells (Goldberg et al., 2000). The first study to implicate WASP family proteins in the control of growth cone motility is from Banzai et al. (Banzai et al., 2000). They showed that an N-WASP mutant unable to bind Cdc42 and another N-WASP mutant unable to bind the Arp2/3 complex are both able to inhibit NGF-induced neurite outgrowth when expressed in PC12 cells. The double mutant has no effect, suggesting that the single mutant forms of N-WASP act as dominant negative protein by sequestering the normal binding partners. The mutant unable to bind to the Arp2/3 complex is also shown to block neurite extension in primary hippocampal neurons. Observations on filopodia formation at growth cones in neuroblastoma cells showed that WAVE isoforms play distinct roles in filopodia formation (Nozumi et al., 2003). WAVE1 is continuously distributed along the leading edge only and is not found in the filopodia. WAVE2 and WAVE3 are discretely localized at the initiation sites of microspikes on the leading edge and also concentrated at the tips of protruding filopodia. These results indicate that WAVE2 and WAVE3 may guide the actin bundles into the

filopodia and promote actin assembly at the tips. The *D. melanogaster* Scar, a member of the WASP family, has been shown to play a role in axonal development. Mutations in Scar disrupt the normal growth and bundling of segmental axons, and mutations in the *D. melanogaster* Arp2/3 proteins also produce abnormal axon development (Zallen et al., 2002). Interestingly, mutations in *D. melanogaster* WASP do not lead to abnormal axon growth, but lead to problems in cell fate determination, suggesting again that the different WASP proteins regulate Arp2/3 activity under different circumstances.

Profilin and Ena/VASP family members facilitate *Listeria* monocytogenes motility. They also regulate growth cone motility, but they appear to have opposing roles. Profilin binds monomeric actin and promotes actin assembly at the barbed ends. Its critical role in promoting growth cone motility is revealed by studies showing that dominant negative profilin blocks axonogenesis in neuroblastoma cells (Suetsugu et al., 1998), and that loss of profilin function results in axon stalling in *D. melanogaster* (Wills et al., 1999). VASP belongs to a family of profilin-binding proteins that also includes mammalian, *D. melanogaster* and *C. elegans* Enabled (Mena, Ena and UNC-34, respectively) (Lanier and Gertler, 2000). They appear to function by binding to the barbed ends of filaments and competing with capping proteins, allowing for longer filament extension (Bear et al., 2002). Axon projection errors in *D. melanogaster* Ena mutants (Wills et al., 1999) and Mena knockout mice (Lanier et al., 1999) reveal the importance of Ena/VASP proteins in controlling growth cone motility. But whereas Ena/VASP proteins promote Listeria motility (Laurent et al., 1999; Loisel et al., 1999), they appear to inhibit cell and growth cone motility in *D. melanogaster*. Ena/VASP proteins have been implicated in growth cone repulsion by Slit and its receptor Robo in *D. melanogaster* (Bashaw et al., 2000). In the *D. melanogaster* embryo, Slit repels growth cones expressing the receptor Robo, preventing them from crossing the midline of the CNS. Genetic and biochemical data suggest that Ena mediates at least part of this repulsive response. Ena binds directly to the Robo cytoplasmic domain. It is not yet known whether the Robo-Ena interaction is dependent on Slit, but an attractive model would be that exposure to Slit results in the local recruitment of Ena, preventing the growth cone from advancing further towards the midline. Ena/VASP have also been implicated in growth cone repulsion by netrin/UNC-6 and its receptor UNC-5 in *C.*

elegans (Colavita and Culotti, 1998). Ena antagonizes the function of Trio in *D. melanogaster* (Liebl et al., 2000). The mechanism by which Ena/VASP proteins reduce motility is unknown.

UNC-115, a putative actin-binding protein similar to human ABLim, has previously been implicated in axon pathfinding in *C. elegans* (Lundquist et al., 1998; Roof et al., 1997). It consists of three N-terminal LIM domains, which are thought to mediate protein-protein interactions (Dawid et al., 1998), and a C-terminal villin headpiece domain (VHD), an actin-binding domain found in a variety of proteins (Vardar et al., 2002). UNC-115 might act as a cytoskeletal adaptor protein that interacts with actin via the VHD and with other molecules via the LIM domains. It was shown to act downstream of Rac1 signaling during axon pathfinding, suggesting that UNC-115 might adapt Rac1 activity to the growth cone actin cytoskeleton (Struckhoff and Lundquist, 2003).

1.4 THE ADAPTOR MOLECULE NCK

The nck gene was originally isolated from a human melanoma cDNA library using monoclonal antibodies produced against the melanoma-associated antigen (Lehmann et al., 1990). The Nck family has two known members in human cells (Nck-1/Nckα and Nck-2/Nckβ), two in mouse cells (mNckα and mNckβ/Grb4) and one in *D. melanogaster* (Dock) (Braverman and Quilliam, 1999; Chen et al., 1998; Coutinho et al., 2000; Tu et al., 1998). Nckα and Nckβ in both humans and mice share only 68% amino acid identity. In contrast, a comparison of mouse Nckα with human Nckα and mouse Nckβ with human Nckβ show over 95% amino acid identity (Chen et al., 1998). The human nck-1 gene is localized to the 3q21 locus of chromosome 3; the nck-2 gene is localized on chromosome 2 at the 2q12 locus (Huebner et al., 1994; Lehmann et al., 1990; Vorobieva et al., 1995). Nck proteins are 47 kDa cytosolic adaptor molecules containing three SH3 domains in the N-terminus and one SH2 domain in the C-terminus (Lehmann et al., 1990). Both human Nck proteins are widely expressed although the pattern of their expression shows some difference.

1.4.1 Function of Nck

It has been well documented that SH2 domains are involved in protein-protein interactions (Schlessinger, 1994). Several groups have shown that Nck associates, via its SH2 domain, with activated EGF and PDGF receptor tyrosine kinases (Li et al., 1992; Park and Rhee, 1992) upon EGF and PDGF stimulation. EGF and PDGF RTK activation induce the phosphorylation of Nck at a single tyrosine (Meisenhelder and Hunter, 1992). Association between Nck and the EGF RTK is not direct but through a linker protein Dok2 (for downstream of tyrosine kinase) (Tang et al., 1997). In response to EGF, Dok2 is tyrosine phosphorylated and binds the SH2 domain of Nck (Jones and Dumont, 1999). It has also been shown that stimulation of EphB1 and EphB2 receptor tyrosine kinases results in a complex formation between Nck, Nck-interacting kinase (NIK), Dok1, RasGAP and an unidentified 145 kDa tyrosine phosphoprotein (Becker et al., 2000).

The presence of three distinct SH3 domains suggests that Nck may be capable of association with multiple proline-rich containing proteins. Indeed, various biochemical analyses have identified around 20 proteins that interact with one or more of the Nck SH3

domains, and the majority of the target proteins bind to the second SH3 domain of Nck. Some *in vivo* evidence has implicated several Nck SH3 domain-associated proteins as physiological effectors of various intracellular signal transduction responses. Williams and colleagues (Hu et al., 1995) first reported that Nck, when exogenously overexpressed in tissue culture cells, directly interacts with the Sos GTP exchange factor, leading to enhanced transcription from a Ras-dependent reporter gene. Nck and Sos constitutively associated via the second SH3 domain of Nck and a C-terminal proline-rich region of Sos. Following PDGF stimulation, Nck and hyperphosphorylated Sos translocate to the activated RTK, presumably leading to Sos-mediated Ras activation. Two members of the PAK family, PAK1 and PAK3, have been shown to be constitutively associated with the second SH3 domain of Nck (Galisteo et al., 1996; Lu et al., 1997). Nck can recruit PAK1 to the plasma membrane in response to growth factors, such as EGF and PDGF. Briefly, Nck associates with autophosphorylated tyrosine kinase receptors via its SH2 domain, whereas its second SH3 domain can interact with the first proline-rich region at the N-terminus of PAK1. Moreover, membrane localization of PAK1, either by direct fusion of a myristylation signal to PAK1 or by translocation mediated by binding to the second SH3 domain of Nck, is sufficient to stimulate its kinase activity and activate MAPK cascade.

Studies over the last few years have indicated that the major cellular function of Nck is to link cell surface receptors to the actin cytoskeleton, which is the prerequisite of various biological responses such as axon pathfinding, migration, and endocytosis. The third SH3 domain of Nck has been shown to interact with WASP (Rivero-Lezcano et al., 1995). Several lines of evidence suggest that Nck/WASP interaction represents another bridge between tyrosine kinases and actin dynamics. In activated T lymphocytes, Nck mediates the phosphotyrosine-dependent assembly of a complex containing WASP, Nck, SLP-76 (SH2 domain containing leukocyte protein of p76) and SLP-76 associated protein (SLAP) (Krause et al., 2000). Mice deficient in WASP show specific defects in T cell activation and actin polymerization in response to antibody cross-linking. It has been recently shown that Nck SH3 domains dramatically stimulate the rate of actin nucleation by purified N-WASP in the presence of Arp2/3 (Rohatgi et al., 2001). Nck-stimulated actin nucleation by N-WASP/Arp2/3 complexes is further stimulated by

phosphatidylinositol 4,5-biphosphate but not by GTP-Cdc42, suggesting synergistic activation of N-WASP by Nck and phosphoinositide. It has been shown that WIP, a proline-rich containing protein that interacts with WASP, associates *in vivo* with Nck via the second SH3 domain (Anton et al., 1998). WIP is known to associate with the actin polymerization regulatory protein profilin and to induce actin polymerization and cytoskeletal reorganization in lymphoid cells (Anton et al., 1998). Recently, WAVE1 has been shown to mediate signals from Nck and Rac1 to actin nucleation (Eden et al., 2002). Nck associates with Nck-associated protein (Nap) via the third SH3 domain leading to release of the active WAVE1 and promotion of actin nucleation.

Inactivation of Nck-1 and Nck-2 genes in mouse provides insight into the *in vivo* functions of Nck (Bladt et al., 2003). In mouse embryos, the two Nck proteins have broadly overlapping expression patterns. They are functionally redundant because mice deficient for either Nck-1 or Nck-2 are viable, whereas inactivation of both Nck genes results in early embryonic lethality and profound defects in mesoderm-derived embryonic structures. Fibroblast cell lines derived from Nck-$1^{-/-}$ Nck-$2^{-/-}$ embryos have defects in cell motility and in the organization of the lamellipodial actin network. These data suggest that Nck have important functions in the development of mesodermal structures during embryogenesis, potentially linked to a role in cell movement and cytoskeletal organization.

Recent genetic data have implicated the *D. melanogaster* adaptor protein, Dock, the homolog of Nck, in the control of axon guidance and target recognition by photoreceptor axons in *D. melanogaster* larvae (Garrity et al., 1996). During a genetic screen to identify effectors involved in axonal pathfinding, *dock* was identified as an essential gene for proper photoreceptor axon targeting and fasciculation. The *dock* gene product is localized primarily to photoreceptor growth cones, suggesting a role in regulating actin cytoskeletal dynamics. It has been reported that Dock interacts with the GCK family member Misshapen (Msn) *in vitro* and in the fly photoreceptor (R cell) growth cones (Ruan et al., 1999; Su et al., 2000). Loss of Msn results in a failure of growth cones to stop at the target, a phenotype similar to loss of Dock, whereas overexpression of Msn induces pretarget growth cone termination. Msn, similar to the other members of group I GCKs, can activate JNK signaling pathway. Although Dock

can interact with Msn *in vitro* and in the *D. melanogaster* photoreceptor growth cones, it is highly likely that in addition to Msn, other downstream targets of Dock may exist, such as PAK kinases (Su et al., 2000). Indeed, Hing et al (Hing, 1999) have recently shown that Dock and PAK colocalize to R cell axons and growth cones, physically interact, and their loss-of-function phenotypes are indistinguishable. Finally, mutations in the gene encoding Trio results in projection defects similar to those observed in both PAK and Dock mutants. It seems that Trio interacts genetically with Rac1, PAK and Dock (Newsome et al., 2000). The studies by Su et al. (Su et al., 2000) showed that Msn was mainly involved in dorsal closure of the embryo, and the activated form of Msn was not sufficient to rescue the *dock* mutant phenotype. They suggest that, although Msn played some role in R cell axon pathfinding, it probably was not the main downstream mediator of Dock signaling in the control of photoreceptor axon guidance and targeting. Recent studies showed that the *D. melanogaster* insulin receptor (DInR) funtions as a guidance receptor upstream of Dock (Song et al., 2003). Using the DInR intracellular domain as bait in a yeast two-hybrid screen, Song et al. identified Dock. DInR interacts with Dock via both the SH2 and SH3 domains. The interaction requires DInR kinase activity and the C-terminal tail that contains SH2 domain binding sites. Animals carrying *dinr* mutations have abnormal retinotopic connections, characterized by gaps and crossing of axon bundles and failure of growth cone expansion in the medulla. These phenotypes are indistinguishable from defects observed in *dock* mutant animals. Thus, DInR functions as a guidance receptor upstream of Dock in the visual system. Dscam is another cell surface receptor upstream of Dock in axon pathfinding in the embryonic CNS and Bolwig's nerve (Schmucker et al., 2000). To search for Dock-SH2-interacting proteins in *D. melanogaster*, Schmucker and colleagues found a protein highly related to human DSCAM (Down syndrome cell adhesion molecule). The cytoplasmic region of the *D. melanogaster* Dscam showed multiple potential phosphotyrosine sites including a Nck/Dock SH2 binding motif, and several putative SH3-binding PxxP sites. Dscam indeed binds to Dock through both SH2 and SH3 domains and acts as an upstream activator of the Dock-Pak guidance pathway. Thus, different cell surface receptors appear to activate the Dock pathway at different developmental stages. KETTE, a member of the HEM family in *D. melanogaster*, has been recently implicated in cytoskeletal

organization during axon pathfinding (Hummel et al., 2000). The KETTE protein is homologous to the transmembrane protein HEM-2/Nap1 (Nck-associated protein 1) whose interaction with Nck has been well documented (Kitamura et al., 1997). Nck/Nap1 interaction is mediated through the first SH3 domain of Nck. In *D. melanogaster*, mutations in the *kette* gene lead to axonal defects similar to mutations in Dock. Furthermore, it has been shown that *kette* and *dock* mutants genetically interact (Hummel et al., 2000). dPTP61F, a protein tyrosine phosphatase in *D. melanogaster*, was also found to bind and colocalize with Dock in the developing *D. melanogaster* nervous system (Clemens et al., 1996).

Since Nck and Dock are highly conserved (50-60% overall amino acid identity), Nck may contribute a similar function during vertebrate nervous system development. The Eph family of RTKs regulate signaling events involving axonal pathfinding and fasciculation during vertebrate development (Orioli et al., 1996). Supporting a functional link between Nck and the Eph RTKs, EphB2 indirectly associates with and induces the tyrosine phosphorylation of Nck-1 following ligand stimulation (Holland et al., 1997), whereas EphB1 binds directly to Nck-1 following the ephrinB1 treatment (Stein et al., 1998). Nck-2 has been shown to transduce B-ephrin reverse signals through binding to the cytoplasmic domain of B-ephrins in a phosphotyrosine-dependent manner (Cowan and Henkemeyer, 2001). The reverse signal transduced by B-ephrins is thought to lead to axonal repulsion (Birgbauer et al., 2000; Henkemeyer et al., 1996). They show that ephrinB1 reverse signaling leads to cell rounding, loss of stress fibres, a redistribution of paxillin away from focal adhesions, and an increased phosphorylation of Tyr397 in FAK. All of these changes are consistent with the repellent roles for B-ephrins reverse signaling. Thus, the interaction of Nck-2 and B-ephrins may have functional significance by regulating the cytoskeleton during axonal pathfinding and cell migration.

RATIONALE AND OBJECTIVES

Rationale

Netrins are bifunctional molecules attracting and repelling different classes of axons. Growth cone attraction mediated by netrin-1 involves the transmembrane receptor DCC. However, the intracellular mechanisms mediating the response of an axon to netrin-1 are not clear. Evidence suggests that extracellular guidance cues instruct the growth cone to advance, retract, or turn by regulating the actin cytoskeleton within the growth cone. Rho family GTPases are key regulators of the actin cytoskeleton in non-neuronal cells. There is now growing evidence showing that Rho GTPases regulate cytoskeletal dynamics in neuronal growth cones as well. Therefore, investigation of whether Rho proteins mediate the DCC signaling and characterization of the signaling components linking DCC to the actin cytoskeleton will provide insight into understanding how the growth cone respond to netrin-1.

Objectives

There are three objectives in this thesis:

1. Given that the mechanisms underlying netrin-1 receptor DCC are still unknown, we investigated the role of the small GTPases, RhoA, Rac1, and Cdc42, in the DCC signaling pathways using *in vitro* model systems.

2. We attempted to identify the linker protein in DCC signaling pathways that link DCC to Rho GTPases.

3. We characterized the function of the linker protein using both biochemical and functional assays.

PREFACE TO CHAPTER 2

The intracellular mechanisms underlying the netrin-1 receptor DCC are still unknown. However, there is now growing evidence showing that Rho GTPases regulate cytoskeletal dynamics in neuronal growth cones. A current model suggests that activation of Rac1 or Cdc42 by attractive guidance cues induces filopodia and lamellipodia formation leading to growth cone extension, whereas activation of RhoA by repulsive cues induces filopodia and lamellipodia retraction leading to growth cone collapse. These suggest that Rho GTPases may mediate DCC-induced signaling pathways. Using spinal cord explants, N1E-115 neuroblastoma cells and Swiss 3T3 fibroblasts as model systems, we investigated (a) whether the Rho family GTPases are involved in mediating the axon outgrowth promoting activity of netrin-1, (b) which GTPase activity is required for neurite outgrowth induced by DCC, (c) whether there is a cross-talk within the Rho GTPases in DCC-induced signaling pathways.

CHAPTER 2

RAC1 AND CDC42 BUT BOT RHOA OR RHO KINASE ACTIVITIES ARE REQUIRED FOR NEURITE OUTGROWTH INDUCED BY THE NETRIN-1 RECEPTOR DCC (DELETED IN COLORECTAL CANCER) IN N1E-115 NEUROBLASTOMA CELLS

Rac1 and Cdc42 but not RhoA or Rho-kinase activities are required for neurite outgrowth induced by the Netrin-1 receptor DCC (deleted in colorectal cancer) in N1E-115 neuroblastoma cells

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ABSTRACT

Netrins are chemotropic guidance cues that attract or repel growing axons during development. DCC (deleted in colorectal cancer), a transmembrane protein that is a receptor for netrin-1 is implicated in mediating both responses. However, the mechanism by which this is achieved remains unclear. Here we report that Rho GTPases are required for embryonic spinal commissural axon outgrowth induced by netrin-1. Using N1E-115 neuroblastoma cells we found that both Rac1 and Cdc42 activities are required for DCC-induced neurite outgrowth. In contrast, downregulation of RhoA and its effector Rho-kinase stimulates the ability of DCC to induce neurite outgrowth. In Swiss 3T3 fibroblasts, DCC was found to trigger actin reorganization through activation of Rac1 but not Cdc42 or RhoA. We detected that stimulation of DCC receptors with netrin-1 resulted in a 4-fold increase in Rac1 activation. These results implicate the small GTPases Rac1, Cdc42, and RhoA as essential components that participate in signaling the response of axons to netrin-1 during neural development.

INTRODUCTION

Netrins are a small family of secreted proteins that guide growing axons during neural development (Kennedy and Tessier-Lavigne, 1995; Tessier-Lavigne and Goodman, 1996). The first netrin cloned, UNC-6, was identified using a genetic screen for mutations affecting axon guidance in *C. elegans* (Ishii et al., 1992). Netrins were first identified in vertebrates on the basis of their ability to promote commissural axon outgrowth from explants of embryonic spinal cord (Kennedy et al., 1994; Serafini et al., 1994). Netrin family members have now been identified in multiple vertebrate and invertebrate species and shown to have a highly conserved function as axon guidance cues (Chisholm and Tessier-Lavigne, 1999). Netrins are bifunctional molecules attracting and repelling different classes of axons. Growth cone attraction mediated by netrin-1 involves the transmembrane netrin receptor DCC (Culotti and Merz, 1998; Keino-Masu et al., 1996). In *C. elegans*, the identification of UNC-5 first implicated it as a receptor required for the repellent response to UNC-6 (Leung-Hagesteijn et al., 1992). Three UNC-5 homologs have now been identified in mammals (Ackerman et al., 1997; Leonardo et al., 1997). Current evidence suggests that netrin mediated repulsion requires the function of both UNC-5 and DCC family members in some, and perhaps all cases, suggesting that UNC-5 and DCC may form a netrin receptor complex (Hong et al., 1999).

The intracellular mechanisms mediating the response of an axon to netrin-1 are currently unclear. Previous studies indicate that extracellular guidance cues induce the neuronal growth cone to advance, retract, or turn by regulating the actin cytoskeleton within the growth cone (Suter and Forscher, 1998). The Rho family of small GTPases- in particular, RhoA, Rac1, and Cdc42- are well-established regulators of actin reorganization in non-neuronal cells (Hall, 1998), and there is now compelling evidence demonstrating a role for RhoA, Rac1, and Cdc42 as signaling elements within the neuronal growth cone (Dickson, 2001a; Luo, 2000). Here, we report that members of the Rho family of GTPases are required for commissural axon outgrowth produced by netrin-1 from explants of embryonic rat spinal cord. Both Rac1 and Cdc42 are required for neurite outgrowth promoted by DCC in N1E-115 neuroblastoma cells, and in contrast, inhibition of RhoA and Rho-kinase increases the ability of DCC to induce neurite outgrowth. In Swiss 3T3 cells, DCC was found to trigger actin reorganization through

activation of Rac1 in a netrin-1-dependent manner. In fibroblasts, DCC did not activate Cdc42 or RhoA.

EXPERIMENTAL PROCEDURES

Explant Assay

Embryonic day 13 rat dorsal spinal cord and floor plate explants were dissected and cultured in three dimensional collagen gels as described (Serafini et al., 1994). Recombinant chick netrin-1 protein was produced and purified as described (Serafini et al., 1994). Toxin B was purified as previously described (Just et al., 1995). Both netrin-1 protein and toxin B were added to the culture media at the beginning of the culture period. Explants were cultured for 14 hours, then fixed with 4% paraformaldehyde, and photographed with an Optronics MagnaFire camera and a Carl Zeiss Axiovert microscope using a 20X objective lens and phase contrast optics. The length of axon fascicles growing out of the explants were quantified using Northern Eclipse Software (Empix Imaging). The total length of fascicle growth was then calculated for each explant.

DNA Constructs

Standard DNA protocols were used as described (Sambrook et al., 1989). pRK5-DCC-C (3062-4335 bp) was generated by digestion of pBS-DCC with *Eco*RI and *Bgl*II followed by ligation of *Eco*RI-*Bgl*II fragment into pRK5 digested with *Eco*RI and *Bam*HI. To generate pRK5 encoding full-length DCC, a fragment (3061 bp) from the start codon to the *Eco*RI site of pBS-DCC was amplified by PCR and subcloned into pRK5-DCC-C digested with E*co*RI. pDCC-E encoding the amino terminus of DCC (1122 amino acids) comprising the extracellular and transmembrane domains tagged with green fluorescent protein (GFP) at its C-terminus was kindly provided by Dr. Tim Kennedy (McGill University). DNA was purified using Qiagen kit. For microinjection studies, purified plasmids were filtered through a 0.2μm cellulose acetate membrane (Corning) before microinjection into cells.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was purified using Trizol (Invitrogen) and polyA (+) RNA was isolated using the Oligotex mRNA purification kit (Qiagen). First strand cDNA was synthesized using superscript reverse transcriptase (Invitrogen). PCR was used to amplify cDNA using the following primers: UNC5h1: GGA ATT CCC TCC CTC GAT CCC

AAT GTG T; TCC CCG CGG GGC AGG GAA CGA AAG TAG T, 909 bp; UNC5h2: GCT CTA GAG TCG CGG CAG CAG GTG GAG GAA; GGA ATT CAG GGG GCG GCT TTT AGG GTC GTT, 771bp; DCC: CCG CTC GAG TGG TCA CCG TGG GCG TTC TCA; GGC TGG ATC CTC TGT TGG CTT GTG, 938 bp. Primers were annealed at 60°C and 35 cycles of amplification carried out. The size of the predicted amplification product is indicated.

Cell Culture and Microinjection

Mouse fibroblast Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and antibiotics and maintained in an atmosphere of 10% CO_2. Confluent serum-starved Swiss 3T3 cells were prepared as described (Ridley et al., 1992). Briefly, cells were plated in 5% serum at a density of 6 x 10^4 onto acid-washed coverslips. 7-10 days later, the cells became quiescent and were subjected to serum starvation for 16 h in DMEM containing 2g/l $NaHCO_3$. The eukaryotic expression vector pRK5 encoding full-length DCC or truncated DCC (pRK5-DCC-C) was microinjected alone or with pRK5 encoding myc-tagged Cdc42N17, RacN17, or pEFmyc-C3 transferase at 0.1 mg/ml into the nucleus of ~100 cells over a period of 20 min in CO_2-independent medium (Gibco) using an Eppendorf microinjection system 5246. pDCC-E or pEGFP were microinjected at 0.1 mg/ml into the nucleus of ~ 100 cells. During microinjection, cells were maintained at 37°C within a humidified atmosphere. Cells were returned to the incubator for a further 5 hr followed by the addition of purified netrin-1 at 500ng/ml for up to 30 min.

Mammalian Cell Transfection

N1E-115 neuroblastoma cells and COS-7 cells were grown in DMEM supplemented with 10% FCS and antibiotics at 10% CO_2. N1E-115 cells were plated onto coverslips previously coated with laminin (20 μg/ml; VWR Canlab) for 24 h at 37°C, washed twice with water, and left to air dry. Transfection was carried out with Lipofectamine transfection reagent (Gibco) according to the manufacturer's protocol. Briefly, cells were incubated in serum-free medium for 1 h. During this time, pRK5, pRK5-DCC-C, pDCC-E, pRK5-DCC (0.4 μg) with or without pRK5myc-RacN17 or -

Cdc42N17 or pEFmyc-C3 transferase (0.2 μg) were mixed with Lipofectamine reagent and incubated for 15 min at room temperature followed by the addition of the transfection mix to the cells. Six hours later, the transfection mix was replaced with DMEM containing 5% FCS and incubated for 12h with or without the blocking antibodies PN3 against netrin-1 (25 μg/ml) (Manitt et al., 2001) (provided by Dr. Tim Kennedy) and DCC (10 μg/ml) (AF5, Cedarlane laboratories LTD) or mouse IgG (25 μg/ml) before fixation in freshly prepared 4% (w/v) paraformaldehyde for 10 min. When indicated, cells transfected with pRK5 or pRK5-DCC were incubated with 10 μM Y-27632 compound for 2 hours before fixation. COS-7 cells were transfected using the DEAE-dextran method as described previously (Olson et al., 1995). The amounts of plasmid used per 100mm dish were as follows: pRK5, 5 μg; pRK5-DCC, 5 μg; pRK5myc-Rac1 or -Cdc42, 1.5 μg. Twenty-four hours after transfection, cells were serum-starved overnight and treated with netrin-1 (500 ng/ml) for different period of time.

Immunofluorescence Microscopy

At the indicated times, microinjected Swiss 3T3 cells or transfected N1E-115 cells were rinsed with PBS and fixed for 10 min in freshly prepared 4% (w/v) paraformaldehyde. All steps were carried out at room temperature, and coverslips were rinsed in PBS between each of the step. Cells were permeabilized in 0.2% Triton X-100 for 5 min, free aldehyde groups were reduced with 0.5 mg/ml sodium borohydride for 10 min. Cells were double-labeled following the procedure previously described (Nobes et al., 1995). Briefly, cells were incubated with the primary monoclonal antibodies anti-DCC (Pharmingen, G97-449), anti-myc (a generous gift from Dr. Nicole Beauchemin, McGill University), or anti-GFP (Molecular Probe) diluted in PBS for 60 min. Then, coverslips were transferred to a secondary antibody mixture composed of FITC-conjugated goat anti-mouse antibody (Sigma) and TRITC-conjugated phalloidin (Sigma) for 60 min. In N1E-115 cells, a neurite was defined as a process that measured at least the length of the cell body and stained positively for neurofilament M using a polyclonal anti-neurofilament 150 (Chemicon). Coverslips were mounted by inverting them onto 8 μl of mowiol (Calbiochem) mountant containing p-phenylenediamine as an anti-bleach reagent. After 2 hr at room temperature, the coverslips were examined on a Zeiss

Axiovert 135 microscope using Zeiss oil immersion 63x objective lens. Fluorescence images were recorded using a digital camera (DVC) and analyzed with Northern Eclipse software (Empix Imaging Inc.).

Purification of GST-PAK and GST-WASP

GST-PAK (aa 56-272) and GST-WASP (aa 201-321) were used to isolate GTP-bound Rac1 and Cdc42, respectively. *E. coli* transformed with GST-PAK and GST-WASP constructs were grown at 37°C to an absorbance of 0.5. Expression of the fusion proteins was induced by isopropyl-β-D-thiogalactopyranoside (1 mM) for 3h at 37°C. Cells were washed once in STE buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA) prior to sonication in buffer A (20 mM Hepes pH 7.5, 120 mM NaCl, 2 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were cleared by centrifugation and NP-40 was added to a final concentration of 0.5%. The proteins were stored at -80°C until use. Protein concentration was determined by comparison with different amounts of BSA using SDS-PAGE. For each sample, 10-15 μg of GST-PAK or GST-WASP was purified using glutathione-Sepharose beads (Sigma) for 30 min at 4°C. Beads were washed twice with buffer A and the protein lysates were added as described below.

Rac/Cdc42 GTP-loading assay

COS-7 cells co-transfected with pRK5 encoding DCC and myc-tagged Rac1 or Cdc42 were serum-starved overnight and treated with purified netrin-1 for different periods of time. Cells were lysed in 25 mM Hepes pH 7.5, 1% NP-40, 10 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Protein concentrations were determined using BioRad protein assay kit. An equal amount of proteins was incubated for 1h at 4°C with GST-PAK or GST-WASP (10-15 μg) purified on glutathione-Sepharose beads in binding buffer (25 mM Hepes pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl, 0.5% NP-40, 1 mM DTT) as described (Royal et al., 2000). Beads were washed three times with washing buffer (25 mM Hepes pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl, 1 mM DTT) and twice with washing buffer containing 1% NP-40 before being boiled in SDS-sample buffer. Proteins were separated

on 12% SDS-PAGE. GTP-bound Rac1 and Cdc42 were revealed by immunoblotting using the anti-myc antibody and ECL reagent detection kit (Amersham). Levels of GTP-bound Rac1 and Cdc42 in each sample were assessed using densitometry.

RESULTS

Rho GTPases are required for commissural axon outgrowth evoked by netrin-1.

To determine if Rho GTPases are involved in mediating the axon outgrowth promoting activity of netrin-1, we examined the effect of adding the Rho GTPase inhibitor toxin B to explants of E13 rat dorsal spinal cord cultured in a three dimensional collagen gel in the presence of netrin-1. Explants of dorsal spinal cord cultured in the presence of recombinant netrin-1 (200 ng/ml) produced maximal commissural axon outgrowth from these explants as previously reported (Kennedy et al., 1994) (Fig. 2.1A, C). Addition of increasing concentrations of toxin B from 0.001ng/ml to 1 ng/ml in the presence of maximal concentrations of netrin-1 resulted in increasing inhibition of commissural axon outgrowth from explants (Fig. 2.1A, D-G). In the presence of toxin B, cells at the edge of the explants were clearly rounded (Fig. 2.1E, see arrow), a characteristic effect of toxin B on other cell types (Aktories et al., 2000). In addition, although a small amount of axon outgrowth occurred at higher concentrations of toxin B, the axons were much less fasciculated than normal (compare Fig. 2.1C with 2.1D, E). These findings indicate that netrin-1 mediated commissural axon outgrowth requires the activity of one or more Rho GTPases.

DCC-induced neurite outgrowth requires Rac1 and Cdc42 but not RhoA or Rho-kinase activities in N1E-115 neuroblastoma cells.

Mouse N1E-115 neuroblastoma cells exhibit neurite outgrowth in response to serum deprivation (Jalink et al., 1994). Using immunoblotting analyses, N1E-115 cells were found to constitutively express netrin-1 either in the presence (data not shown) or in the absence of serum. However, these cells did not express DCC (Fig. 2.2A). RT-PCR analysis revealed the expression of mRNAs encoding the netrin-1 receptors UNC5h1 and UNC5h2 but not DCC (Fig. 2.2B). In the presence of 5% serum, cells are round and extend lamellipodia and multiple filopodia (Fig. 2.3G). When DCC is expressed in N1E-115 cells in the presence of 5% serum, 62% of transfected cells exhibited neurite outgrowth (Fig. 2.4). The majority of DCC-expressing cells contained one long neurite (~ 30 μm) per cell with thin filopodia along the neurite (Fig. 2.3A and B). DCC protein was consistently enriched at the extending tip of the neurite as shown in Fig. 2.3A (see arrow).

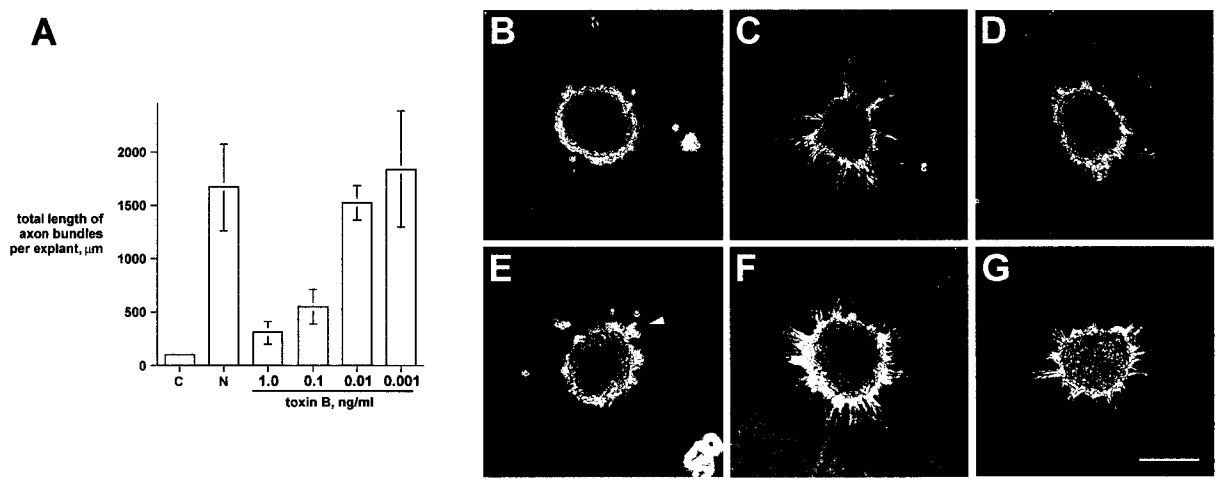

A
total length of axon bundles per explant, µm
2000
1500
1000
500
0
C
N
1.0
0.1
0.01
0.001
toxin B, ng/ml
B
C
D
E
F
G

Figure 2.1. Toxin B inhibits netrin-1-dependent outgrowth of commissural axons. Panel A illustrates the quantification of axon outgrowth from explants of E13 rat dorsal spinal cord shown in B-G. C: control, N: netrin-1 (200ng/ml) (B) A negative control explant cultured in the absence of netrin-1. (C) Axon outgrowth in the presence of 200 ng/ml netrin-1 protein. Netrin-1-dependent (200ng/ml) axon outgrowth was reduced by the addition of toxin B to the cultures (D: 1.0 ng/ml; E: 0.1 ng/ml; F: 0.01 ng/ml; G: 0.001 ng/ml). The arrowhead in E indicates a rounded cell body present at the edge of the explant. Scale bar in G corresponds to 100 μm and is the same in B-G. Error bars represent the SEM and n=4 in each case.

A

B

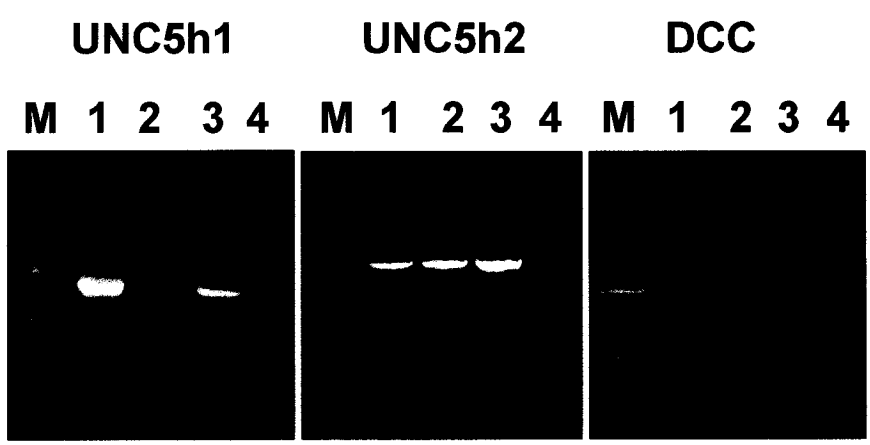


Figure 2.2. Expression of netrin-1 and netrin-1 receptors in N1E-115 cells and Swiss 3T3 fibroblasts.

A) The expression of DCC and netrin-1 proteins was detected by western blot analyses using monoclonal anti-DCC (upper panel) and polyclonal PN3 anti-netrin-1 (lower panel) antibodies, respectively. Upper panel: Lane 1: 50 μg of protein lysate from DCC-expressing COS-7 cells, Lane 2: 100 μg of Swiss 3T3 protein cell lysate, lane 3: 100 μg of serum-starved N1E-115 protein cell lysate. Lower panel: Lane 1: 100 ng purified netrin-1; Lane 2: 100 μg of Swiss 3T3 protein cell lysate, lane 3: 100 μg of serum-starved N1E-115 protein cell lysate. B) RT-PCR amplification of cDNA from polyA (+) RNA isolated from newborn mouse brain (lane 1), Swiss 3T3 cells (lane 2), or serum-starved N1E-115 cells (lane 3). Lane 4 is a negative control containing no cDNA. DNA markers (M) are the 100 bp DNA ladder (New England Biolabs).

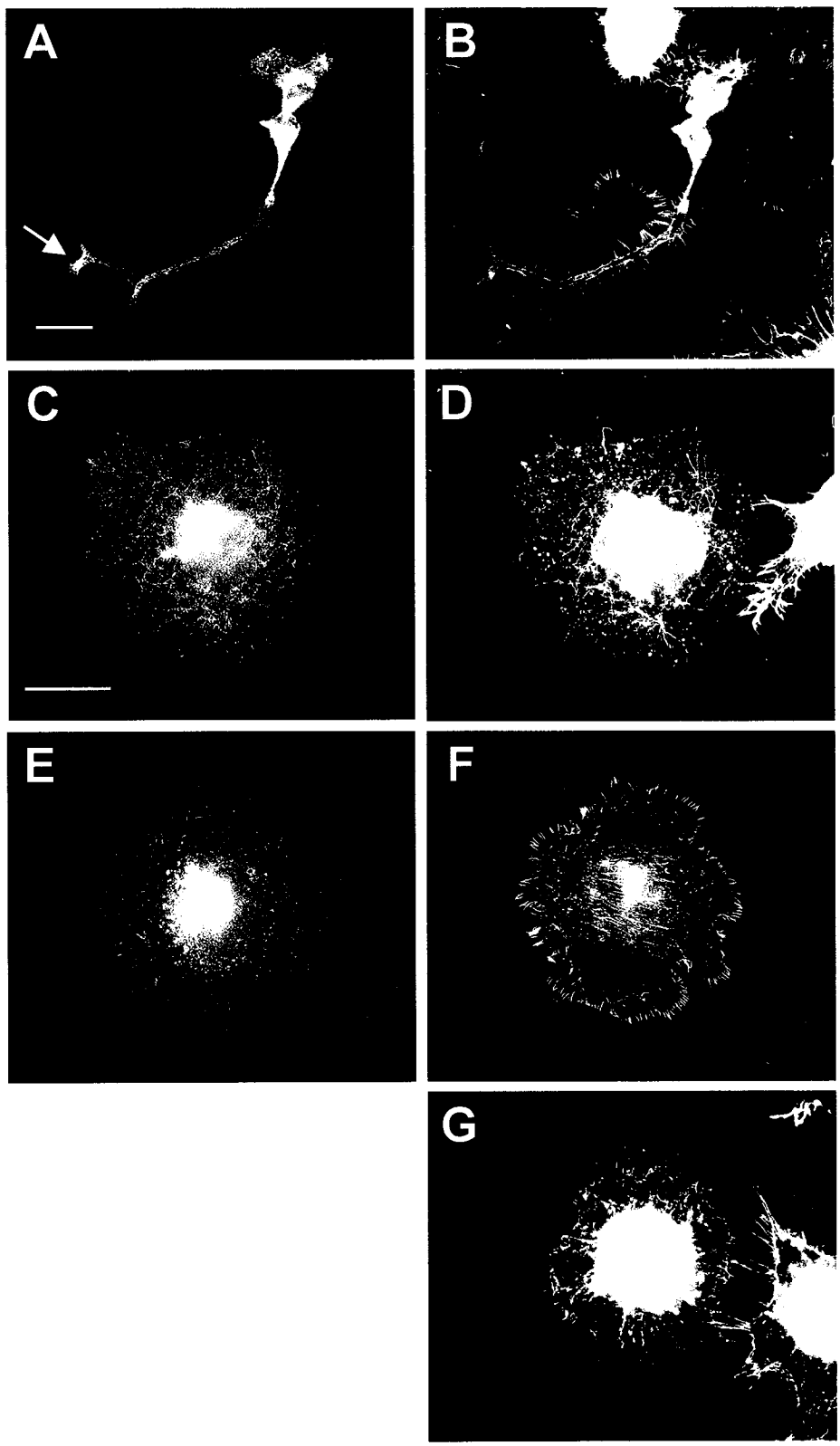

A
B
C
D
E
F
G

Figure 2.3. DCC-induced neurite outgrowth requires Rac1 and Cdc42 activity in N1E-115 neuroblastoma cells.

N1E-115 neuroblastoma cells were transfected either with empty vector pRK5 (G), pRK5-DCC alone (A and B), or with pRK5myc-RacN17 (C and D) or pRK5myc-Cdc42N17 (E and F). F-actin (B, D, F, and G) was visualized with fluorescently tagged phalloidin and DCC (A, C, and E) was revealed by co-staining with an anti-DCC antibody and by indirect immunofluorescence. The expression of myc-tagged RacN17 and Cdc42N17 was detected using anti-myc antibodies and by indirect immunofluorescence. Scale bars, 10 μm. The scale in panels A and B is different from that in panels C to G.

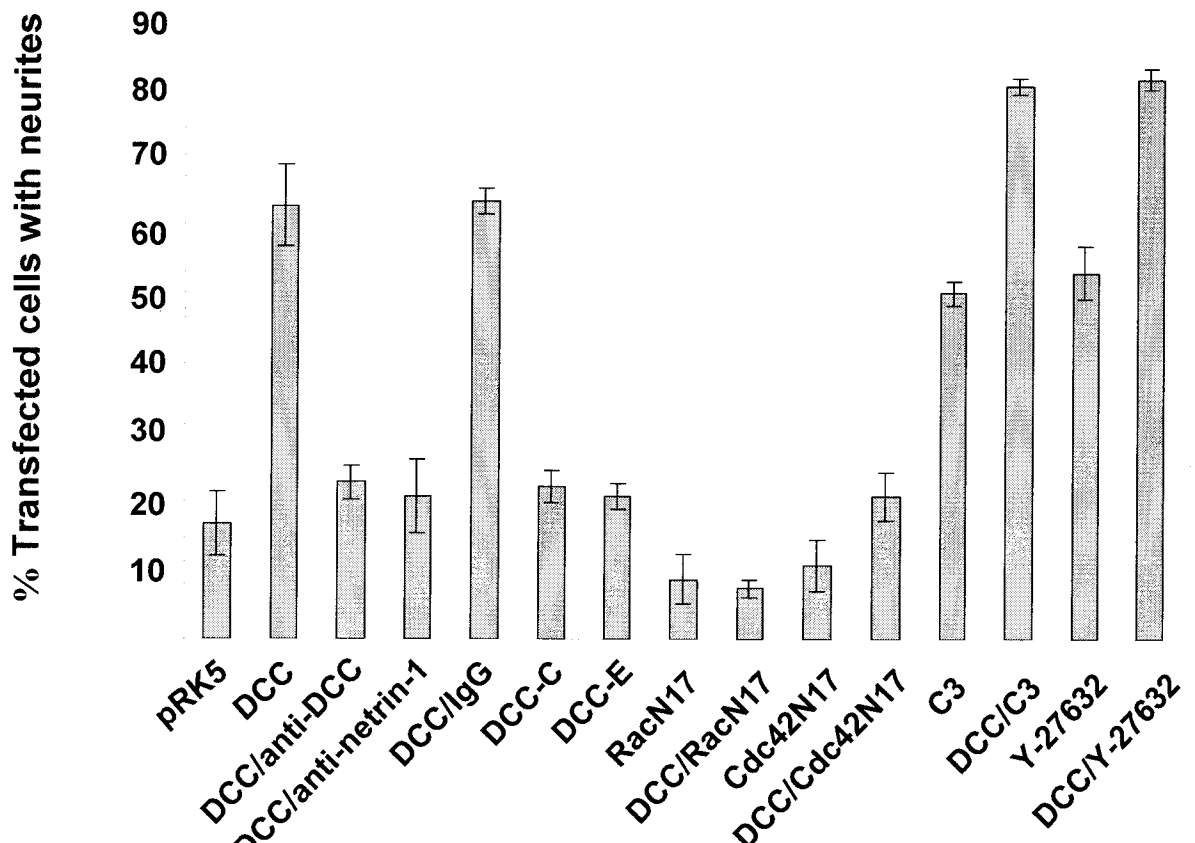

% Transfected cells with neurites
90
80
70
60
50
40
30
20
10
pRK5
DCC
DCC/anti-DCC
DCC/anti-netrin-1
DCC/IgG
DCC-C
DCC-E
RacN17
DCC/RacN17
Cdc42N17
DCC/Cdc42N17
C3
DCC/C3
Y-27632
DCC/Y-27632

Figure 2.4. Inhibition of RhoA and Rho-kinase stimulates the ability of DCC to induce neurite outgrowth in N1E-115 cells.

N1E-115 neuroblastoma cells were transfected either with empty vector pRK5, pRK5myc-RacN17, pRK5myc-N17cdc42, pEFmyc-C3 transferase, pRK5-DCC-C, pDCC-E, pRK5-DCC alone, or with pEFmyc-C3 transferase, pRK5myc-RacN17 or -Cdc42N17. When indicated, cells transfected with pRK5 or pRK5-DCC were treated with 10 μM of Y-27632 compound for 2 hours prior fixation, or with the blocking antibodies against netrin-1 (PN3) and DCC (AF5), or with mouse IgG as a negative control. F-actin, DCC, and myc-tagged proteins were visualized as described in Figure 3. DCC lacking the majority of the extracellular domain (DCC-C) was revealed with the anti-DCC antibody (Pharmingen) raised against the intracellular domain of DCC. DCC lacking the cytoplasmic domain (DCC-E) tagged at its C-terminus with GFP was visualized with anti-GFP antibodies. Values indicate the percentage of transfected cells with neurite extension, and correspond to the average of at least 3 independent experiments.

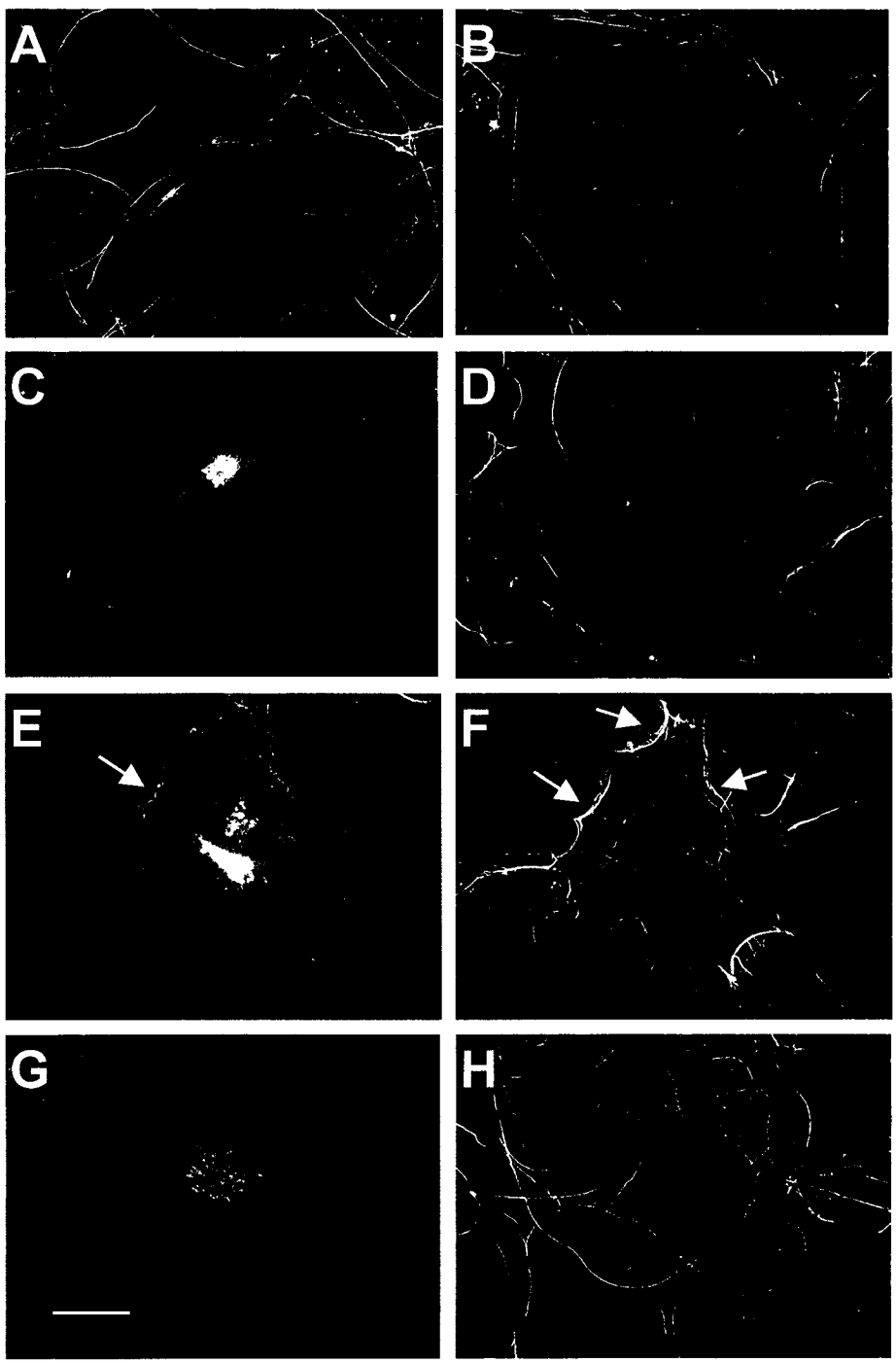
The presence of antibodies blocking the function of DCC or netrin-1 inhibited the ability of DCC to induce neurite outgrowth in N1E-115 cells (Fig. 2.4). In addition, truncated DCC proteins lacking the majority of the extracellular domain (DCC-C) or the cytoplasmic domain (DCC-E) of DCC were unable to produce neurite outgrowth (Fig. 2.4). These results strongly suggest that netrin-1 binding to DCC is necessary to mediate intracellular signaling events leading to neurite outgrowth in N1E-115 cells.

C3 transferase has been shown to inactivate RhoA by ADP-ribosylation at residue Asn41 (Aktories et al., 2000). When C3 transferase is expressed in N1E-115 cells, 50% of transfected cells showed neurite outgrowth as previously reported (Kozma, 1997) (Fig. 2.4). When DCC is expressed in the presence of C3 transferase, the number of transfected cells with neurite outgrowth increased to 80% (Fig. 2.4). Similarly, when DCC-expressing cells are incubated with the Y-27632 compound that inhibits the Rho effector Rho-kinase, neurite outgrowth is stimulated in more than 80% of transfected cells (Fig. 2.4). These results suggest that inhibition of RhoA and its effector Rho-kinase known to mediate the effects of RhoA on neurite retraction in N1E-115 cells (Hirose et al., 1998) increases the ability of DCC to stimulate neurite extension in N1E-115 cells.

Expression plasmids encoding dominant negative RacN17 or Cdc42N17 were transfected together with pRK5-DCC into N1E-115 cells. As shown in Fig. 2.3D and F, both dominant negative Rac1 and Cdc42 significantly inhibited neurite outgrowth induced by DCC. In DCC-expressing cells, the dominant negative Rac1 and Cdc42 mutants reduced neurite extension by 55 and 45%, respectively (Fig. 2.4). Cells expressing both RacN17 and DCC were rounded and flattened, exhibited long filopodia but not lamellipodia. These findings suggest that although Rac1 has been inhibited, Cdc42 remained activated in these cells (Fig. 2.3D). Cells expressing both Cdc42N17 and DCC exhibited lamellipodia and short microspikes at the plasma membrane, suggesting that Rac1 remained activated in these cells (Fig. 2.3F). Therefore, both Rac1 and Cdc42 activities are required for neurite outgrowth induced by DCC in N1E-115 cells.

The netrin-1 receptor DCC activates Rac1 but not Cdc42 or RhoA in Swiss 3T3 fibroblasts.

To further dissect the mechanisms used by netrin-1 to signal through Rho GTPases, we reconstituted the phenomenon in Swiss 3T3 fibroblasts by transiently expressing DCC and using the organization of the actin cytoskeleton as a functional read-out. Following serum-starvation, Swiss 3T3 cells lose most of the actin-based structures usually found in a fibroblast: lamellipodia, filopodia, stress fibers. However, the cells do remain attached to the supporting extracellular matrix (Fig. 2.5A). Microinjection of constitutively active Cdc42L61, RacL61, and RhoL63 proteins into quiescent, serum-starved Swiss 3T3 cells has been shown to rapidly induce the formation of three distinct actin based structures: filopodia, lamellipodia and stress fibers, respectively (Kozma et al., 1995; Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992). In addition, in some cell types, such as fibroblasts and epithelial cells, activation of Cdc42 leads to rapid activation of Rac1, which in turn leads to activation of RhoA (Nobes and Hall, 1995). Netrin-1 and DCC proteins were undetectable by western blot analyses of Swiss 3T3 cell lysates (Fig. 2.2A). RT-PCR analyses detected the expression of mRNAs encoding UNC5h2 but not UNC5h1 or DCC. As shown in Fig. 2.5B, the addition of recombinant netrin-1 protein does not affect the reorganization of polymerized actin in uninjected cells. Microinjection of the eukaryotic expression vector, pRK5, encoding full-length rat DCC into quiescent, serum-starved Swiss 3T3 cells led to the expression of DCC (Fig. 2.5C) and no spontaneous reorganization of actin was observed (Fig. 2.5D). However, ten minutes after the addition of 500 ng/ml of purified netrin-1 to the media, assemblies of polymerized actin were detected at the leading edge of the plasma membrane in DCC-expressing cells. These developed into lamellipodia and membrane ruffles as shown in Fig. 2.5F (see arrows). The minimum concentration of netrin-1 required to cause actin reorganization in DCC expressing cells was 100 ng/ml (data not shown). However, optimal effects were obtained at 500 ng/ml of netrin-1. Thirty minutes after the addition of netrin-1, in cells expressing DCC, actin assembled into stress fibers that traverse the cell (Fig. 2.5H). Recently, it has been reported that netrin-1 binds to the extracellular domain of DCC (Stein, 2001). A truncated DCC protein lacking the majority of the extracellular domain did not induce actin reorganization after the addition of netrin-1 for 30 minutes, suggesting that netrin-1 binding to DCC is essential to activate Rho GTPase signaling pathways (Fig. 2.6B). Similarly, the expression of a truncated DCC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H

Figure 2.5. Cytoskeletal changes in Swiss 3T3 cells induced by netrin-1 receptor DCC.

Serum-starved Swiss 3T3 cells microinjected with pRK5-DCC (C to H) were fixed after either no addition (A, C, and D) or addition of 500 ng/ml recombinant netrin-1 for 10 min (E and F) or 30 min (B, G, H). F-Actin (A, B, D, F, H) and DCC (C, E, G) were visualized as in Figure 2.3. Approximately 100 cells were microinjected per coverslip, and 5 hr after injection, 90% of the injected cells showed expression of DCC. Scale bars, 10 μm. Arrows indicate localization of DCC at the plasma membrane (E) and membrane ruffles (F).

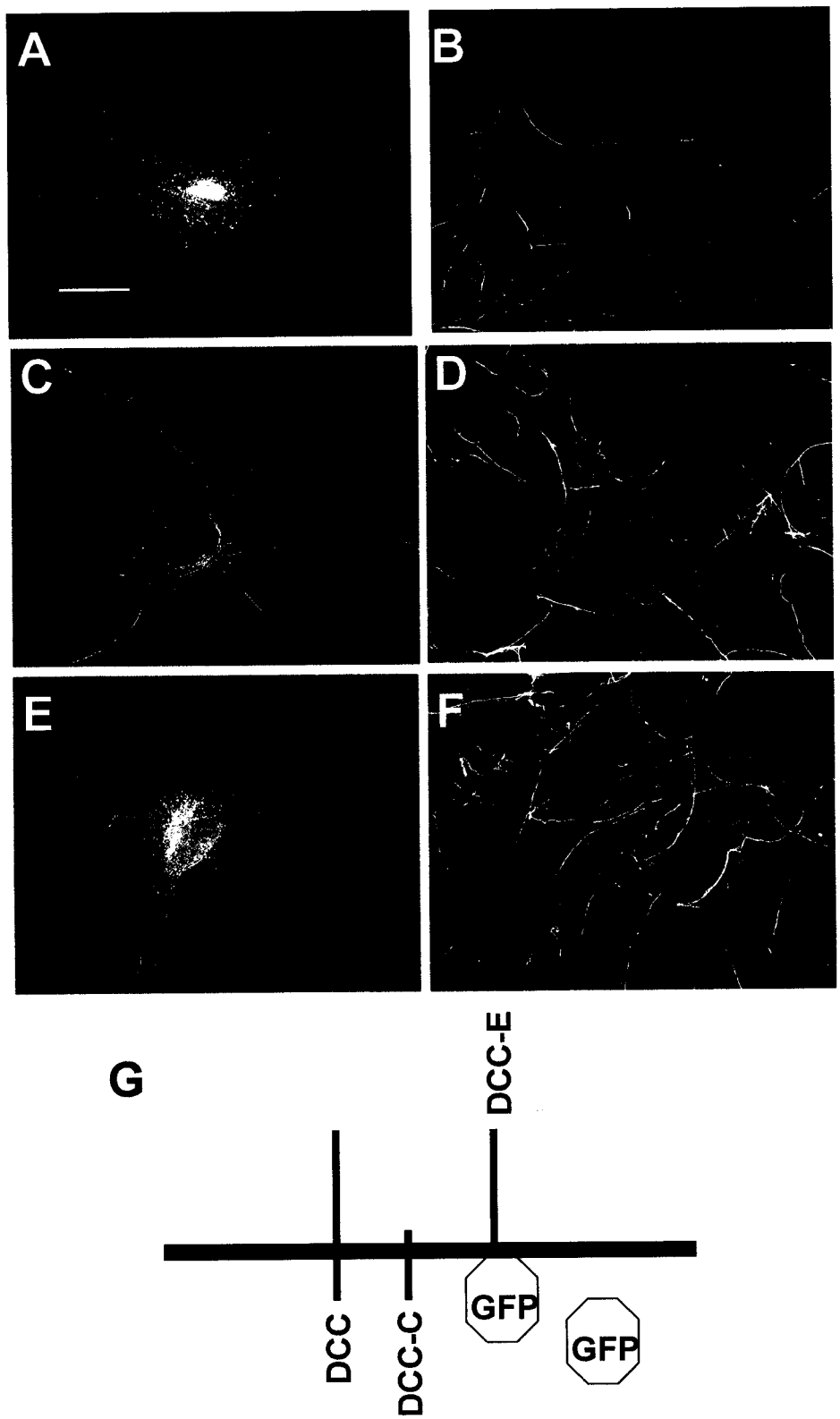

A
B
C
D
E
F
G
DCC-E
DCC
DCC-C
GFP
GFP

Figure 2.6. Netrin-1 binding to DCC is essential to mediate actin reorganization in Swiss 3T3 fibroblasts.

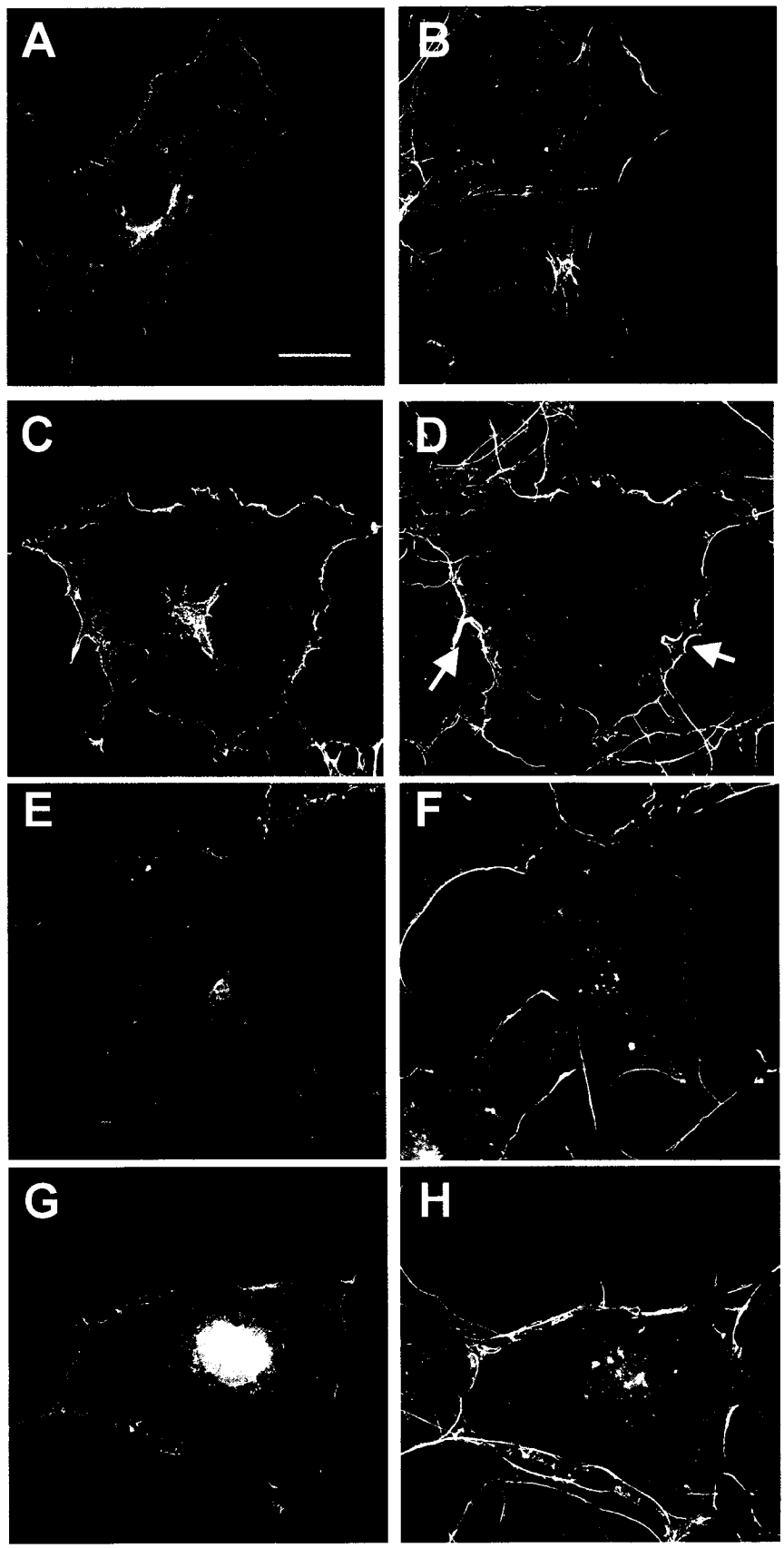
Serum-starved Swiss 3T3 cells microinjected with pRK5-DCC-C (A,B), or with pDCC-E (C,D), or with pEGFP (E,F) were fixed after addition of 500 ng/ml recombinant netrin-1 for 30 min. F-Actin (B,D, F) and DCC-C (A) were visualized as in Figure 2.3. DCC-E fused to GFP at its C-terminus (C) and GFP (E) were visualized using anti-GFP antibodies and indirect fluorescence. Approximately 100 cells were microinjected per coverslip, and 5 hr after injection, 90% of the injected cells showed expression of DCC. Scale bars, 10 μm. (G) Schematic view of the DCC expression constructs.

protein lacking the cytoplasmic domain and coupled to green fluorescent protein did not lead to actin reorganization after the addition of netrin-1 (Fig. 2.6D). We conclude that DCC is essential to activate the cascade of Rho GTPases in Swiss 3T3 cells in a ligand-dependent manner.

To determine if DCC activates the cascade of Rho GTPases through Cdc42 or Rac1 in Swiss 3T3 fibroblasts, we microinjected quiescent, serum-starved Swiss 3T3 cells with pRK5-DCC together with eukaryotic vectors encoding either myc-tagged dominant negative RacN17 or Cdc42N17 or C3 transferase. As shown in Fig. 2.7, the expression of dominant negative Cdc42N17 did not inhibit actin reorganization induced by netrin-1 in DCC-expressing cells (compare Fig. 2.7D with 2.7B), whereas dominant negative RacN17 inhibited the formation of both lamellipodia and stress fibers (Fig. 2.7F). C3 transferase blocked the formation of stress fibers but not the formation of polymerized actin at the leading edge of the plasma membrane (Fig. 2.7H). Hence, DCC activates Rac1-induced signaling pathways but not Cdc42-dependent signals in Swiss 3T3 fibroblasts. These findings indicate that in these cells, activation of RhoA by DCC is a consequence of crosstalk between Rac1 and RhoA.

The Netrin-1 receptor DCC promotes Rac1 GTP-loading.

Pull-down assays were carried out in which Rac1 and Cdc42 GTP-loading were assessed by specific binding of the active GTPases to the CRIB domain (Cdc42/Rac interactive binding) (Burbelo, 1995) of $p65^{PAK}$ or WASP (Wiskott-Aldrich syndrome protein) fused to glutathione S-transferase (GST-PAK or GST-WASP), respectively. DCC and myc-tagged Rac1 or Cdc42 were coexpressed in COS-7 cells for 24 hours, cells were serum-starved overnight followed by the addition of netrin-1 to the media. Lysates were prepared, and the amount of Rac1 or Cdc42 precipitated with GST-PAK or GST-WASP, respectively, was determined by western blot analysis. Netrin-1 stimulated a 4-fold increase in the level of activated Rac1 (Fig. 2.8A,B) whereas no increase in GTP-Cdc42 was observed after stimulation with netrin-1 (Fig. 2.8C,D). Cells expressing Rac1 in the absence of DCC showed no increase in Rac1-GTP after 5 minute stimulation with netrin-1, suggesting that DCC is required for Rac1 activation. These data are consistent


A
B
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D
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H

Figure 2.7. Netrin-1 receptor DCC activates Rac1 but not RhoA or Cdc42 in Swiss 3T3 fibroblasts.

Serum-starved Swiss 3T3 cells were microinjected with pRK5-DCC alone (A and B) or with pRK5 encoding myc-tagged Cdc42N17 (C and D), RacN17 (E and F), or pEF-mycC3 transferase (G and H). 5 hours after microinjection, netrin-1 (500 ng/ml) was added to the media for 30 min. F-actin (B, D, F, and H) and DCC (A, C, E, and G) were visualized as in Figure 2.3. Myc-tagged proteins were revealed by co-staining with an anti-Myc antibody and by indirect immunofluorescence (not shown). Approximately 100 cells were microinjected per coverslip. Scale bar, 10 μm. Arrows indicate membrane ruffles.

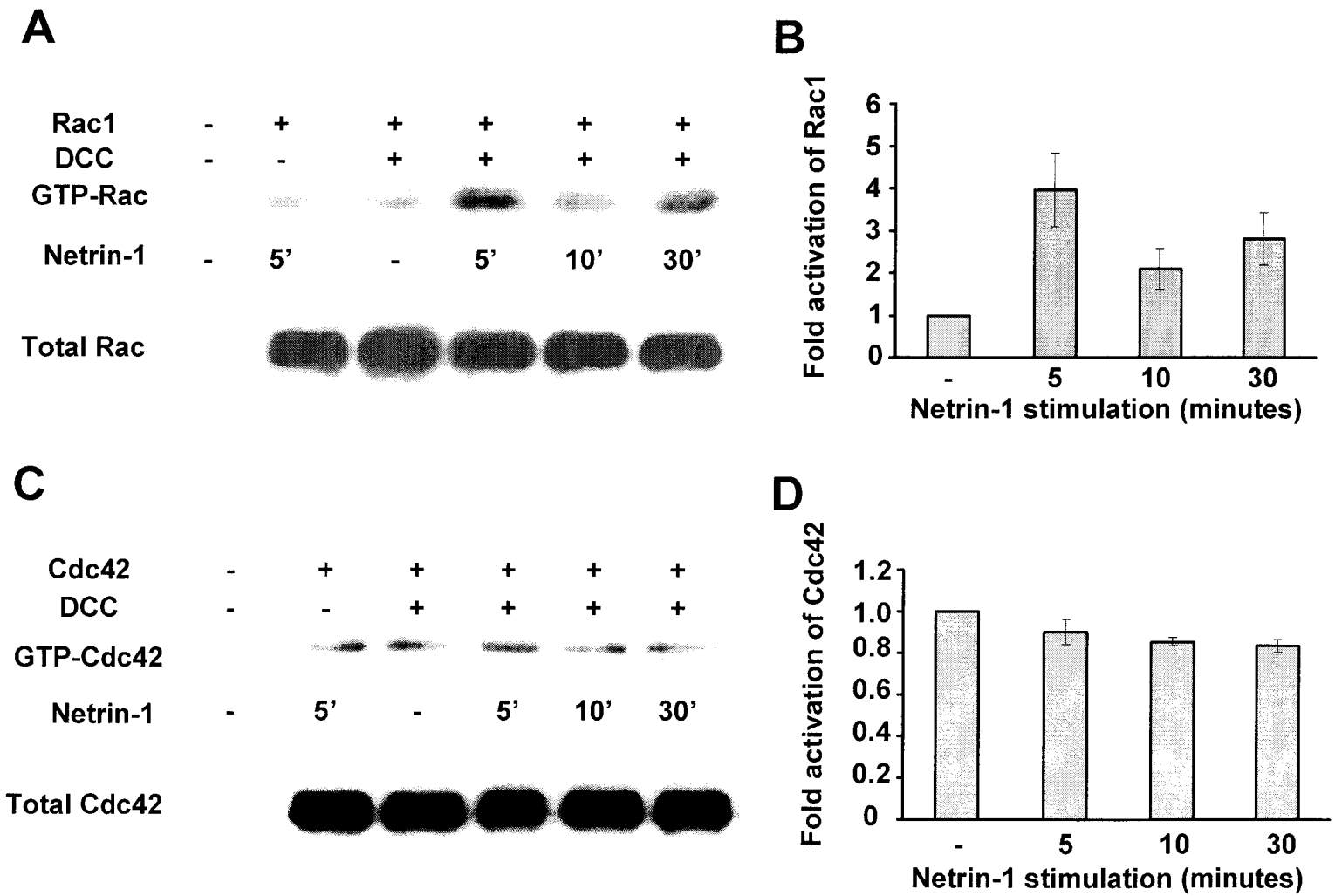
A
Rac1 - + + + + +
DCC - - + + + +
GTP-Rac
Netrin-1 - 5' - 5' 10' 30'
Total Rac
B
Fold activation of Rac1
0 1 2 3 4 5 6
- 5 10 30
Netrin-1 stimulation (minutes)
C
Cdc42 - + + + + +
DCC - - + + + +
GTP-Cdc42
Netrin-1 - 5' - 5' 10' 30'
Total Cdc42
D
Fold activation of Cdc42
0 0.2 0.4 0.6 0.8 1.0 1.2
- 5 10 30
Netrin-1 stimulation (minutes)

Figure 2.8. Netrin-1 receptor DCC promotes Rac GTP-loading.

COS-7 cells were transfected with 5 μg of pRK5 or pRK5-DCC together with 1.5 μg of pRK5myc-Rac1 (A,B) or pRK5myc-Cdc42 (C,D) and treated with netrin-1 (500 ng/ml) for the indicated times. Cells were lysed and an equal amount of protein was incubated with GST-PAK (aa 56-272) (A,B) or GST-WASP (aa 201-321) (C,D) protein coupled to glutathione-Sepharose beads. Upper panel: GTP-bound Rac1 (A) or GTP-bound Cdc42 (C) was detected by western blotting using anti-myc antibodies. Lower panel: total cell lysates probed for Rac1 (A) or Cdc42 (C) demonstrated equal amounts of GTPase. Panels B and D illustrate a time course of the fold change of Cdc42 and Rac1 activation following the addition of netrin-1 to cells expressing DCC. The fold activation of GTPase was determined by densitometry and values correspond to the averages of at least three independent experiments.

DISCUSSION

Netrin-1 and its receptor, DCC, are widely expressed in embryonic and adult tissues (Fearon et al., 1990; Kennedy et al., 1994; Manitt et al., 2001; Meyerhardt et al., 1999; Reale et al., 1994). Their function in many cell types is poorly understood, but in the embryonic CNS they act as attractive and repulsive cues that guide the migration of developing axons (Culotti and Merz, 1998). Here we demonstrated that toxin B inhibits commissural axon outgrowth evoked by netrin-1, thereby implicating Rho GTPases in mediating the effect of netrin-1 on these axons. Both Rac1 and Cdc42 were found to be necessary for DCC-induced neurite outgrowth in N1E-115 neuroblastoma cells. When RhoA and Rho-kinase were inhibited respectively, by C3 transferase or Y-27632 in N1E-115 cells, 80% of DCC-expressing cells exhibit neurite outgrowth, suggesting that downregulation of RhoA and Rho-kinase is required for DCC to induce neurite outgrowth in N1E-115 cells. In fibroblasts, the expression of DCC triggered actin reorganization in a netrin-1-dependent manner through the activation of Rac1 but not RhoA or Cdc42. Netrin-1 stimulation of DCC resulted in a 4-fold increase of Rac1 activation without affecting the level of activated Cdc42. Interestingly, these results suggest that a neuronal-specific guanine nucleotide exchange factor (GEF) required for DCC to activate Cdc42 may be absent in fibroblasts. Alternatively, a specific coreceptor for netrin-1 that is required for DCC to activate Cdc42 may not be expressed in fibroblasts. Altogether, this study provide compelling evidence for a key role of regulated activities of Rac1, Cdc42, and RhoA in the cytosolic signaling mechanisms induced by DCC when it binds to netrin-1. Consistent with our findings, it has been reported that some of the defects caused by an activated form of the *C. elegans* DCC homologue, UNC-40, could be partly suppressed by mutations in ced-10, a member of the Rac family in *C. elegans* (Dickson, 2001b).

In addition to the formation of lamellipodia in DCC-expressing fibroblasts, DCC also induced the formation of stress fibers as a result of cross-talk between Rac1 and RhoA. A current model suggests that attractive guidance cues activate Rac1 or Cdc42 and inhibit RhoA to promote directed axonal outgrowth, whereas repulsive cues inhibit Rac1 or Cdc42 and stimulate RhoA to induce retraction (Dickson, 2001a; Luo, 2000). In support of this model, Wahl et al. (Wahl et al., 2000) showed that ephrin-A5 activates RhoA and inhibits Rac1 in cultured retinal ganglion cells. Here we propose that when a

growth cone is attracted by netrin-1, DCC may activate Rac1 while inhibiting RhoA in neuronal cells. It may be the case that the activation of RhoA by Rac1 in fibroblasts reported here is restricted to non-neuronal cells.

The implication that second messengers-Ca^{2+} and cAMP-modulate the response to netrin-1 has emerged from *in vitro* studies of growth cone turning using *Xenopus* spinal neurons (de la Torre et al., 1997; Hong et al., 2000; Ming et al., 1997; Zheng, 2000). Using the same assay, co-activation of phosphatidylinositol-3 kinase and phospholipase Cγ pathways were shown to be required for the turning response of the growth cone (Ming et al., 1999). Phosphatidylinositol-3 kinase mediates activation of Rac1 downstream of many tyrosine kinase receptors (Bar-Sagi, 2000). However, it has not yet been determined if phosphatidylinositol-3 kinase links DCC to activation of Rac1 upon binding to netrin-1. Protein kinase A phosphorylation of RhoA and the intracellular level of cAMP negatively regulate the activity of RhoA in different cell types (Lang et al., 1996) and the inhibition of RhoA and of its effector, Rho-kinase is required for cAMP-induced outgrowth of dendrites in B16 cells (Busca et al., 1998). The effects shown here mediated by DCC may be a consequence of a coordinated activation of Rac1 leading to actin polymerization at the advancing edge of the growth cone and inactivation of RhoA through the maintenance of the intracellular levels of cAMP in neurons.

The cytoplasmic domain of DCC did not interact physically with Rac1 (data not shown), suggesting an indirect link between DCC and Rac1. A candidate protein that may link DCC to activation of Rac1 is the UNC-73 ortholog Trio, a GEF with activity toward both Rac1 and RhoA (Bellanger, 1998), found to play a major role in axonal development and pathfinding (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000; Steven, 1998). The cytoplasmic tail of DCC contains several putative SH3 binding motifs PXXP (Yu, 1994) which may interact with the two SH3 domains of Trio or to an SH3-containing adaptor molecule.

Before its discovery as an axon guidance receptor in the development of the nervous system, DCC was identified as a tumor suppressor gene in colorectal cancer and appears to activate signaling pathways affecting both cell proliferation and differentiation (Fearon, 1996; Fearon et al., 1990; Meyerhardt et al., 1999). Consistent with Rho proteins as potential oncogenes playing important roles in the development of cell transformation

and metastasis (Bar-Sagi, 2000), the identification of Rho GTPase activities in DCC-induced signaling pathways now provides new insight into unraveling the molecular mechanisms underlying the tumor suppressor function of DCC.

PREFACE TO CHAPTER 3

The study presented in Chapter 2 suggests that netrin-1 receptor DCC activate Rac1 but not RhoA or Cdc42. However, the cytoplasmic domain of DCC did not interact physically with Rac1 (Fig. 3a), suggesting an indirect link between DCC and Rac1. Therefore, the second objective of this thesis was to identify the linker protein in the DCC signaling pathways that link DCC to Rac1 activation. The cytoplasmic domain of DCC contains several putative SH3-binding motifs, PxxP, which may interact with an SH3-containing adaptor molecule. Nck is an adaptor protein composed of a single SH2 and three SH3 domains. When we tested the interaction of DCC with Nck-1, we found that DCC interact with Nck-1 independently of netrin-1. The third objective of this thesis was to characterize the function of Nck-1 in the DCC signaling pathways by both biochemical and functional assays.

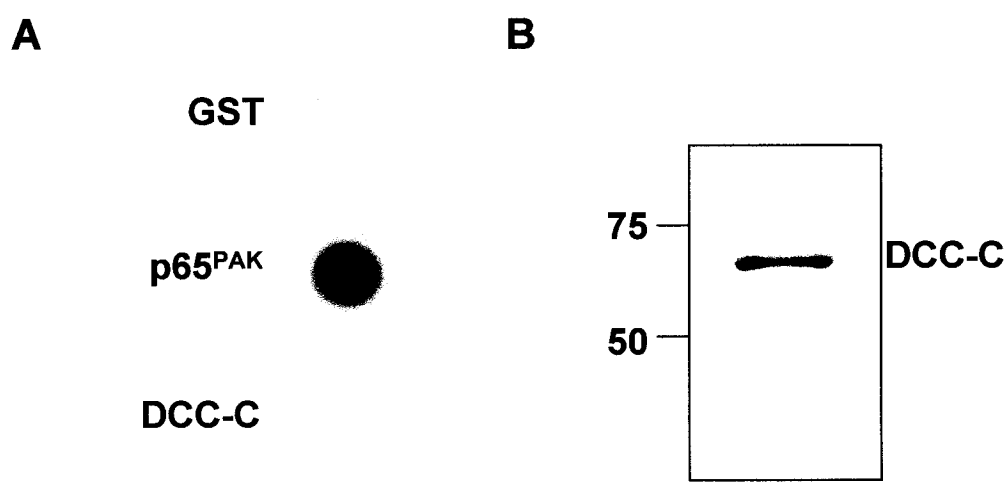
A
GST
p65PAK
DCC-C
B
75
50
DCC-C

Figure 3a. DCC does not interact directly with Rac1.

A, GST (5 μg), GST-$p65^{PAK}$ (5 μg), and GST-DCC-C (5 μg) were spotted onto a nitrocellulose membrane in a volume of 5 μl and incubated with 0.1 μg of [γ^{32}P]-GTP-loaded RacL61. GTPase interactions were visualized by autoradiography. B, GST-DCC-C was submitted to 7.5% SDS-PAGE, and DCC was detected by Coomassie blue staining.

CHAPTER 3

THE ADAPTOR PROTEIN NCK-1 COUPLES THE NETRIN-1 RECEPTOR DCC (DELETED IN COLORECTAL CANCER) TO THE ACTIVATION OF THE SMALL GTPASE RAC1 THROUGH AN ATYPICAL MECHANISM

The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism

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Running title: Nck-1 binds to the netrin-1 receptor DCC

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ABSTRACT

Netrins are a family of secreted proteins that guide the migration of cells and axonal growth cones during development. DCC (deleted in colorectal cancer) is a receptor for netrin-1 implicated in mediating these responses. Here, we show that DCC interacts constitutively with the SH3/SH2 adaptor Nck in commissural neurons. This interaction is direct and requires the SH3 but not SH2 domains of Nck-1. Moreover, both DCC and Nck-1 associate with the actin cytoskeleton and this association is mediated by DCC. A dominant negative Nck-1 inhibits the ability of DCC to induce neurite outgrowth in N1E-115 cells and to activate Rac1 in fibroblasts in response to netrin-1. These studies provide evidence for an important role of mammalian Nck-1 in a novel signaling pathway from an extracellular guidance cue to changes in the actin-based cytoskeleton responsible for axonal guidance.

INTRODUCTION

In the developing nervous system, newborn neurons extend their axons to their targets in response to attractive and repulsive cues. The neuronal growth cone, located at the tip of the growing axon, is a highly motile structure that can be viewed as a sophisticated signal transduction device, capable of recognizing extracellular guidance cues and translating them into directed neurite outgrowth (Mueller, 1999; Tessier-Lavigne and Goodman, 1996). Over the past years, a combination of cellular and genetic studies has led to the identification of several proteins playing a critical role in guiding axons along their pathways, including members of the ephrin, Semaphorin, netrin, Slit, Reelin, and NGF families of proteins (Song and Poo, 2001). Several classes of transmembrane proteins have also been identified and characterized as their respective binding receptors (Patel and Van Vactor, 2002). For instance, two classes of receptors, DCC and the UNC-5 proteins, have been described for the netrins. Netrins are bifunctional molecules attracting and repelling different classes of axons. The DCC family of receptors mediates growth cone attraction by netrins (Chan et al., 1996; Fazeli et al., 1997; Keino-Masu et al., 1996; Kolodziej et al., 1996), whereas the UNC-5 proteins are required for the repulsive effect of the netrins (Ackerman et al., 1997; Leonardo et al., 1997; Leung-Hagesteijn et al., 1992). However, the pathway is more complicated since UNC-5-mediated repulsion requires the function of DCC proteins in some cases (Hedgecock et al., 1990; Hong et al., 1999; Merz et al., 2001).

We recently determined that the small GTPases Rac1, Cdc42, and RhoA play a key role in the cytosolic signaling events induced by the netrin-1 receptor DCC (Li et al., 2002b; Shekarabi and Kennedy, 2002). Several putative Src homology 3 (SH3) binding motifs PXXP (Yu, 1994) are found in the cytoplasmic tail of DCC, suggesting a possible interaction with an SH3-containing adaptor molecule to mediate netrin-1 signaling to Rho GTPases. A role for the fly protein Dock in axon guidance has been well characterized during *D. melanogaster* eye and nervous system development (Desai et al., 1999; Garrity et al., 1996; Hummel et al., 2000; Schmucker et al., 2000). Nck, the mammalian homolog of Dock, is a ubiquitously expressed protein composed of a single SH2 and three SH3 domains and is represented by two genes (Li et al., 2001; Li and She, 2000).

In this paper, we provide biochemical and functional evidence for the involvement of Nck in mediating the netrin-1 receptor DCC signaling. We demonstrate that DCC associates with Nck in embryonic spinal commissural neurons. The interaction of DCC with Nck is independent of netrin-1 and involves the direct binding of DCC to two SH3 domains of Nck-1. Furthermore, DCC associates with the actin cytoskeleton and is responsible for the relocalization of Nck-1 to the Triton-insoluble fraction. In N1E-115 neuroblastoma cells, a dominant negative Nck-1 inhibits the ability of DCC to induce neurite outgrowth. In fibroblasts, dominant negative Nck-1 blocks the activation of Rac1 by DCC in the presence of netrin-1. Thus, we propose that Nck-1 is constitutively associated with DCC through two SH3 domains. In the presence of netrin-1, Nck-1 bound to DCC is now able to interact with downstream effectors, via the SH2 domain, to mediate changes in the actin-based cytoskeleton through activation of Rac1.

EXPERIMENTAL PROCEDURES

Plasmids

pRK5 encoding full length DCC (pRK5-DCC) was as described previously (Li et al., 2002b). pGEX4T-2 encoding the carboxyl terminus of DCC (425 amino acids) comprising the cytoplasmic, transmembrane domain and 61 amino acids of the extracellular domain was produced by PCR amplification of pRK5-DCC-C (Li et al., 2002b) followed by ligation of the PCR product into pGEX4T-2 digested with *Eco*RI and *Xho*I. pcDNAmyc encoding Nck-1 or the various mutant proteins and GST fusion proteins of Nck-1 and mutant proteins were as described previously (Kebache et al., 2002). GST fusion proteins encoding the C-terminal SH3 of Grb2 (aa 159-217), the SH3 domains of Src, intersectin, and the PRD of CdGAP were described elsewhere (Jenna et al., 2002; Yamabhai et al., 1998). DNA was purified using Qiagen kit. For microinjection studies, purified plasmids were filtered through a 0.2μm cellulose acetate membrane (Corning) before microinjection into cells.

Preparation of Total Cell Lysates from New-born Rat Brains and Commissural Neurons

Whole brains were extracted from new-born rats and incubated in 10ml (per brain) of a buffer containing 20mM Hepes pH 7.2, 1% Triton X-100, 10% glycerol, 1mg/ml BSA, 100mM NaCl, 20mM sodium fluoride, 1mM Na_3VO_4, 10μg/ml aprotinin, 10μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Brain tissues were homogenized using a Dounce homogenizer and incubated for 30 min on ice. Lysates were cleared by a 10 min centrifugation at 14000 rpm and supernatants were passed several times through 25-gauge needles. The supernatants were then subjected to immunoprecipitation and immunoblotting. Commissural neurons were obtained by dissecting E13 rat embryo dorsal spinal cords as described previously (Shekarabi and Kennedy, 2002). Briefly, 5 X 10^3 cells were plated onto 13mm coverslips coated with poly-D-Lysine and laminin-1 or on laminin-1-coated 60 mm dishes, and were maintained for 48 hours after dissection in Neurobasal medium (Invitrogen) containing 10 % iFBS. After treatment or not for 10min at 37°C with 160 ng/ml netrin-1, commissural neurons plated on coverslips were

subjected to immunofluorescence whereas 60mm plated cells were lysed and subjected to imunoprecipitation and immunoblotting.

Cell Culture and Microinjection

Mouse fibroblast Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics and maintained in an atmosphere of 10% CO_2. Confluent serum-starved Swiss 3T3 cells were prepared as described (Ridley and Hall, 1992). Briefly, cells were plated in 10% serum at a density of 6×10^4 onto acid-washed coverslips. 7-10 days later, the cells became quiescent and were subjected to serum starvation for 16 h in DMEM containing 2 g/l NaHCO3. The eukaryotic expression vector pRK5 encoding full-length DCC was microinjected alone or with GST, GST-Nck-$(SH3_1/SH3_2/SH3_3)^{1-251}$, GST-Grb2-SH3 into the nucleus of ~100 cells over a period of 20 min in CO2-independent medium (Gibco) using an Eppendorf microinjection system 5246. During microinjection, cells were maintained at 37°C within a humidified atmosphere. Cells were returned to the incubator for a further 5 hours before the addition of purified netrin-1 at 500 ng/ml for 10 min.

Mammalian Cell Transfection

N1E-115 neuroblastoma cells and COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics at 10% CO_2. N1E-115 cells were plated onto coverslips previously coated with laminin (20 μg/ml; VWR Canlab) for 24 h at 37°C, washed twice with water, and left to air dry. Transfection was carried out with Lipofectamine transfection reagent (Gibco) according to the manufacturer's protocol and as previously described (Li et al., 2002b). 0.4 μg of pRK5, pRK5-DCC or with either 0.4μg of pcDNAmyc-Nck-1, -Nck(R308K)$^{1-377}$, -Nck(W38,143,229R)$^{1-377}$, or -Nck-$(SH3_1/SH3_2/SH3_3)^{1-251}$ were used. COS-7 Cells were transfected using the DEAE-dextran method as described previously (Olson et al., 1995). The amount of plasmids used per 100mm dish was as follows: pRK5, 5 μg; pRK5-DCC, 2.5 μg; pcDNAmyc-Nck-1, 2.5 μg. Twenty-four hours after transfection, cells were either or not serum-starved overnight and treated with netrin-1 at 500 ng/ml for different period of time.

Immunofluorescence Microscopy

At the indicated times, microinjected Swiss 3T3 cells or transfected N1E-115 cells were rinsed with PBS and fixed for 10 min in freshly prepared 4% (w/v) paraformaldehyde. All steps were carried out at room temperature, and coverslips were rinsed in PBS between each of the steps. Cells were permeabilized in 0.2% Triton X-100 for 5 min, free aldehyde groups were reduced with 0.5 mg/ml sodium borohydride for 10 min. Cells were double-labelled following the procedure previously described (Li et al., 2002b). Briefly, cells were incubated with monoclonal anti-DCC antibodies (Pharmingen, G97-449), polyclonal anti-Nck antibodies (Upstate Biotechnology, #06-288) or anti-vinculin (Sigma) diluted in PBS for 60 min. Then, coverslips were transferred to a secondary antibody mixture composed of fluorescein isothiocyanate-conjugated goat anti-mouse (Sigma), or goat anti-rabbit (Sigma) and tetramethyrhodamine isothiocyanate-conjugated phalloidin (Sigma) for 60 min. For vinculin staining, cells were incubated with a tertiary fluorescein isothiocyanate-conjugated donkey anti-goat antibodies for an additional 30 min. Coverslips were mounted by inverting them onto 8 µl of mowiol mountant containing p-phenylenediamine as an anti-bleach reagent. After 2 hrs at room temperature, the coverslips were examined on a Zeiss Axiovert 135 microscope using Zeiss oil immersion 63X objective lens. Fluorescence images were recorded using a digital camera (DVC) and analyzed with Northern Eclipse software (Empix Imaging Inc.). In N1E-115 cells, a neurite was defined as a process that measured at least the length of a cell body.

Dissected E13 rat dorsal commissural neurons, treated or not for 10 min at 37°C with 160 ng/ml netrin-1, were rapidly rinsed with PBS, fixed in 4% paraformaldehyde for 10 min and then permeabilized with 0.25%Triton X-100 in PBS for 5 min at room temperature. Blocking was performed during 1 hour in 5% heat-inactivated normal goat serum. Coverslips were then incubated overnight at 4°C with monoclonal anti-DCC antibody (2µg/ml) and polyclonal anti-Nck (2µg/ml, Upstate biotechnology). Cells were then gently washed with PBS and incubated with tetramethyrhodamine isothiocyanate-conjugated goat anti-mouse and fluorescein isothiocyanate–conjugated goat anti-rabbit (Sigma) antibodies for 1 hour at room temperature. Coverslips were mounted as described

above. Coverslips were analyzed using a Zeiss LSM410 invert laser scanning confocal microscope and images were captured with a 63X objective lens. Acquired images were analyzed using Zeiss LSM software.

Immunoprecipitation

New-born rat brains, dissociated commissural neurons treated or not with 160ng/ml netrin-1, or COS-7 cells transfected with pcDNAmyc-Nck-1 alone or together with pRK5-DCC were lysed in buffer containing 20 mM Hepes pH 7.2, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mg/ml BSA, 20 mM sodium fluoride, 1mM Phenymethysulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. 1 mg of protein lysates were precleared with 20 μl of protein G-Sepharose beads (Pharmacia) at 4°C overnight. The supernatants were incubated with normal mouse IgGs (Pierce) or with monoclonal anti-Nck (Upstate Biotechnology, #05-160) antibody at 4°C for 2 hours and with 10 μl of protein G-Sepharose beads for an additional hour. Beads were washed three times in lysis buffer and boiled in SDS-sample buffer. Proteins were separated on 7.5% SDS-PAGE and revealed by immunoblotting using anti-DCC and polyclonal anti-Nck antibodies (Lussier and Larose, 1997) and the ECL reagent detection kit (NEN). The level of immunoprecipitated DCC and Nck proteins obtained from commissural cell lysates was determined by densitometry (Biorad imaging densitometer GS-700).

Purification of GST Fusion Proteins

E. coli transformed with various GST fusion proteins were grown at 37°C for one hour. Expression of the fusion proteins was induced by isopropyl-β-D-thiogalactopyranoside (1mM) for 3 hours at 37°C, except for GST-$p65^{PAK}$ that was induced at room temperature. Cells were sonicated in buffer A (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The lysates were cleared by centrifugation and Triton X-100 was added to a final concentration of 1% followed by incubation with glutathione-Sepharose beads (Pharmacia) for 1h at 4°C. Beads were washed three times with buffer B (50 mM Tris-HCl pH7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1mM phenylmethylsulfonyl fluoride). GST fusion proteins were eluted in buffer D (50 mM Tris pH 8, 150 mM NaCl, 5 mM $MgCl_2$,

1mM DTT, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 5 mM glutathione (Sigma).

Dot Blot Assay

The interaction of the cytoplasmic tail of DCC expressed as a GST fusion protein (DCC-C) and Nck-1 was assessed using a dot blot assay. In brief, GST (5 or 50μg), GST-$p65^{PAK}$ (5μg), GST-CdGAP-PRD (5μg), and GST-DCC-C (5μg) were spotted onto nitrocellulose membranes. The filter was air dried and incubated with blocking buffer (TBS containing 5% milk and 0.05% tween 20) for 2 hours at room temperature. The cDNA encoding Nck-1 has been subcloned into pGEX-2TK (Amersham Pharmacia) containing a protein kinase site located between the GST domain and the multi cloning site that allows *in vitro* phosphorylation of GST-Nck-1. According to the manufacturer's protocol, GST-Nck-1 coupled to glutathione-Sepharose beads was incubated for 30 min at 4°C in kinase buffer containing 20mM Tris-HCl pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, 10μl [(^{32}P]-ATP (10mCi/ml), and 10μl protein kinase catalytic subunit from bovine heart (50μg/ml) (Sigma, #P2645). The reaction was stopped by adding 10 mM sodium phosphate pH 8, 10 mM EDTA, 10 mM sodium pyrophosphate, and 1 mg/ml BSA. Beads were washed 5 times with PBS and radiolabeled GST-Nck-1 was eluted with 50 mM Tris-HCl pH 8 and 10 mM glutathione. The membrane was incubated overnight at 4°C with blocking buffer containing 10^6cpm of radiolabeled GST-Nck-1. The membrane was then washed with TBS containing 0.05% tween 20. Nck-1 binding to the different GST fusion proteins was visualized by autoradiography.

GST Pull-down Assay

COS-7 cells transfected with pRK5-DCC were lysed in buffer A (10 mM Hepes pH 7.4, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). 1 mg of protein lysate was precleared with 20 μl of glutathione-Sepharose beads (Pharmacia) at 4°C overnight. The supernatant was incubated with 20μg GST fusion proteins and 10 μl of glutathione-Sepharose beads at 4°C for 2 hrs. Beads were washed three times with buffer A and boiled in SDS-sample buffer. Proteins were separated on 10% SDS-PAGE and DCC was revealed by immunoblotting using anti-DCC

antibody and ECL reagent detection kit. The amount of GST fusion proteins was detected by Ponceau S (Sigma) staining.

Detergent Extraction Assay

COS-7 cells transfected with pRK5, pRK5myc-Rac1, pRK5-DCC, pcDNAmyc-Nck-1 or co-transfected with pRK5-DCC and pcDNAmyc-Nck-1 were serum-starved overnight and treated with netrin-1 at 500 ng/ml for different periods of time. Cells were washed 3 times with cold PBS followed by incubation on ice for 10 minutes with gentle shaking in 200 µl extraction buffer (15 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 150 mM NaCl, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM Phenylmethylsulfonyl fluoride containing 0.1 to 1% Triton X-100). The extracted material (soluble fraction) was collected and 100 µl of SDS-sample buffer was added to each sample. Dishes were rinsed twice with cold PBS and the insoluble fraction was collected in 300 µl SDS-sample buffer. The proteins were separated by 7.5% SDS-PAGE. DCC, Rac1 and Nck-1 were revealed by immunoblotting using anti-DCC, monoclonal anti-myc (Li et al., 2002b) or polyclonal anti-Nck antibodies and the ECL reagent detection kit. The level of DCC and Nck-1 proteins in the soluble and insoluble fractions was determined by densitometry.

For immunofluorescence detection of DCC associated with the actin cytoskeleton after detergent extractions, Swiss 3T3 cells microinjected with pRK5-DCC and treated with netrin-1 for 30 min, were incubated with cytoskeleton (CSK) buffer (10 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, and 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1mM phenylmethylsulfonyl fluoride) for 10 min on ice, rinsed with PBS, and fixed with 4% paraformaldehyde for 10 min followed by immunofluorescence detection as described.

RESULTS

The netrin-1 receptor DCC interacts with Nck in embryonic spinal commissural neurons independently of netrin-1.

To determine whether Nck plays a role in the netrin-1 receptor DCC signaling, we investigated if endogenous Nck and DCC could be co-precipitated from rat day 1 brain lysate. In this experiment, DCC was found to co-precipitate with Nck in rat whole brain protein lysates with no band corresponding to DCC observed in immunoprecipitates using normal mouse IgGs (Fig. 3.1A). To assess whether the interaction between endogenous Nck and DCC is similar in neurons that respond to netrin-1, we immunoprecipitated Nck from dissociated commissural neurons obtained from the dorsal half of micro-dissected embryonic day 13 rat spinal cords. After two days in culture, cells were incubated with or without 160ng/ml netrin-1 for 10 min prior to cell lysis. DCC was found to co-precipitate with Nck both in the absence or in the presence of netrin-1 and DCC was not significantly detected in the negative control using normal mouse IgGs (Fig. 3.1B). Following netrin-1 treatment, the higher amount of DCC proteins co-precipitated with Nck correlated with the increased amount of immunoprecipitated Nck (Fig. 3.1D). Time-course incubation with netrin-1 from 5 to 20 min did not show any increase in the level of DCC interacting with Nck (data not shown). Furthermore, when DCC was co-expressed with Nck-1 in COS-7 cells that do not express endogenous DCC or netrin-1 (data not shown), DCC also co-immunoprecipitated with Nck-1 in the absence of netrin-1 (Fig. 3.1C).

As previously found (Shekarabi and Kennedy, 2002), DCC was observed in the cell bodies, along the axons of the commissural neurons, and as a punctate staining enriched in the growth cones and at the tip of the filopodia (Fig. 3.2A,B). Nck was also highly enriched in the cell bodies and along the axons. Similar to the distribution of DCC, Nck was present in the filopodia and lamellipodia of the growth cones (Fig. 3.2A,B). Confocal microscopy analysis revealed that DCC and Nck highly colocalized in the cell bodies and along the axons both in the absence (Fig. 3.2A) or in the presence of netrin-1 (Fig. 3.2B). They were also found to be associated at discrete sites at the tip of the filopodia in the growth cones (Fig. 3.2A,B). Hence, DCC interacts with Nck *in vivo* and this interaction is independent of netrin-1 in embryonic commissural neurons.

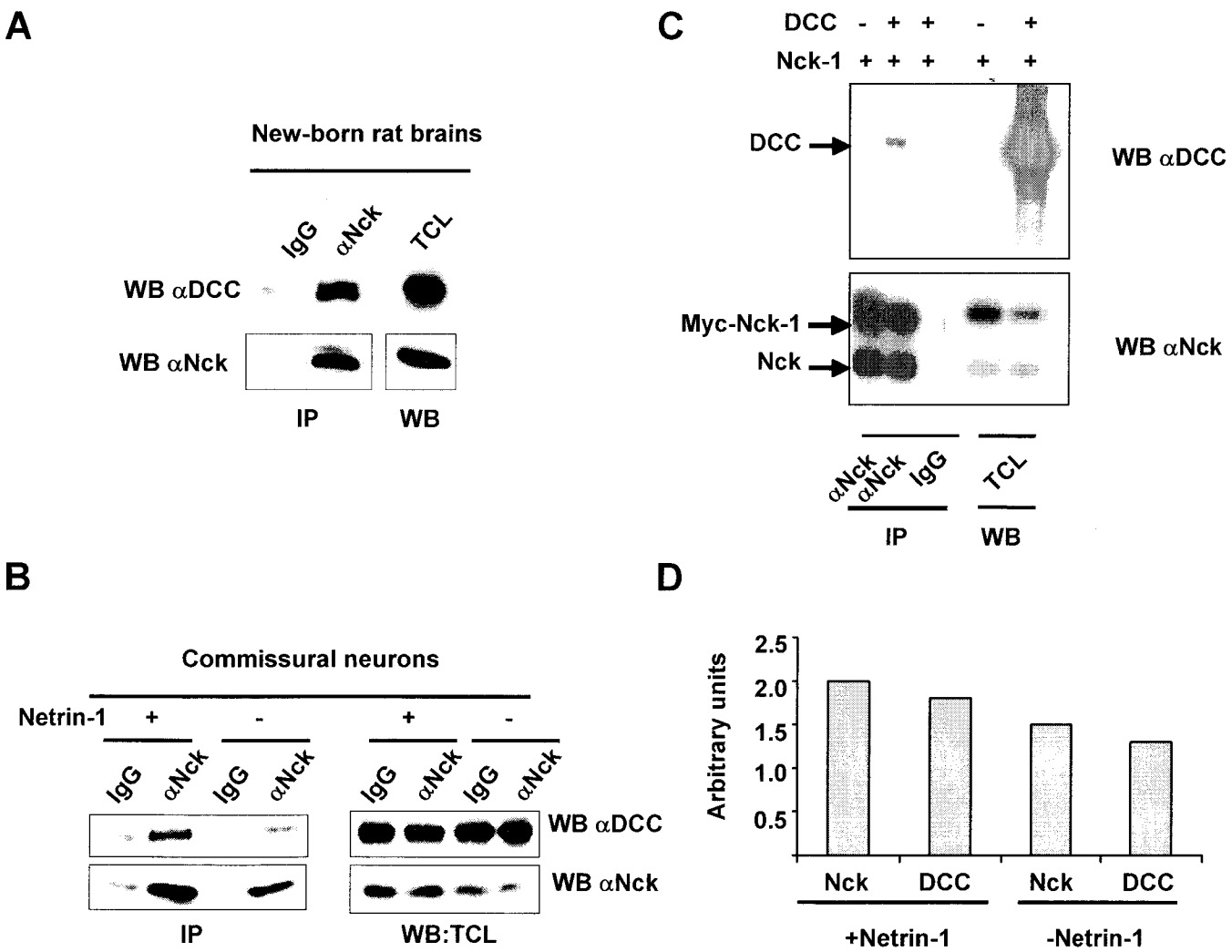

A
New-born rat brains
IgG
αNck
TCL
WB αDCC
WB αNck
IP
WB
B
Commissural neurons
Netrin-1
+
-
+
-
IgG
αNck
IgG
αNck
IgG
αNck
IgG
αNck
WB αDCC
WB αNck
IP
WB:TCL
C
DCC - + + - +
Nck-1 + + + + +
DCC
WB αDCC
Myc-Nck-1
Nck
WB αNck
αNck
αNck
IgG
TCL
IP
WB
D
Arbitrary units
2.5
2.0
1.5
1.0
0.5
Nck
DCC
Nck
DCC
+Netrin-1
-Netrin-1

Figure 3.1. DCC associates with Nck in commissural neurons from E13 rat spinal cord.

Lysates of newborn rat brains (A), or commissural neurons incubated with or without netrin-1 for 10 minutes at 160ng/ml (B), or COS-7 cells transfected with pcDNA-myc-Nck-1 alone or together with pRK5-DCC (C) were submitted to immunoprecipitation using monoclonal anti-Nck antibodies (IP αNck) or with mouse immunoglobulin G coupled to protein G-Sepharose beads (IP IgG). Total cell lysates (TCL) and immunoprecipitated proteins (IP) were submitted to SDS-PAGE and DCC and Nck were detected by western blotting using monoclonal anti-DCC (WB αDCC) and polyclonal anti-Nck (WB αNck) antibodies, respectively. (D) The level of DCC and Nck proteins co-immunoprecipitated from commissural cell lysates in (B) was quantified by densitometry and is expressed as arbitrary units. Values are representative of at least three independent experiments.

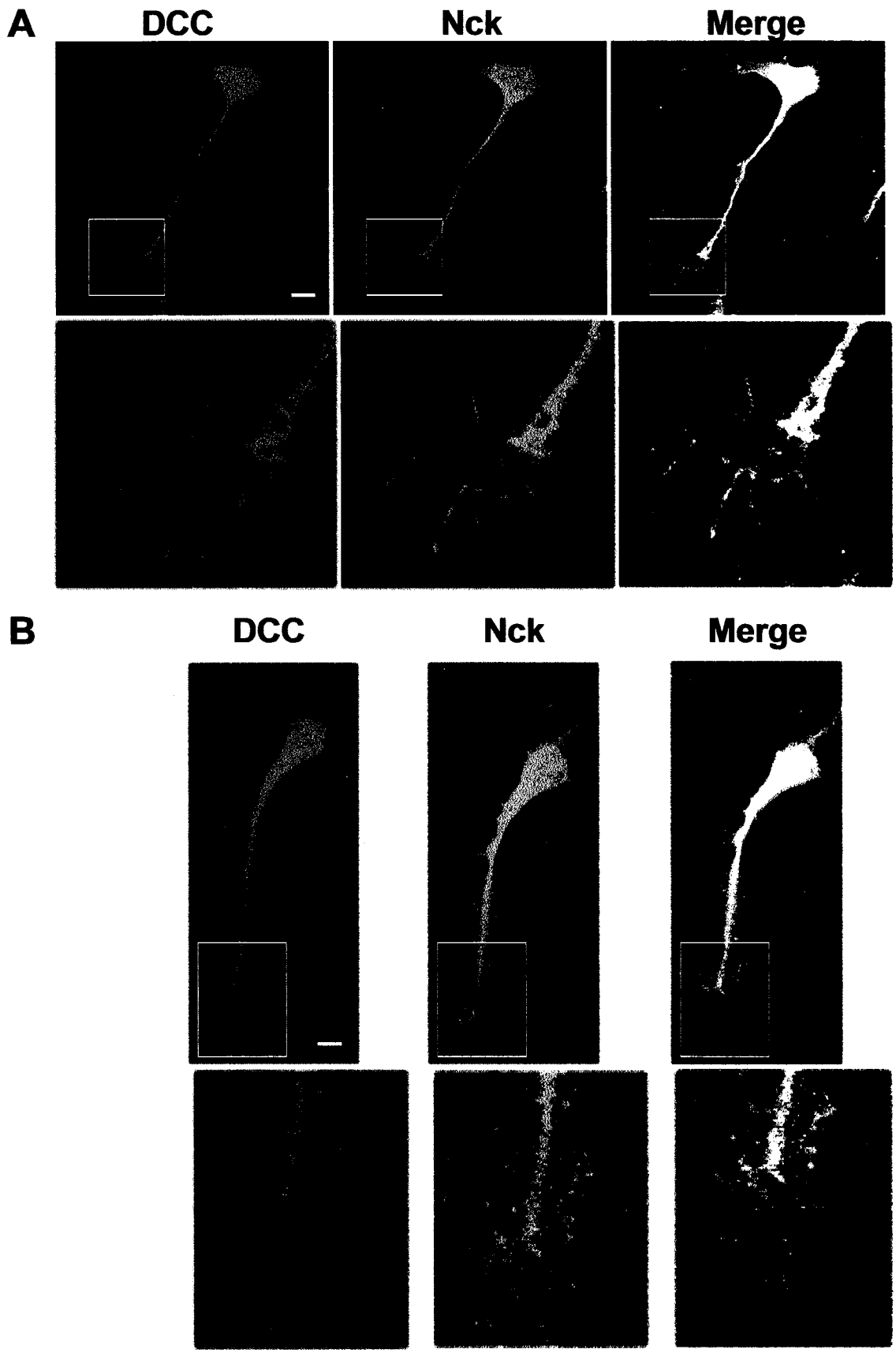

A
DCC
Nck
Merge
B
DCC
Nck
Merge

Figure 3.2. DCC and Nck colocalize in the cell bodies, along the axons, and in growth cones of commissural neurons.

DCC and Nck were visualized in commissural neurons treated with (B) or without netrin-1 (A) by co-staining with monoclonal anti-DCC and polyclonal anti-Nck antibodies, respectively, and by indirect immunofluorescence using a Zeiss 410 confocal microscope (upper panels). Lower panels represent enlargement of growth cones. Picture merge was obtained using Zeiss LSM software. Scale bar, 10 μm.

Nck-1 binds directly to the cytoplasmic tail of DCC through the first and third SH3 domains.

To characterize the interaction of DCC with Nck, GST pull-down experiments were performed using lysates of COS-7 cells expressing DCC incubated with wild-type or mutated Nck-1 expressed as GST fusion proteins (Table 3.1). As shown in Fig. 3.3A, wild-type Nck-1 or the three SH3 domains of Nck-1 showed binding to DCC, whereas the Nck-1 SH2 domain showed no interaction. To dissect the interaction of DCC with the SH3 domains of Nck-1, each of the SH3 domains of Nck-1 were expressed as GST fusion proteins and incubated with lysates of DCC-expressing cells. The third SH3 domain of Nck-1 was found to interact with DCC. In addition, the first SH3 domain of Nck-1 also bound to DCC but to a lower extent. No significant binding was observed with the second SH3 domain (Fig. 3.3A). Full length Nck-1 containing amino acid substitutions W38R, W143R, and W229R in each of the SH3 domains predicted to inhibit interactions with proline-containing proteins (Pawson and Gish, 1992; Tanaka et al., 1995) did not interact with DCC (Fig. 3.3A). However, Nck-1 protein mutants containing these amino acid substitutions in individual SH3 domains were still able to interact with DCC (Fig. 3.3B). These results suggest that the first and third SH3 domains of Nck-1 are sufficient to mediate the interaction with DCC.

To assess the specificity of the interaction between DCC and Nck-1, we incubated lysates of DCC-expressing cells with the SH3 domains of Grb-2, Src and the endocytic protein intersectin. As shown in Fig. 3.3C, no binding was observed with the C-terminal SH3 domain of Grb2, the SH3 domain of Src, or the SH3A and D domains of intersectin, suggesting a specific interaction between Nck-1 and DCC. To determine whether Nck-1 interacts directly with the cytoplasmic tail of DCC, we have expressed a GST fusion protein with the complete intracellular domain of DCC (DCC-C) in *E.coli*. Direct binding between DCC-C and Nck-1 was assessed in a dot blot assay using purified Nck-1 expressed as a GST fusion protein and phosphorylated *in vitro* with [γ^{32}P]-ATP (Fig. 3.3D). Nck-1 was able to bind directly to the cytoplasmic domain of DCC and to the Serine/Threonine kinase $p65^{PAK}$ known to interact with the second SH3 domain of Nck-1 (Bokoch et al., 1996; Galisteo et al., 1996). No binding was observed with the proline-

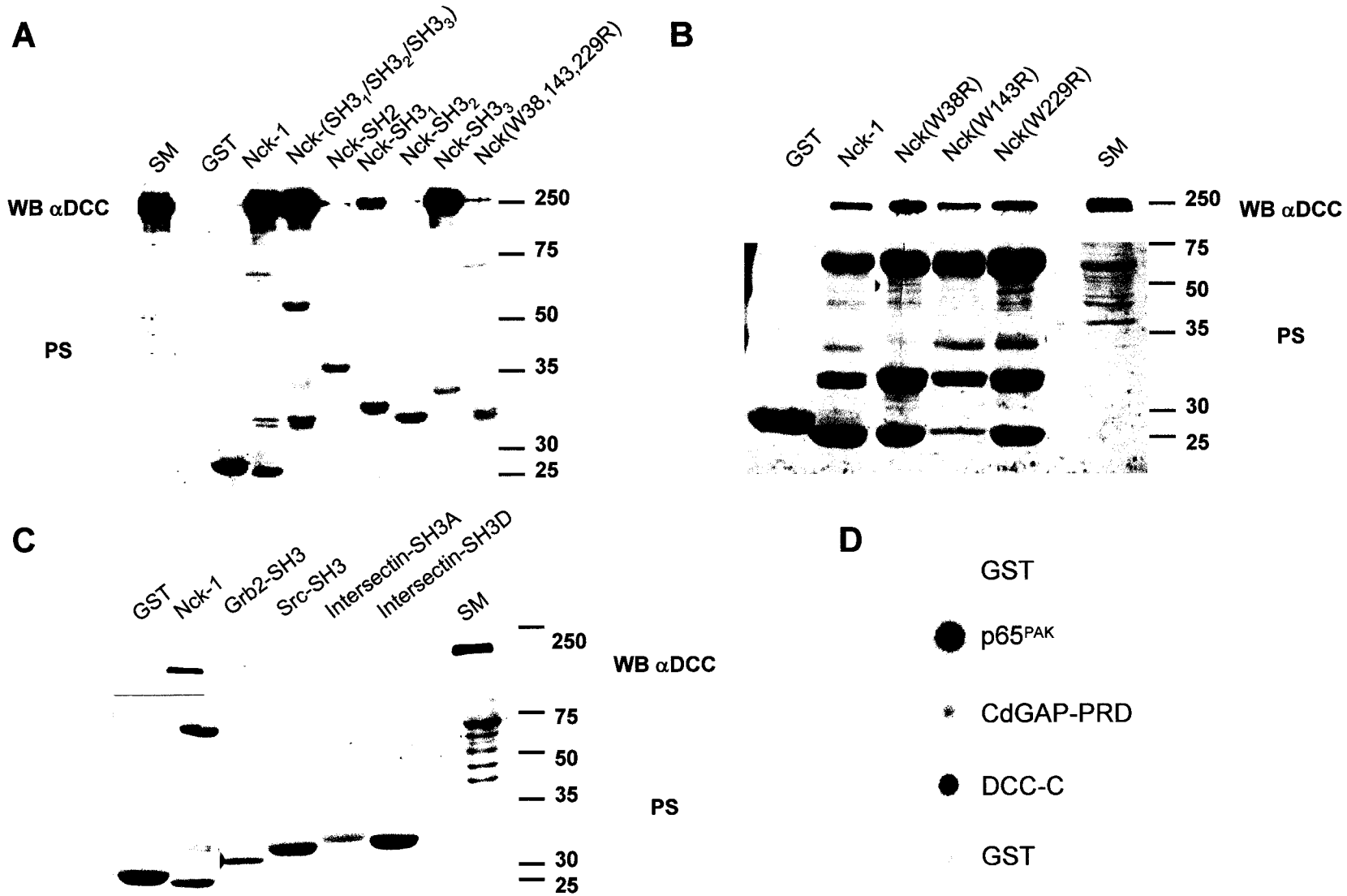

A
SM
GST
Nck-1
Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
Nck-SH2
Nck-SH3$_1$
Nck-SH3$_2$
Nck-SH3$_3$
Nck(W38,143,229R)
WB αDCC
PS
250
75
50
35
30
25
B
GST
Nck-1
Nck(W38R)
Nck(W143R)
Nck(W229R)
SM
WB αDCC
PS
250
75
50
35
30
25
C
GST
Nck-1
Grb2-SH3
Src-SH3
Intersectin-SH3A
Intersectin-SH3D
SM
WB αDCC
PS
250
75
50
35
30
25
D
GST
p65PAK
CdGAP-PRD
DCC-C
GST

Figure 3.3. DCC interacts directly with Nck-1.

COS-7 cells transfected with pRK5-DCC were lysed and 1 mg of protein lysates were incubated with either 20 μg of GST or GST fusion proteins of various Nck-1 mutant proteins (A, B and C) or proteins containing the SH3 domains of Grb2, Src, and intersectin (C) coupled to glutathione-Sepharose beads. 40 μg of starting material (SM) and Nck-1-associated proteins were submitted to SDS-PAGE and DCC was detected by western blotting analysis using anti-DCC antibodies (WB αDCC). Ponceau S (PS) staining shows the level of GST fusion proteins in each sample. (D), GST (5 μg), GST-$p65^{PAK}$ (5 μg), GST-CdGAP-PRD (5 μg), GST-DCC-C (5 μg), and GST (50 μg) were spotted onto a nitrocellulose membrane in a volume of 5 μl and incubated with *in vitro* phosphorylated GST-Nck-1 by bovine heart protein kinase. Nck-1 interactions were visualized by autoradiography.

rich domain of CdGAP or with high amount of GST proteins. Therefore, Nck-1 interacts directly *in vitro* with the intracellular domain of DCC.

The SH2 domain of Nck-1 is required for DCC to induce neurite outgrowth in N1E-115 neuroblastoma cells.

Mouse N1E-115 neuroblastoma cells express netrin-1 but not DCC (Li et al., 2002b). When DCC is ectopically expressed in N1E-115 cells, the majority of DCC-expressing cells show neurite outgrowth in a netrin-1-dependent manner (Li et al., 2002b). To address the cellular function of Nck-1 in the context of netrin-1 mediated DCC signaling, we expressed full length Nck-1 or various Nck-1 mutant proteins alone or together with DCC into N1E-115 cells and measured the number of transfected cells with neurite outgrowth (Fig. 3.4A and Table 3.1). 60% of transfected cells with the expression vector pRK5 encoding DCC exhibited neurite outgrowth while Nck-1 induced neurite outgrowth in more than 50% of transfected cells. When DCC and Nck-1 were expressed together in N1E-115 cells (Fig. 3.4B), 80% of transfected cells now showed neurite outgrowth (Fig. 3.4A). When full length Nck-1 containing a R308K substitution to block the interaction between the SH2 domain of Nck-1 and phosphotyrosine-containing proteins (Pawson and Gish, 1992; Tanaka et al., 1995) was expressed in N1E-115 cells, it was unable to induce neurite outgrowth. This suggested that the SH2 domain is required for Nck-1 to induce neurite outgrowth in N1E-115 cells. Similarly, a deletion mutant protein of Nck-1 lacking the SH2 domain was unable to induce neurite outgrowth. When DCC was expressed in the presence of Nck-1 mutant proteins containing a R308K substitution or lacking the SH2 domain, the formation of neurite outgrowth was almost completely abolished. Alternatively, Nck-1 containing the W38R, W143R, and W229R substitutions in each of the SH3 domains was unable to induce neurite outgrowth but did not interfere in the formation of neurite outgrowth provoked by the expression of DCC in N1E-115 cells. Therefore, the SH3 and SH2 domains of Nck-1 are required for Nck-1 to induce neurite outgrowth in N1E-115 cells. Furthermore, Nck-1 lacking a functional SH2 domain acts as a dominant negative protein by interacting with DCC but is unable to mediate the downstream signaling pathways leading to induction of neurite outgrowth.

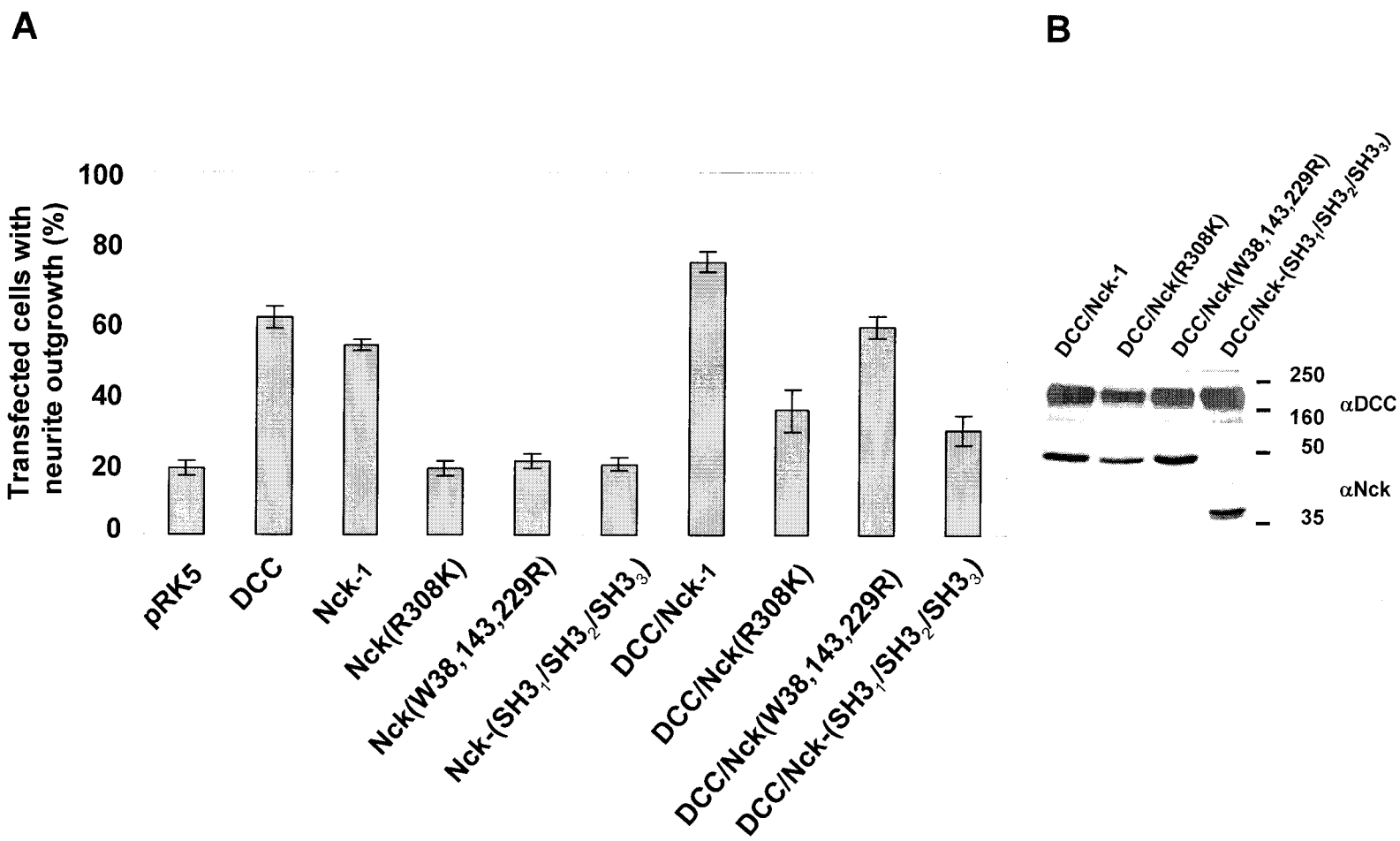

A
Transfected cells with neurite outgrowth (%)
100
80
60
40
20
0
pRK5
DCC
Nck-1
Nck(R308K)
Nck(W38,143,229R)
Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
DCC/Nck-1
DCC/Nck(R308K)
DCC/Nck(W38,143,229R)
DCC/Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
B
DCC/Nck-1
DCC/Nck(R308K)
DCC/Nck(W38,143,229R)
DCC/Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
250
160
50
35
αDCC
αNck

Figure 3.4. A dominant negative Nck-1 inhibits DCC-induced neurite outgrowth in N1E-115 cells.

(A) N1E-115 neuroblastoma cells were transfected either with pRK5, pRK5-DCC, pcDNAmyc-Nck$^{1-377}$, -Nck(R308K)$^{1-377}$, -Nck(W38,143,229R)$^{1-377}$, or -Nck-($SH3_1$/$SH3_2$/$SH3_3$)$^{1-251}$ alone, or with pRK5-DCC and pcDNAmyc encoding the various Nck-1 protein mutants. Values indicate the percentage of transfected cells with neurite extension, and correspond to the average of at least 3 independent experiments. (B) Total cell lysates were submitted to SDS-PAGE and level of DCC and wild type or mutant Nck-1 proteins were detected by western blotting using monoclonal anti-DCC and polyclonal anti-Nck antibodies, respectively.

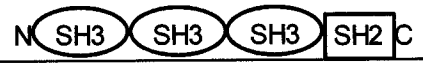
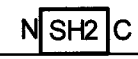
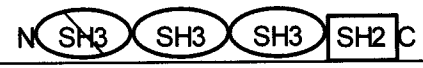
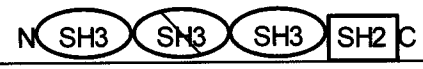
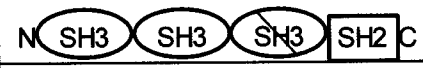
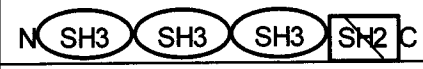
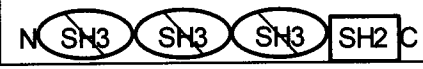
		DCC binding	Inhibition of neurite outgrowth
Nck^{1-377}		+	-
$Nck\text{-}SH2^{260-377}$		-	ND
$Nck\text{-}(SH3_1/SH3_2/SH3_3)^{1-251}$		+	+
$Nck\text{-}SH3_1^{1-65}$		+	ND
$Nck\text{-}SH3_2^{108-165}$		-	ND
$Nck\text{-}SH3_3^{188-266}$		+	ND
$Nck(W38R)^{1-377}$		+	ND
$Nck(W143R)^{1-377}$		+	ND
$Nck(W229R)^{1-377}$		+	ND
$Nck(R308K)^{1-377}$		ND	+
$Nck(W38,143,229R)^{1-377}$		-	-

Table 3.1. Summary of the binding of DCC to the various Nck-1 protein mutants and their effect on DCC-induced neurite outgrowth.

The numbers in superscript indicate amino acid residues of Nck-1. W38R, W143R, W229R, and R308K represent amino acid substitutions in the first, second, third SH3, and SH2 domains of Nck-1, respectively. ND, not determined.

Thus, the SH2 domain of Nck-1 plays an essential role in mediating neurite outgrowth induced by DCC.

Nck-1 couples the netrin-1 receptor DCC to activation of Rac1 in Swiss 3T3 fibroblasts.

Stimulation of DCC receptors with netrin-1 triggers actin reorganization through activation of Rac1 but not Cdc42 or RhoA in Swiss 3T3 fibroblasts (Li et al., 2002b). Expression of DCC stimulates the formation of lamellipodia and membrane ruffles after the addition of netrin-1 for 10 minutes in a Rac1-dependent effect. To determine whether Nck-1 mediates the activation of Rac1 by DCC, we co-injected the expression vector pRK5 encoding full-length DCC alone or together with dominant negative Nck-1 containing the three SH3 domains expressed as a GST fusion protein into quiescent, serum-starved Swiss 3T3 cells. As expected, in the presence of netrin-1, DCC induced the formation of lamellipodia and stress fibers (Fig. 3.5C). In contrast, the presence of dominant negative Nck-1 inhibited the effect of DCC on actin whereas the presence of GST proteins had no effect on the formation of lamellipodia in cells expressing DCC (Fig. 3.5E, G). To confirm that the inhibitory effect on Rac1 activation is specific to the SH3 domains of Nck-1, we co-injected DCC-encoding vector together with the SH3 domain of Grb2 expressed as a GST fusion protein into Swiss 3T3 fibroblasts. As shown in Fig. 3.5I, Grb2-SH3 previously shown not to bind to DCC (Fig. 3.3C) was unable to block the formation of lamellipodia induced by the netrin-1 receptor DCC. Therefore, we propose that the interaction of Nck-1 with DCC is required for Rac1 activation leading to actin reorganization in fibroblasts. These results also demonstrate that the SH2 domain of Nck-1 is necessary to mediate the activation of Rac1 by DCC in the presence of netrin-1.

DCC associates with the actin cytoskeleton and re-localizes Nck-1 to the Triton-insoluble fraction in a ligand-independent manner.

DCC expressed in Swiss 3T3 fibroblasts in the presence of netrin-1 was found to cluster within lamellipodia and co-localize with regions enriched in polymerized actin (Fig. 3.6C). COS-7 cells expressing DCC in the presence or absence of netrin-1 for 30 minutes were extracted using Triton X-100 and proteins present in the detergent-soluble

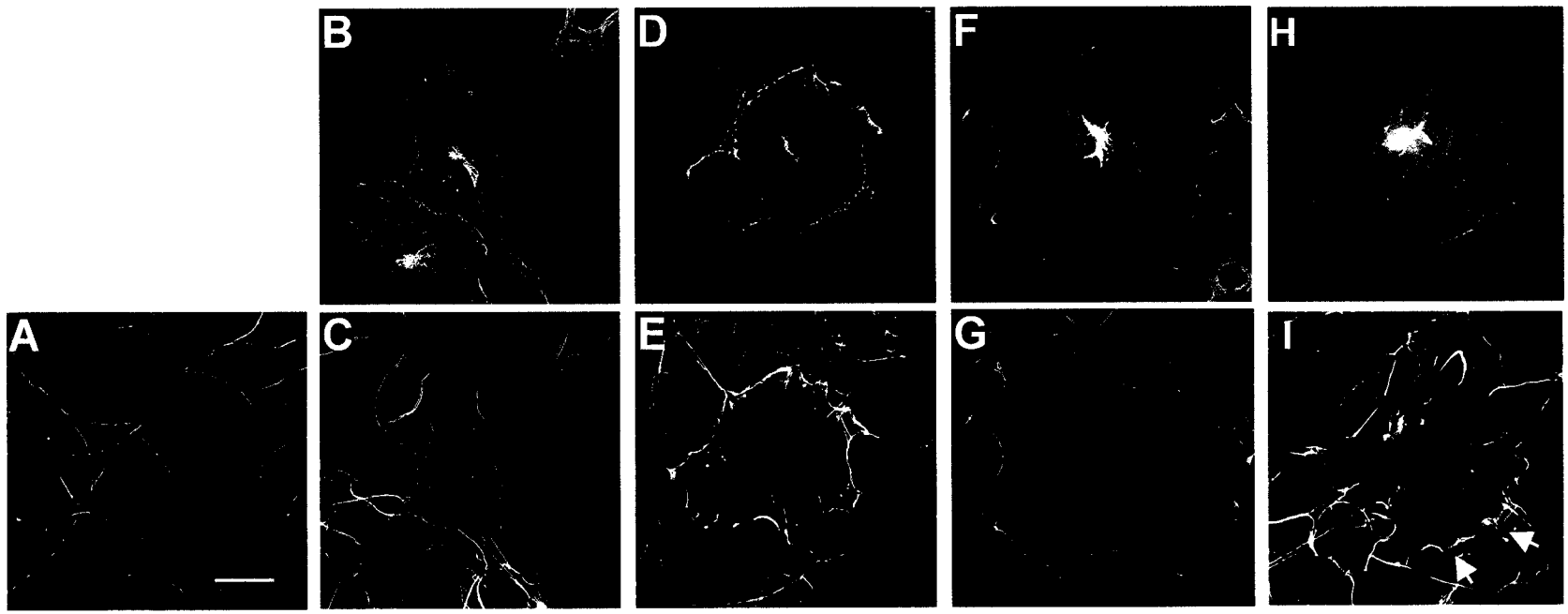

A
B
C
D
E
F
G
H
I

Figure 3.5. A dominant negative Nck-1 inhibits activation of Rac1 by the netrin-1 receptor DCC.

Serum-starved Swiss 3T3 cells were microinjected with pRK5-DCC (B and C) alone or with GST (D and E), GST-Nck-$(SH3_1/SH3_2/SH3_3)^{1-251}$ (F and G), or GST-Grb2-SH3 (H and I). 5 hours after microinjection, netrin-1 (500 ng/ml) was added to the media for 10 min. Panel A shows uninjected cells. DCC (B, D, F, and H) was revealed by staining with an anti-DCC antibody and by indirect immunofluorescence. F-actin (A, C, E, G, and I) was visualized with fluorescence-tagged phalloidin. Arrows in panel I indicate lamellipodia. Scale bar, 10 μm.

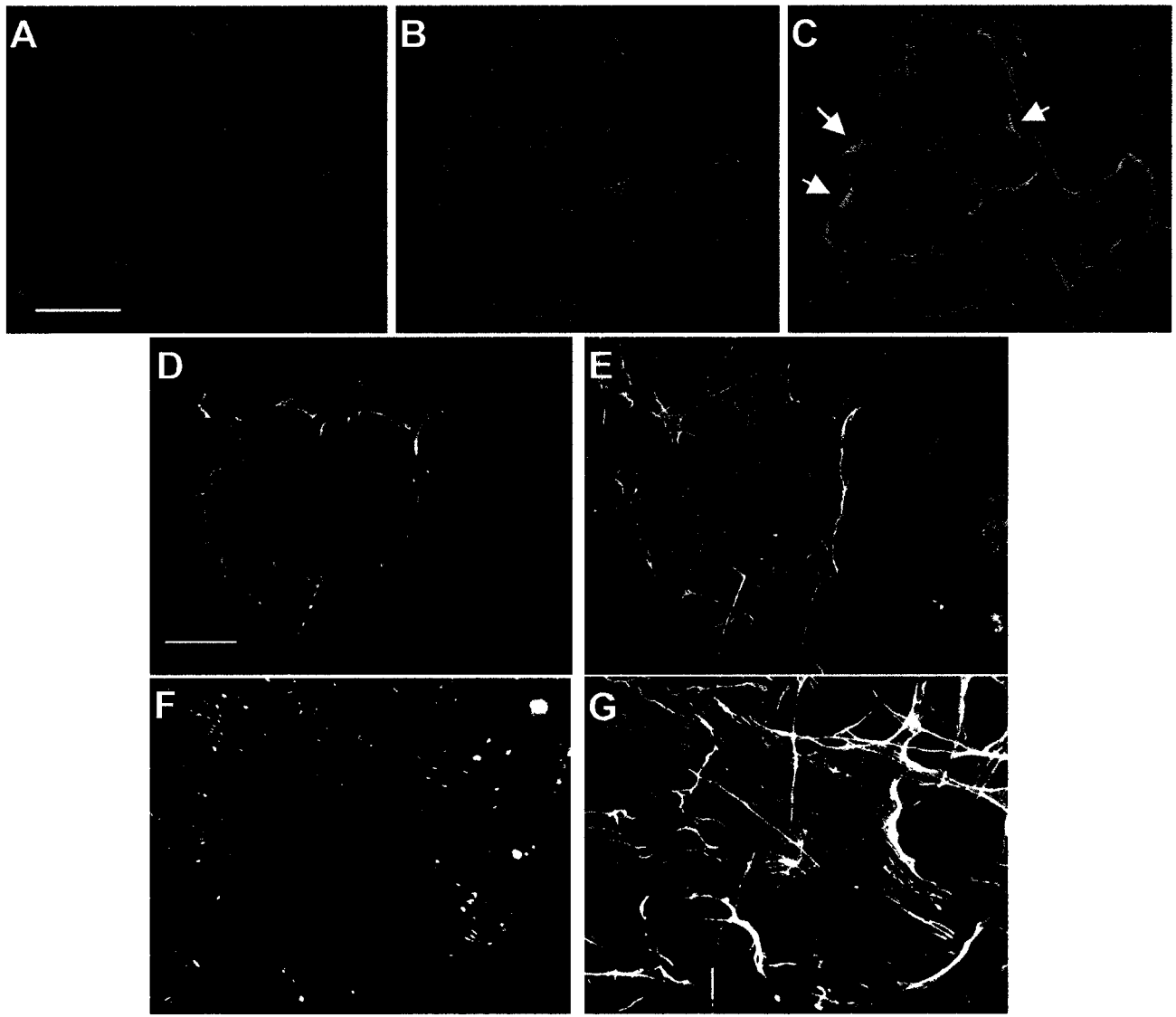

A
B
C
D
E
F
G

Figure 3.6. DCC associates with the actin cytoskeleton.

Serum-starved Swiss 3T3 cells microinjected with pRK5-DCC and stimulated with netrin-1 for 30 min were fixed and detected by indirect immunofluorescence on a Zeiss 410 confocal microscope (A-C). F-actin (A) and DCC (B) were visualized as in Figure 3.5. Panel C is a merged image of A and B. Arrows in panel C indicate co-localization of lamellipodia and DCC. Serum-starved Swiss 3T3 cells microinjected with pRK5-DCC and stimulated with netrin-1 (D and E) for 30 min were subjected to a treatment of CSK buffer containing 0.5% Triton X-100 for 10 min before fixation. Serum-starved Swiss 3T3 cells stimulated with 15% fetal bovine serum for 30 min and subjected to CSK buffer treatment (F and G) were used as a control. The cells were fixed and visualized by immunostaining using anti-DCC antibody (D), anti-vinculin antibody (F) and fluorescently tagged phalloidin (E and G). Scale bar, 10 μm. The scale in panels D-G is different from the one in panels A to C.

and -insoluble fractions were identified by immunoblotting. 32% of DCC was resistant to extraction with 0.4-1.0% Triton X-100 in cells either treated or untreated with netrin-1 (Fig. 3.7A,B, and D). Rac1 was used as a control in these experiments, and upon extraction, was found in the soluble fraction, even at low Triton X-100 concentrations (Fig. 3.7B). Thus, in these experimental conditions, a significant fraction of DCC was associated with the underlying actin cytoskeleton independent of netrin-1. In a similar fashion, after the treatment with a CSK buffer containing 0.5% Triton X-100, DCC was found to remain present in lamellipodia structures in netrin-1-stimulated Swiss 3T3 cells expressing DCC (Fig. 3.6D). This is consistent with the presence of DCC in the insoluble compartment upon detergent extraction. Vinculin, a focal adhesion protein associated with actin filaments, was used as a positive control and was also found associated with the insoluble compartment in cells stimulated with serum (Fig. 3.6F).

To determine whether Nck-1 associates with DCC in the insoluble compartment, COS-7 cells expressing myc-tagged Nck-1 alone or together with DCC were treated with netrin-1 for various periods of time before extraction using 1% Triton X-100 (Fig. 3.7C,D). In the absence of DCC, Nck-1 was found in the detergent-soluble fraction. Interestingly, when Nck-1 is expressed together with DCC, 51% of Nck-1 proteins were now found in the detergent-insoluble fraction in the presence or absence of netrin-1. Thus, these results provide further evidence that the netrin-1 receptor DCC interacts with Nck-1 in the same cellular compartment coincident with actin changes and axon outgrowth.

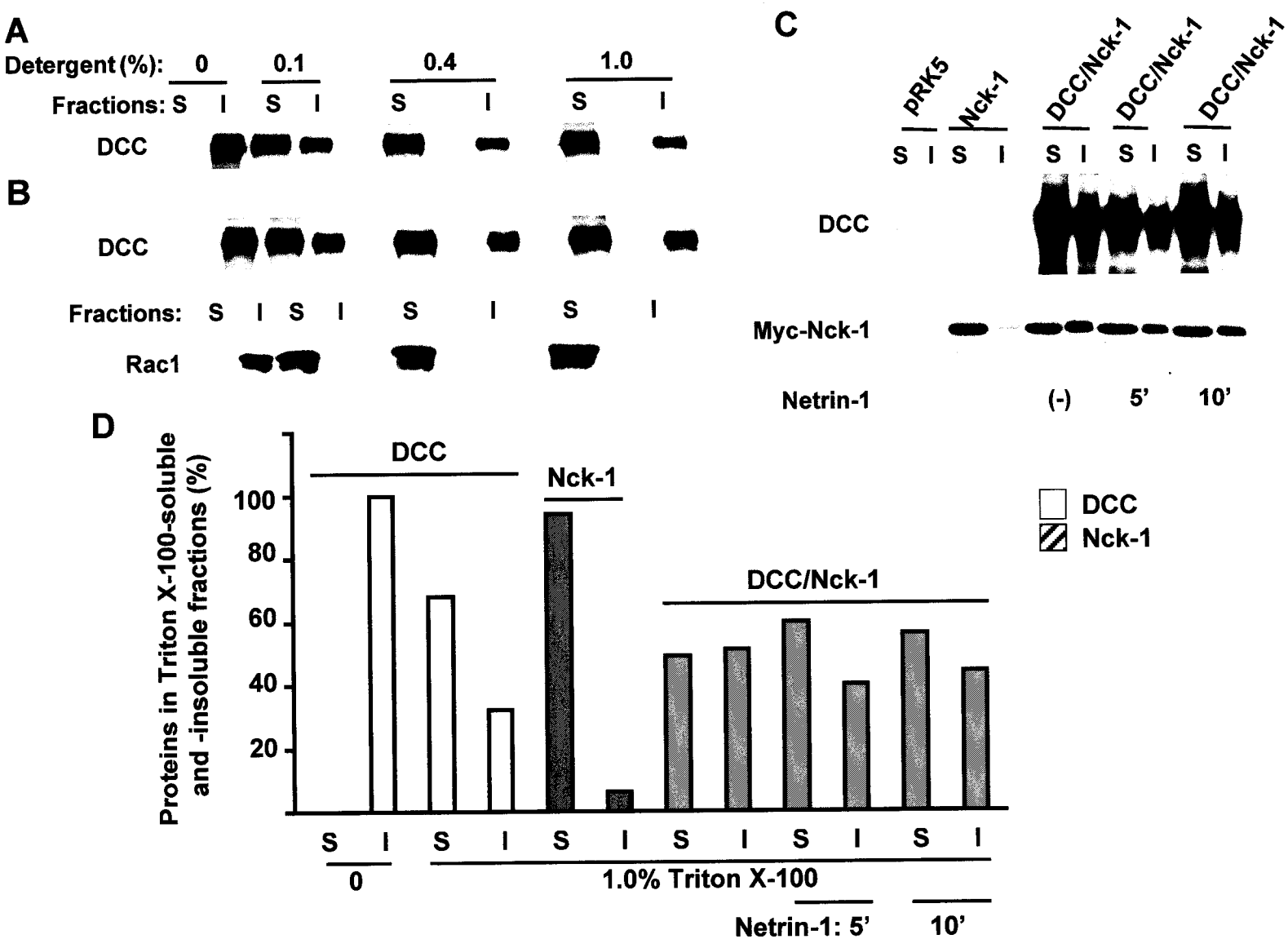

A
Detergent (%): 0 0.1 0.4 1.0
Fractions: S I S I S I S I
DCC
B
DCC
Fractions: S I S I S I S I
Rac1
C
pRK5
Nck-1
DCC/Nck-1
DCC/Nck-1
DCC/Nck-1
S I S I S I S I S I
DCC
Myc-Nck-1
Netrin-1
(-) 5' 10'
D
Proteins in Triton X-100-soluble and -insoluble fractions (%)
DCC
Nck-1
DCC/Nck-1
100
80
60
40
20
DCC
Nck-1
S I S I S I S I S I S I
0
1.0% Triton X-100
Netrin-1: 5' 10'

Figure 3.7. DCC relocalizes Nck-1 to the Triton-insoluble fraction independently of netrin-1.

COS-7 cells transfected either with pRK5-DCC (A and B-upper panel), pRK5 (C), pcDNAmyc-Nck-1 (C) alone, or co-transfected with pRK5-DCC and pcDNAmyc-Nck-1 (C) were serum-starved overnight and stimulated with 500 ng/ml netrin-1 for different time (C), or for 10 min (B-upper panel) or no stimulation (A) followed by incubation on ice with buffers containing different concentrations of Triton X-100 detergent as indicated (A and B) or 1% Triton X-100 (C). Triton X-100 soluble (S) and insoluble (I) protein fractions were separated on SDS-PAGE and transferred to nitrocellulose membrane. The presence of DCC (A, B and C-upper panels) and Nck-1 (C-lower panel) were detected by western blotting using anti-DCC and anti-myc antibodies and ECL detection system. Myc-tagged Rac1 overexpressed in COS-7 cells was used as a control (B-lower panel). The cells were processed under identical conditions, and Rac1 was revealed using an anti-myc antibody. (D) The level of DCC and Nck-1 proteins presents in the Triton X-100 insoluble and soluble fractions after treatment of cells with 1% Triton X-100 was estimated by densitometry. Values are representative of at least three independent experiments.

DISCUSSION

Axon guidance results from coordinated cell movements in which the neuronal growth cone receives signals from the environment and translates them into changes in the actin- and microtubule-based cytoskeleton that lead ultimately to directed neurite outgrowth (Tessier-Lavigne and Goodman, 1996). Growth cone movements require the expression of axon guidance receptors responsive to extracellular guidance cues with attractive or repulsive signals, specifying the direction of axon outgrowth. Recent studies have demonstrated a role for the cytoplasmic domains of transmembrane guidance receptors and the implication of the Rho GTPases to effect changes in motility (Patel and Van Vactor, 2002). Furthermore, the small GTPases Rac1, Cdc42, and RhoA act as essential components in the cytosolic signaling mechanisms induced by DCC when it binds to netrin-1 (Li et al., 2002b; Shekarabi and Kennedy, 2002). The cytoplasmic domain of DCC is essential for neurite outgrowth in N1E-115 cells and to activate Rac1 in fibroblasts (Li et al., 2002b).

A role for the SH3/SH2 adaptor Nck-1 in netrin-1 mediated DCC signaling is now indicated. Nck and DCC physically interact in new-born rat brains and in commissural neurons from E13 rat spinal cord. DCC and Nck colocalize in the cell bodies as well as in the axons and growth cones of commissural neurons. The association of DCC with Nck was present in the absence of netrin-1 both in commissural neurons and in COS-7 cells overexpressing DCC and Nck-1. The *in vitro* interaction between Nck-1 and DCC is direct and mediated by the first and third SH3 domains of Nck-1. Therefore, the interaction between the SH3 domains of Nck-1 and putative PXXP motifs in the receptor DCC is likely to be constitutive. This is not surprising since most, but not all, SH3-PXXP interactions are often constitutive whereas SH2-pY binding are always induced by extracellular signals (Li et al., 2001).

A role for Dock, the fly homologue of mammalian Nck, in axon guidance is well documented (Li et al., 2001). During a genetic screen to identify effectors involved in axonal pathfinding, Dock was identified as an essential gene for proper photoreceptor axon targeting and fasciculation (Garrity et al., 1996). In addition to its role in the adult fly visual system, Dock has been shown to play an essential role in synapse formation in the embryonic nervous system. Dock protein is expressed in most or all central nervous

system (CNS) axons and cell bodies (Desai et al., 1999). Interestingly, Dock and Frazzled, the fly homologue of DCC, are both expressed on commissural and longitudinal axons in the developing CNS (Desai et al., 1999; Kolodziej, 1996). Whether Dock and Frazzled interact together in the fly system is not known and will be of great interest to demonstrate. Recently, Dock was shown to interact with the axon guidance receptors, Dscam and Kette (Hummel et al., 2000; Schmucker et al., 2000). Dock directly interacts with Dscam through both its SH2 and SH3 domains. Genetic studies showed that Dscam, Dock, and the Rac/Cdc42 effector $p65^{PAK}$ act together to direct axon pathfinding (Schmucker et al., 2000). Similar to its mammalian counterpart, HEM-2/NAP, which has been shown to interact with the first SH3 domain of Nck-1 (Kitamura et al., 1996), Kette and Dock interact genetically (Hummel et al., 2000). Whether Dock interacts constitutively with Dscam or Kette is not known and will have to await the identification of the ligands to understand better the precise mechanisms.

An essential role for the SH2 domain of Nck-1 in mediating DCC-induced neurite outgrowth in N1E-115 cells and the activation of Rac1 in Swiss 3T3 cells was demonstrated here. The expression of the three SH3 domains of Nck-1 blocked the ability of DCC to induce neurite outgrowth in N1E-115 cells or to induce lamellipodia in the presence of netrin-1 in fibroblasts. Whereas the first and third SH3 domains of Nck-1 are required to bind to DCC, the SH2 domain appears to be functionally essential to mediate the immediate downstream signaling mechanisms induced by the netrin-1 receptor DCC. Studies in mammalian systems demonstrate that Nck-1 links cell surface tyrosine kinase receptors through its SH2 domain, to downstream effectors, via the SH3 domains (Li et al., 2001). Here, we propose an atypical "coupling mechanism" in which Nck-1 links the growth cone guidance receptor DCC, via SH3 domains, to intracellular effectors through its SH2 domain leading to activation of Rac1 (Fig. 3.8). This "coupling mechanism" may represent a general mechanism of action of Dock/Nck-1 coupled to axon guidance receptors including Frazzled/DCC, Dscam and kette/HEM-2/NAP in *D. melanogaster melanogaster* and in mammalian tissues.

DCC associates constitutively with the actin cytoskeleton. In the absence of DCC, most of Nck-1 was found in the soluble fraction whereas the presence of DCC relocalized 51% of Nck-1 into the Triton-insoluble fraction. Therefore, in the absence of ligand, DCC

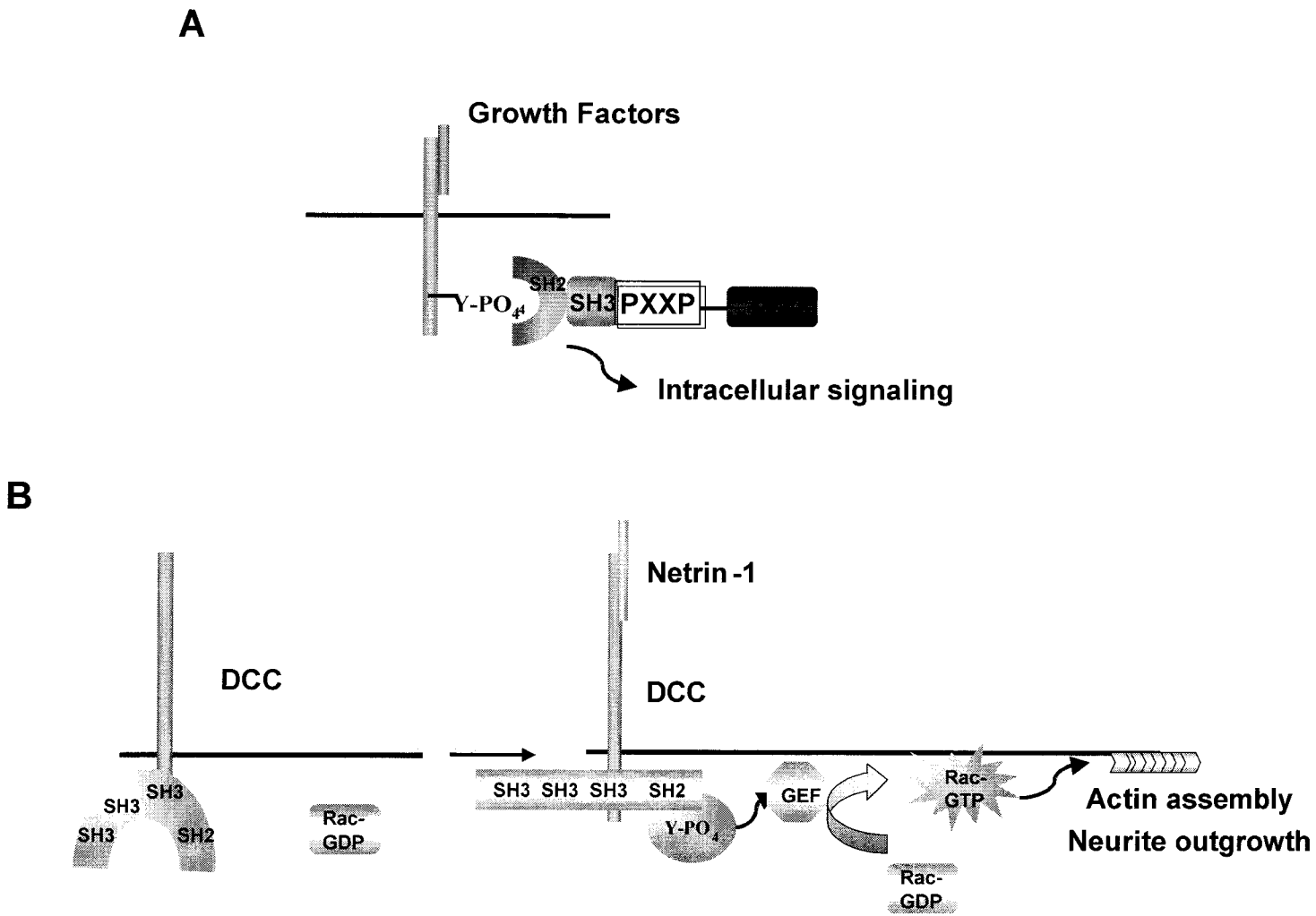

A
Growth Factors
SH2
Y-PO4
SH3
PXXP
Intracellular signaling
B
DCC
SH3
SH3
SH3
SH2
Rac-
GDP
Netrin -1
DCC
SH3 SH3 SH3 SH2
Y-PO4
GEF
Rac-
GTP
Actin assembly
Neurite outgrowth
Rac-
GDP

Figure 3.8. The "coupling mechanism".

(A) SH3/SH2 adaptor links cell surface tyrosine kinase receptors, via SH2-pY interaction, to downstream effectors, through SH3-PXXP binding. (B) Nck-1 is constitutively bound to DCC via the first and third SH3 domains. Following netrin-1 binding to DCC, a conformational change in Nck-1 promotes interaction between the SH2 domain and pY-containing protein (s), leading to activation of a RhoGEF followed by Rac1 stimulation. The activation of Rac1 results in actin assembly at the cell membrane leading to neurite outgrowth.

may be responsible for the association of Nck-1 with the underlying actin cytoskeleton. DCC binding to netrin-1 may result in a conformational change of Nck-1 that is now able to interact with specific effectors via its SH2 domain to promote neurite outgrowth and activation of Rac1 (Fig. 3.8). The formation of a DCC/Nck-1 molecular complex associated with the underlying actin cytoskeleton is likely to be a major step involved in growth cone motility and guidance in response to a source of netrin-1.

PREFACE TO CHAPTER 4

As described in Chapter 3, we have shown that DCC interacts directly with the SH2/SH3 adaptor Nck-1. We have mapped the DCC binding sites on Nck-1 and showed that both SH2 and SH3 domains are required for DCC-induced neurite outgrowth. The cytoplasmic domain of DCC contains no obvious catalytic or signaling protein modules. However, there are 10 proline-rich domains containing the minimum SH3-binding site PxxP motif, suggesting that DCC may interact with Nck-1 via the PxxP motifs. Therefore, in Chapter 4, to determine the Nck-1 binding site(s) on the cytoplasmic tail of DCC, we produced protein mutants of the carboxyl terminus of DCC either lacking each of the proline-rich domains or having prolines replaced by alanines. We assessed the interaction of each of the DCC mutant proteins with Nck-1. We also assessed the interaction of DCC with Nck-1 by competition with the peptides corresponding to each of the PxxP motifs in the cytoplasmic domain of DCC.

CHAPTER 4

DISRUPTION OF EACH OF THE PXXP MOTIFS IN DCC DOES NOT BLOCK THE INTERACTION OF DCC WITH NCK-1

ABSTRACT

Netrins are bifunctional molecules attracting and repelling different classes of axons. DCC is a transmembrane receptor for netrin-1 implicated in mediating growth cone attraction by netrin-1. Nck-1 associates directly with DCC via the first and third SH3 domains. Here, we show that mutant proteins lacking each of the PxxP motifs or having prolines replaced by alanines in the carboxyl terminus of DCC still interact with Nck-1 as well as wild-type DCC. Moreover, in a competition assay, peptides overlapping each of the PxxP regions do not block the interaction of DCC with Nck-1. These results indicate that disruption of each of the PxxP motifs does not block the interaction of DCC with Nck-1, suggesting that more than one PxxP motifs are involved in the interaction, or non-PxxP sequences mediate the interaction of DCC with Nck-1.

INTRODUCTION

The complexity of neural connectivity depends upon the precise navigation of axons to their targets in the developing nervous system. The neuronal growth cones are highly motile, hand-like structures at the tip of developing neurites. Signals from the environment are assumed to steer axons in the right direction through their receptors on the growth cone, but the molecular details of this process have been largely unknown. Recently, impressive progress has been made in identifying families of guidance cues and their corresponding receptors that may underlie this process. Netrins are a family of secreted proteins that guide growing axons during neural development (Tessier-Lavigne and Goodman, 1996). They are bifunctional molecules attracting and repelling different classes of axons. DCC family members are transmembrane receptors that mediate growth cone attraction by netrins (Chan, 1996; Keino-Masu et al., 1996), whereas the UNC-5 family members are required for the repulsive response to netrins (Ackerman et al., 1997; Leonardo et al., 1997). Evidence suggests that short-range repulsion mediated by netrin requires UNC-5 family members, whereas long-range repulsion requires both UNC-5 and DCC family members (Keleman and Dickson, 2001).

We have shown that the small GTPases Rac1, Cdc42, and RhoA play a key role in the signaling events induced by the netrin-1 receptor DCC (Li et al., 2002b), and that adaptor protein Nck-1 couples the netrin-1 receptor DCC to the activation of the small GTPase Rac1 (Li et al., 2002a). Nck-1 associates with DCC via the first and third SH3 domains. There are 10 putative SH3- binding motifs, PxxP (Yu, 1994), in the cytoplasmic tail of DCC, suggesting the interaction may be mediated by the PxxP sequences.

In this chapter, we provide biochemical evidence that mutations in each of these PxxP sequences or competition with the peptides spanning each of the PxxP regions do not disrupt the interaction of DCC with Nck-1.

EXPERIMENTAL PROCEDURES

DNA Constructs and Mutagenesis

pRK5 encoding full length DCC (pRK5-DCC) and pGEX4T-2 encoding the carboxyl terminus of DCC (pGEX4T-DCC-C) were described previously (Li et al., 2002a; Li et al., 2002b). pGEX-DCC-C-M1, -M2, -M3, -M5, -M8, -M9, and -M10 were made by PCR using the overlap extension method (Horton et al., 1989). Briefly, a 12-nucleotide deletion corresponding to 4 amino acids was made at each of the 7 PxxP regions in the carboxyl terminus of DCC, respectively. A six-nucleotide insertion corresponding to the *Kas*I restriction site was inserted into the deletion site. The mutation was generated by PCR amplification of pGEX-DCC-C spanning the *Xba*I site and PxxP region both located in DCC coding sequence and PxxP region and *Not*I site in pGEX4T-2 followed by PCR amplification of the two PCR fragments spanning the *Xba*I site and *Not*I site. The second step PCR product was then ligated back into pGEX-DCC-C digested with *Xba*I and *Not*I. pGEX-DCC-C-M4, M6, M7 were made by site-directed mutagenesis kit (Stratagene). pRK5-DCC-M1, -M2, -M3, -M4, -M5, -M6, -M7, -M8, -M9, and -M10 were produced by PCR amplification of corresponding pGEX-DCC-C mutant followed by ligation of the PCR products into pRK5-DCC digested with *Xba*I and *Sal*I. GST fusion proteins of Nck-1 and mutant proteins were described elsewhere (Kebache et al., 2002).

Synthetic Peptides

Peptides corresponding to the PxxP sequences QQQPMLPPAQPEHPSSEE, SRTIPTACVRPTHPLRSFAN, THPLRSFANPLLPPPMSAIE, SAIEPKVPYTPLLSQPG, LLSQPGPTLPKTHVK, SLGLAGKARSPLLPVSVPTA, KARSPLLPVSVPTAPEVSEE were made from Biotechnology Resource Center. The peptides were dissolved in water at a concentration of 10 mM and stored at -20°C.

Purification of GST Fusion Proteins

Escherichia coli transformed with various GST fusion proteins were grown at 37°C for 1 h. Expression of the fusion proteins was induced by isopropyl-β-thiogalactopyranoside (1mM) for 3 h at 37°C. Cells were sonicated in buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride, 10

μg/ml aprotinin, and 10 μg/ml leupeptin). The lysates were cleared by centrifugation after adding Triton X-100 to a final concentration of 1%. The lysates were then incubated with glutathione-Sepharose beads (Amersham Biosciences) for 1 h at 4°C. Beads were washed 3 times with buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride). GST fusion proteins were eluted in buffer D (50 mM Tris-HCl, pH8, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 5 mM glutathione (Sigma).

Dot Blot Assay

The interaction of the cytoplasmic tail of wild-type or mutant DCC expressed as a GST fusion protein (DCC-C) with wild-type or mutant Nck-1 was assessed using a dot blot assay as described previously (Li et al., 2002a). In brief, GST (5 or 50 μg), GST-DCC-C, and GST-DCC-C mutants were spotted onto nitrocellulose membranes. The filter was air-dried and incubated with blocking buffer (Tris-buffered saline containing 5% milk and 0.05% Tween 20) for 2 h at room temperature. The cDNA encoding wild-type or mutant Nck-1 was subcloned into pGEX-2TK (Amersham Biosciences) containing a protein kinase site located between the GST domain and the multi-cloning site that allows *in vitro* phosphorylation of wild-type or mutant GST-Nck-1. According to the manufacturer's protocol, wild-type or mutant GST-Nck-1 coupled to glutathione-Sepharose beads was incubated for 30 min at 4°C in kinase buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, 10 μl [γ-^{32}p]ATP (10 mCi/ml) (Sigma). The reaction was stopped by adding 10 mM sodium phosphate, pH 8, 10 mM EDTA, 10 mM sodium pyrophosphate, and 1 mg/ml bovine serum albumin. Beads were washed 5 times with PBS, and radiolabeled wild-type or mutant GST-Nck-1 was eluted with 50 mM Tris-HCl, pH 8, and 10 mM glutathione. The membrane was incubated overnight at 4°C with blocking buffer containing 10^6 cpm of radiolabeled wild-type or mutant GST-Nck-1. The membrane was then washed with Tris-buffered saline containing 0.05% Tween 20. Wild-type or mutant Nck-1 binding to the different GST fusion proteins was visualized by autoradiography.

Cell Culture and Transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics at 10% CO_2. Cells were transfected using the DEAE-dextran method as described previously (Olson et al., 1995). The amount of plasmids used per 100-mm dish was 5 μg. Two days later, cells were lysed and collected.

GST Pull-down Assay

COS-7 cells transfected with wild-type or mutant pRK5-DCC were lysed in buffer A (10 mM Hepes, pH 7.4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). 1 mg of protien lysate was precleared with 20 μl glutathione-Sepharose beads at 4°C overnight. The supernatant was incubated with 20 μg of GST fusion proteins and 10 μl of glutathione-Sepharose beads at 4°C for 2 h. Beads were washed three times with buffer A and boiled in SDS sample buffer. Proteins were separated in 10% SDS-PAGE, and DCC was revealed by immunoblotting using anti-DCC antibody and ECL reagent detection kit. The amount of GST fusion protein was detected by Ponceau S staining.

Competition Assay

COS-7 cells transfected with pRK5-DCC were lysed in buffer A as described above. 1 mg of protein lysate was precleared as described. GST fusion protein was preincubated for 10 min with the peptides followed by an incubation with the lysate for 15 min. Beads were washed and proteins were separated and detected as described.

RESULTS

Disruption of each of PxxP motifs does not block the interaction of DCC with Nck-1.

We have previously shown that DCC binds directly to Nck-1 in a dot blot assay (Li et al., 2002a). The cytoplasmic domain of DCC contains no obvious catalytic or signaling protein modules. However, we detected 10 proline-rich domains containing the minimum SH3-binding site, PxxP motif (Fig. 4.1), suggesting that PxxP sequence may mediate the interaction of Nck-1 with DCC. To determine the Nck-1 binding site on the cytoplasmic domain of DCC, we have generated mutants of the carboxyl terminus of DCC lacking each of the proline-rich domains by PCR using the overlap extension method (M1, M2, M3, M5, M8, M9, M10; Fig. 4.1) or having prolines replaced by alanines (M4, M6, M7; Fig. 4.1) and expressed them as GST fusion proteins (GST-DCC-C-M1, -M2, -M3, -M4, -M5, -M6, -M7, -M8, -M9, -M10) in *E. coli.* The interaction between Nck-1 and DCC mutant proteins was assessed in dot blot assay using purified Nck-1 expressed as a GST fusion protein and phosphorylated *in vitro* with [γ-^{32}P]-ATP (Fig. 4.2A). Nck-1 was able to bind to all the DCC mutant proteins as well as the wild-type DCC, suggesting that more than one PxxP region may mediate the interaction. To determine if this is the case, dot blot assay was performed using full length Nck-1 containing amino acid substitutions in either the first (W38R) or the third (W229R) SH3 domain (Fig. 4.2B,C). Both Nck-1 mutant proteins bind to DCC mutant proteins as well as wild-type DCC, indicating that disruption of each of the PxxP sequences does not block the interaction of DCC with Nck-1. These results suggest that the interaction may be mediated by more than one PxxP motif, or by non-PxxP regions in the cytoplasmic domain of DCC.

All the DCC mutant proteins bind to the third SH3 domain of Nck-1.

Because the interaction of the SH3 domain and PxxP sequence is of low affinity (Mayer, 2001), the dot blot assay may not be sensitive enough to detect the differences between the interaction of Nck-1 with the various DCC mutant proteins. We decided to use a GST pull-down assay to characterize the interaction between DCC and Nck-1. First, all the DCC mutants were transferred to pRK5 vector by PCR using the pGEX4T-2-DCC-C mutants as templates. pRK5-DCC-M1, -M2, -M3, -M4, -M5, -M6, -M7, -M8, -

	Class II consensus		X	**P**	p	X	**P**	X	**R**
	SH3-binding motif consensus	p	X	**P**	p	X	**P**		
M1: DCCΔ1309-1312	DCC (1307-1312)	Q	Q	**P**	M	L	**P**		
M2: DCCΔ1313-1316	DCC (1311-1316)	L	P	**P**	A	Q	**P**		
M3: DCCΔ1316-1319	DCC (1314-1319)	A	Q	**P**	E	H	**P**		
* M4: DCCA1336A1339	DCC (1334-1341)	V	R	**P**	T	H	**P**	L	**R**
M5: DCCΔ1446-1449	DCC (1344-1349)	A	N	**P**	L	L	**P**		
* M6: DCCA1357A1360	DCC (1355-1360)	I	E	**P**	K	V	**P**		
* M7: DCCA1360A1363	DCC (1358-1363)	K	V	**P**	Y	T	**P**		
M8: DCCΔ1370-1373	DCC (1368-1373)	P	G	**P**	T	L	**P**		
M9: DCCΔ1391-1394	DCC (1389-1394)	R	S	**P**	L	L	**P**		
M10: DCCΔ1398-1401	DCC (1396-1401)	S	V	**P**	T	A	**P**		

Figure 4.1. Proline-rich regions of the netrin-1 receptor DCC.

DCC consists of 1445 amino acids and the intracellular domain is from 1121 to 1445 amino acids. This figure shows sequence alignment of the proline-rich regions of the cytoplasmic tail of DCC with the SH3-binding motif consensus sequence (Yu et al., 1994). Numbers in brackets refer to the amino acid sequence of DCC. The critical arginine (R, in class II consensus) and prolines (P) are in bold; p, proline-preferred; X, nonconserved residues. Asterisks indicate the three DCC mutants having prolines replaced by alanines in the proline-rich regions of the cytoplasmic tail of DCC. The rest DCC mutants have each of the PxxP sequences deleted.

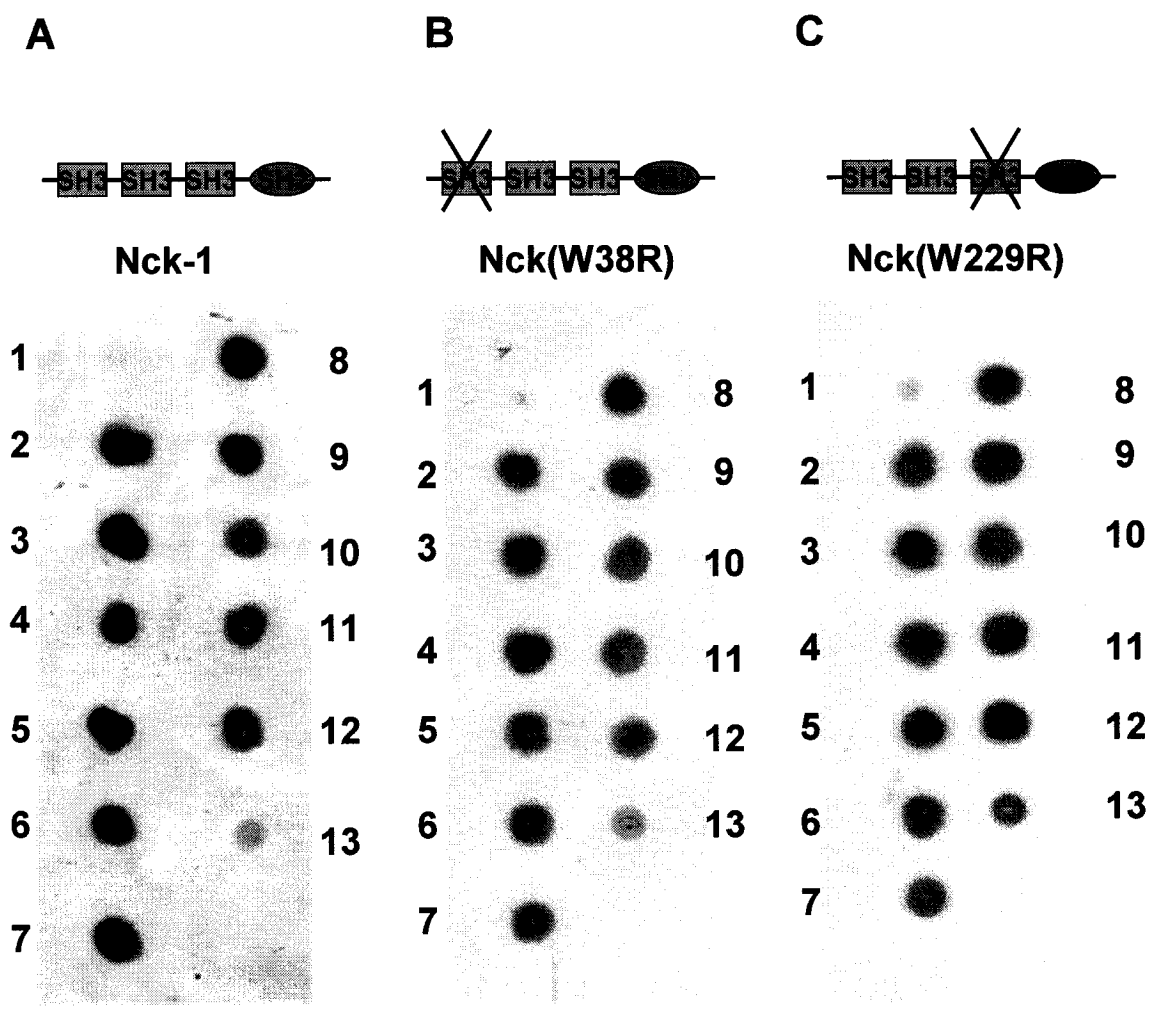

A
B
C
SH3
SH3
SH3
SH3
Nck-1
Nck(W38R)
Nck(W229R)
1
2
3
4
5
6
7
8
9
10
11
12
13

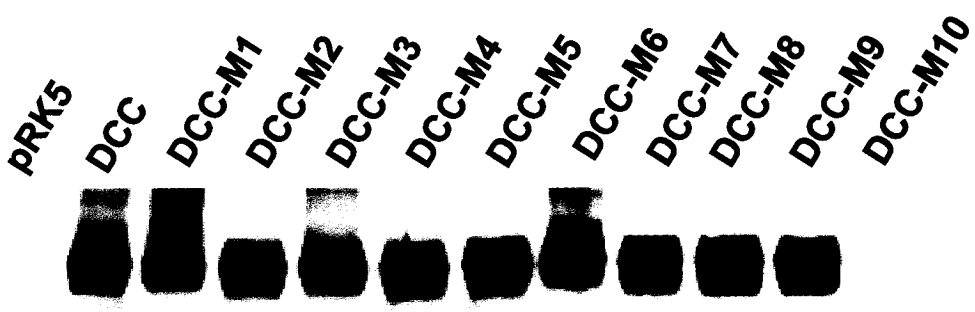
Figure 4.2. Both wild-type and DCC mutant proteins interact with Nck-1.

5 μg of GST (1), GST-DCC-C (2), GST-DCC-C M1 (3), GST-DCC-C M2 (4), GST-DCC-C M3 (5), GST-DCC-C M4 (6), GST-DCC-C M5 (7), GST-DCC-C M6 (8), GST-DCC-C M7 (9), GST-DCC-C M8 (10), GST-DCC-C M9 (11), GST-DCC-C M10 (12), and 50 μg of GST (13) were spotted onto a nitrocellulose membrane in a volume of 5 μl and incubated with *in vitro* phosphorylated GST-Nck-1 (A), GST-Nck(W38R) (B), or GST-Nck(W229R) (C) by bovine heart protein kinase. Nck-1 interactions were visualized by autoradiography. Crosses indicate mutation in the first (B) or the third (C) SH3 domain of Nck-1.

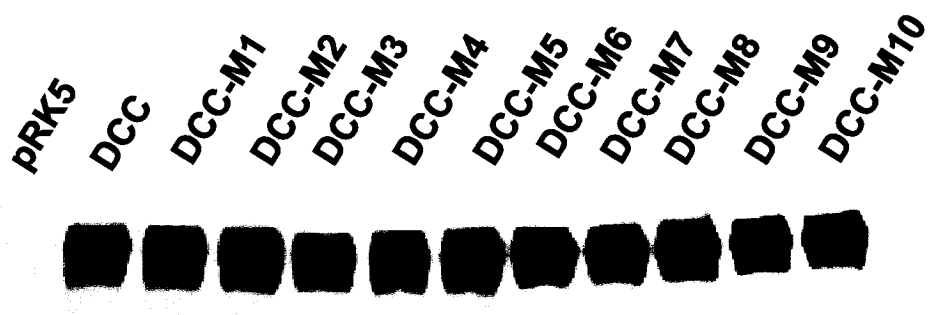
M9, and -M10 were transfected into COS-7 cells and protein lysates were submitted to 7.5% SDS-PAGE. Levels of DCC mutant proteins were detected by Western blotting using anti-DCC antibodies recognizing either the C-terminus (αDCC, Fig. 4.3A) or the N-terminus of DCC (αDCC-N, Fig. 4.3B). αDCC recognizes all the DCC mutant proteins except for M10, whereas αDCC-N recognizes all the DCC mutant proteins. The levels of the mutant proteins are similar to the level of wild-type DCC. Since αDCC-N has a low affinity for DCC compared to αDCC, we used αDCC for the following experiments. GST pull-down experiments were performed using lysates of COS-7 cells expressing either wild-type or DCC mutant proteins incubated with the third SH3 domain of Nck-1 expressed as a GST fusion protein. As shown in Fig. 4.4B, all the DCC mutant proteins showed interaction with the third SH3 domain of Nck-1. The three SH3 domains of Nck-1 was used as a positive control. These results implicate that disruption of each of the PxxP sequences does not block the interaction of DCC with Nck-1, which is consistent with the results from the dot blot assay.

PxxP peptides do not block the interaction of DCC with Nck-1.

DCC lacking each of the PxxP regions may not be sufficient to disrupt the interaction of DCC with Nck-1. To determine which PxxP region(s) is involved in the binding with Nck-1, seven peptides corresponding to the 10 PxxP sequences were designed and used in binding assays (Fig. 4.5). The interaction of the peptides with Nck-1 was assessed in a dot blot assay using purified Nck-1 expressed as a GST fusion protein and phosphorylated *in vitro* with [γ-^{32}P]-ATP (Fig. 4.6A). However, none of the 7 peptides interacted with Nck-1, suggesting that the affinity of the interaction is too low to be detedcted by dot blot assay. In a competition asssay, the third SH3 domain of Nck-1 expressed as a GST fusion protein was preincubated for 10 min with 1mM of each peptide followed by an incubation for 15 min with protein lysates from COS-7 cells expressing wild-type DCC. None of the 7 peptides inhibited the interaction of DCC with Nck-1, although a small reduction in the binding is observed with P1 and P5 (Fig. 4.6B).



WB αDCC



WB αDCC-N

Figure 4.3. Expression level of DCC muant proteins.

COS-7 cells were transfected with empty vector pRK5, pRK5-DCC, or the various DCC mutants. Protein lysates were submitted to SDS-PAGE and DCC proteins were detected by Western blotting (WB) analysis using anti-DCC (upper panel, WB αDCC) and anti-DCC-N (lower panel, WB αDCC-N) antibodies, respectively.

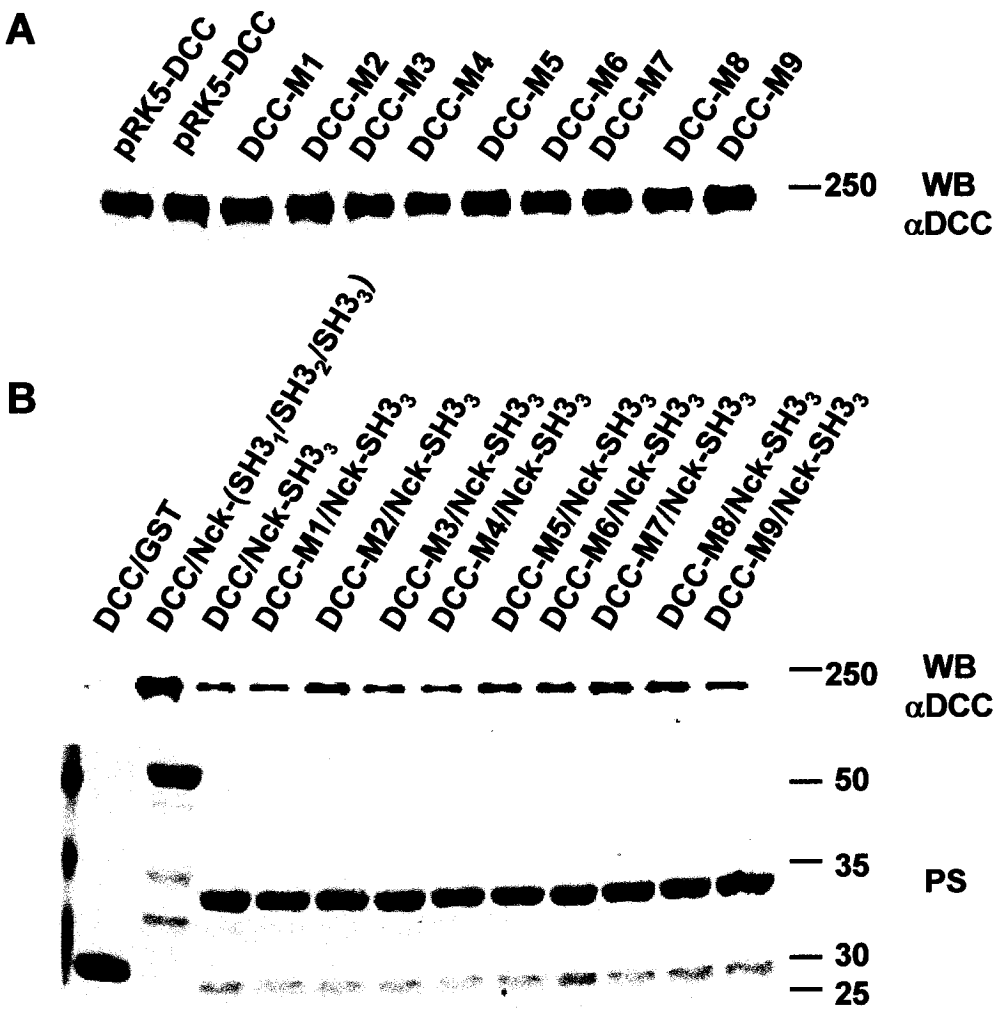

A
pRK5-DCC
pRK5-DCC
DCC-M1
DCC-M2
DCC-M3
DCC-M4
DCC-M5
DCC-M6
DCC-M7
DCC-M8
DCC-M9
—250
WB
αDCC
B
DCC/GST
DCC/Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
DCC/Nck-SH3$_3$
DCC-M1/Nck-SH3$_3$
DCC-M2/Nck-SH3$_3$
DCC-M3/Nck-SH3$_3$
DCC-M4/Nck-SH3$_3$
DCC-M5/Nck-SH3$_3$
DCC-M6/Nck-SH3$_3$
DCC-M7/Nck-SH3$_3$
DCC-M8/Nck-SH3$_3$
DCC-M9/Nck-SH3$_3$
—250
WB
αDCC
— 50
— 35
PS
— 30
— 25

Figure 4.4. DCC mutant proteins interact with Nck-1.

COS-7 cells transfected with pRK5-DCC or DCC mutants were lysed, and 1mg of protein lysates was incubated with 20 μg of either GST or GST fusion proteins of Nck-($SH_1/SH_2/SH_3$) or Nck-$SH3_3$ coupled to glutathione-Sepharose beads. 40 μg of starting material (A) and Nck-1-associated proteins (B) were submitted to SDS-PAGE, and DCC was detected by Western blotting (WB) analysis using anti-DCC antibodies (WB, αDCC). Ponceau S (PS) staining shows the level of GST fusion proteins in each sample.

Figure 4.5. Peptides containing the PxxP regions.

This figure shows the 7 peptides containing the individual PxxP region in the cytoplasmic domain of DCC. P1 contains the PxxP regions corresponding to M1, M2, and M3 (Fig. 4.1). P2 contains the PxxP region corresponding to M4. P3 contains the PxxP region corresponding to M5. P4 contains the PxxP regions corresponding to M6 and M7. P5 contains the PxxP region corresponding to M8. P6 contains the PxxP region corresponding to M9. P7 contains the PxxP region corresponding to M10. The amino acids in the PxxP regions are underlined.

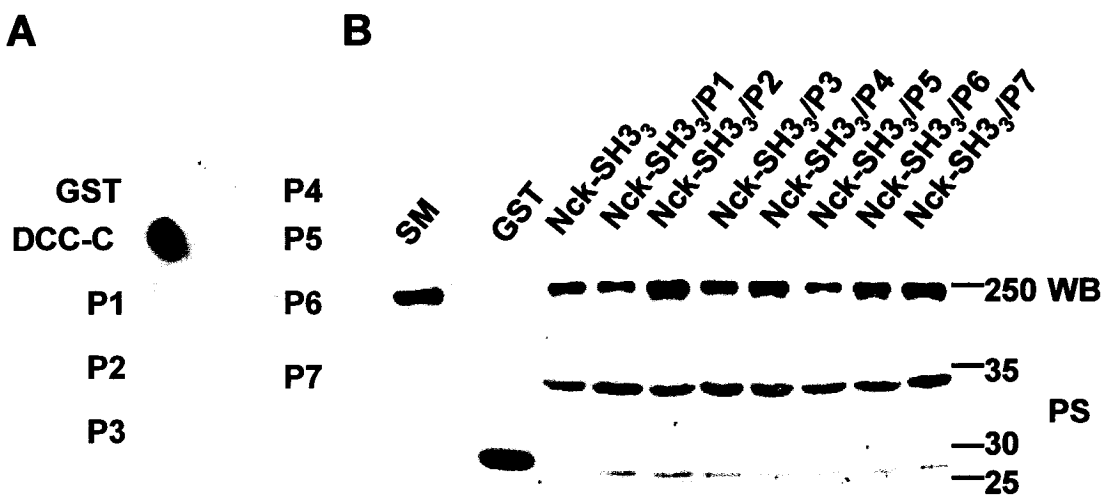

A
B
GST
DCC-C
P1
P2
P3
P4
P5
P6
P7
SM
GST
Nck-SH3$_3$
Nck-SH3$_3$/P1
Nck-SH3$_3$/P2
Nck-SH3$_3$/P3
Nck-SH3$_3$/P4
Nck-SH3$_3$/P5
Nck-SH3$_3$/P6
Nck-SH3$_3$/P7
250
WB
35
PS
30
25

Figure 4.6. PxxP peptides do not block the interaction of DCC with Nck-1.

A, GST (5 μg), GST-DCC-C (5 μg), and peptides (10 μg) flanking the PxxP regions in the cytoplasmic domain of DCC were spotted onto nitrocellulose membrane in a volume of 5 μl and incubated with *in vitro* phosphorylated GST-Nck-1 by bovine heart kinase. Nck-1 interactions were visualized by autoradiography. B, COS-7 cells transfected with pRK5-DCC were lysed, and 1 mg of protein lysates was incubated with 20 μg of either GST or GST-Nck-$SH3_3$ preincubated with 1 mM of the peptides for 10 min and coupled to glutathione-Sepharose beads. 40 μg of starting material (SM) and Nck-1-associated proteins were submitted to SDS-PAGE, and DCC was detected by Western blotting (WB) analysis using anti-DCC antibody. Ponceau S (PS) staining shows the level of GST fusion proteins in each sample.

DISCUSSION

We have previously shown that the netrin-1 receptor DCC activates Rac1 in fibroblasts and is required for neurite outgrowth in N1E-115 neuroblastoma cells (Li et al., 2002b). We have also shown that Nck-1 couples DCC to the activation of Rac1. Nck interacts directly with DCC independently of netrin-1 both in commissural neurons and in COS-7 cells overexpressing DCC and Nck-1 (Li et al., 2002a). The interaction is mediated by the first and third SH3 domains of Nck-1. However, the region(s) in DCC that mediate the interaction with Nck-1 has not been determined. In this chapter we investigated the mechanism of the interaction of DCC with Nck-1 by introducing deletion of each of the PxxP motifs in the carboxyl terminus of DCC. In a dot blot assay or in a GST pull-down assay, using either full length or the third SH3 domain of Nck-1 as a GST fusion protein, all the DCC mutant proteins bound to Nck-1 as well as the wild-type DCC. Since the interaction between SH3 domain and PxxP motif is of low affinity (Mayer, 2001), we thought that the use of peptides in a competition assay may be more sensitive to detect differences in the interaction of Nck-1 with DCC. Therefore, we designed 7 peptides overlapping each of the 10 PxxP regions. However, none of the peptides interacted with Nck-1, and none of the peptides competed significantly for the binding of the third SH3 domain of Nck-1 with DCC, although we cannot rule out the possibility that the peptides do not fold properly *in vitro*. These results suggest that more than one PxxP region in the cytoplasmic tail of DCC are able to mediate the interaction with Nck-1. Additional deletion mutants of DCC will need to be produced to determine which of the PxxP motif(s) are required to mediate the interaction of Nck-1 with DCC.

The SH3-binding peptides are characterized by the presence of a general PxxP consensus sequence, which adopts a polyproline type II helix upon binding to the SH3 domain (Mayer, 2001). Positive charges adjacent to the proline-rich motif contribute to the interaction with SH3 binding with negatively charged residues in the SH3 domain. However, SH3-binding motifs have now been extended to sequences lacking this typical PxxP motif. Consensus sequences including PxxDY (Mongiovi et al., 1999), RKxxYxxY (Kang et al., 2000), and Px(V/I)(D/N)RxxKP (Kato et al., 2000) have now been shown to interact with SH3 domain. Therefore, it is possible that other regions in DCC are involved in mediating the interaction with Nck-1. Indeed, there are several recent reports

supporting the interaction between SH3 domain and non-PxxP sequences (Latour et al., 2003; Lewitzky et al., 2000; Pires et al., 2003). For example, the adaptor Grb2 is an important mediator of normal cell proliferation and oncogenic signal transduction events. It consists of a central SH2 domain flanked by two SH3 domains. The C-terminal SH3 domain has been shown to bind to Gab1 (a receptor tyrosine kinase substrate) and to the adaptor protein SLP-76 via an unusual consensus motif PxxxRxxKP (Lewitzky et al., 2000). Pex13p is an essential component of the peroxisomal protein import machinery and interacts via its C-terminal SH3 domain with the import receptor Pex5p. The SH3-binding sites in Pex5p were mapped to a non-PxxP sequence QPWTDQFEKLEKEV (Pires et al., 2003). Signaling lymphocyte activation molecule (SLAM)-associated protein (SAP), an adaptor-like molecule expressed in immune cells, is composed almost exclusively of a SH2 domain. Through its SH2 domain, SAP associates with the cytoplasmic domain of the SLAM family of immune cell receptors which resulting in recruitment and activation of Fyn (a cytoplasmic Src-related protein tyrosine kinase) (Latour et al., 2001). It was recently shown that SAP interacts with the SH3 domain of Fyn via the non-PxxP sequence RFFRxVLN in the SH2 domain (Latour et al., 2003). We carefully checked the sequences of the cytoplasmic domain of DCC, but none of the non-PxxP motifs mentioned above was found in the cytoplasmic domain of DCC. Therefore, a novel SH3-binding motif in the cytoplasmic domain of DCC may mediate the interaction with Nck-1, and proof of this will need more deletion mutants in the cytoplasmic domain of DCC.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 MAJOR FINDINGS

1. Before these studies, very little was known about the signaling mechanisms mediating the response of an axon to a guidance cue. In this thesis, we provided novel findings in the intracellular mechanisms in axon guidance. We have demonstrated the role of the Rho GTPases in the netrin-1 receptor DCC-induced signaling pathways (Li et al., 2002b). Specifically, Rho GTPases are required for embryonic commissural axon outgrowth evoked by netrin-1. Using N1E-115 neuroblastoma cells, we found that both Rac1 and Cdc42 are required for DCC-induced neurite outgrowth. In contrast, down-regulation of RhoA and its effector Rho kinase stimulates the ability of DCC to induce neurite outgrowth. In Swiss fibroblasts, DCC was found to trigger actin reorganization through activation of Rac1 but not Cdc42 and RhoA. These findings provide compelling evidence for a key role of regulated activities of Rac1, Cdc42, and RhoA in the cytosolic signaling mechanisms induced by DCC in a netrin-1-dependent manner.

2. We have identified the adaptor protein Nck-1 in the DCC-induced signaling pathways that link DCC to the activation of Rac1 (Li et al., 2002a). DCC was found to interact constitutively and directly with Nck in both commissural neurons and COS-7 cells overexpressing DCC and Nck-1. Moreover, a dominant negative Nck-1 inhibits the ability of DCC to induce neurite outgrowth in N1E-115 cells and to activate Rac1 in fibroblasts in response to netrin-1. These findings provide evidence for the important role of the SH3/SH2 adaptor protein Nck-1 in the netrin-1-mediated DCC signaling.

3. We have proposed an atypical "coupling mechanism" in which Nck-1 links the growth cone guidance receptor DCC via the SH3 domains to the intracellular effectors through its SH2 domain (Li et al., 2002a). This coupling mechanism may represent a general mechanism of the action of Dock/Nck-1 coupled to axon guidance receptors in both *D. melanogaster* and mammalian systems. Both DCC and Nck-1 were found to associate with the actin cytoskeleton, and this association is mediated by DCC (Li et al., 2002a). The formation of a DCC/Nck-1 complex associated with the underlying actin cytoskeleton is likely to be a major step involved in growth cone motility and guidance in response to a source of netrin-1. These findings provide important evidence for a role of Nck-1 in a novel signaling pathway from an extracellular guidance cue to changes in the actin-based cytoskeleton responsible for axonal guidance.

4. We have demonstrated that disruption of single PxxP motif in the carboxyl terminus of DCC does not block the interaction of DCC with Nck-1. In both dot blot and GST pull-down assays, DCC protein mutants lacking each of the PxxP motifs in the carboxyl terminus of DCC are still able to bind to Nck-1 as well as wild-type DCC. Peptides overlapping each of the PxxP motifs are also not able to block the interaction of DCC with Nck-1. These findings suggest that more than one PxxP motif or non-PxxP motifs in the cytoplasmic domain of DCC may mediate the interaction of DCC with Nck-1.

5.2 REGULATED ACTIVITIES OF RHO GTPASES IN DCC-INDUCED SIGNALING PATHWAYS

It has been well described that in some cell types such as fibroblasts, there is a cross-talk within the Rho GTPases. Activation of Cdc42 leads to activation of Rac1, which in turn leads to activation of RhoA (Nobes et al., 1995). As described in Chapter 2, we have shown that netrin-1 receptor DCC activates Rac1 in Swiss 3T3 fibroblasts as shown by the formation of lamellipodia in DCC-expressing cells (Li et al., 2002b). DCC also induced the formation of stress fibers, however, it was blocked by both dominant negative Rac1 and C3 transferase, suggesting a cross-talk between Rac1 and RhoA in DCC-induced signaling pathways. Rac1 is not downstream of Cdc42 because dominant negative Cdc42 was not able to block DCC-induced actin reorganization (Li et al., 2002b). Ras has been shown to activate Rac1 in some signaling pathways (Kjoller, 1999; Sarner et al., 2000); However, Rac1 does not seem to be activated by Ras in DCC-induced signaling pathways since dominant negative Ras did not block the lamellipodia formation in Swiss 3T3 cells (Fig. 5.1) and neurite outgrowth in N1E-115 neuroblastoma cells (Fig. 5.2). In N1E-115 cells, both Rac1 and Cdc42 activities were found to be required for DCC-induced neurite outgrowth (Li et al., 2002b). Consistent with our findings, Shekarabi and Kennedy (Shekarabi and Kennedy, 2002) have shown that both Rac1 and Cdc42 are activated by DCC upon netrin-1 binding in HEK293 cells. These results suggest that a specific GEF may be absent in fibroblasts. Alternatively, a specific co-receptor for netrin-1 that is required for DCC to activate Cdc42 may not be expressed in fibroblasts.

A current model suggests that attractive guidance cues activate Rac1 or Cdc42 and inhibit RhoA to promote directed axonal outgrowth, whereas repulsive cues inhibit Rac1 or Cdc42 and stimulate RhoA to induce growth cone retraction (Mueller, 1999), even though there are some exceptions (Jin and Strittmatter, 1997; Vastrik et al., 1999). Our data from neuronal cells fit well with this model. We have demonstrated that both Rac1 and Cdc42 are required for DCC-induced neurite outgrowth in N1E-115 cells. When RhoA and Rho kinase were inhibited, respectively, by C3 transferase or Y-27632 compound, DCC-expressing cells with neurites increase to 80%, suggesting that down-regulation of RhoA and Rho kinase is required for DCC to induce neurite outgrowth in

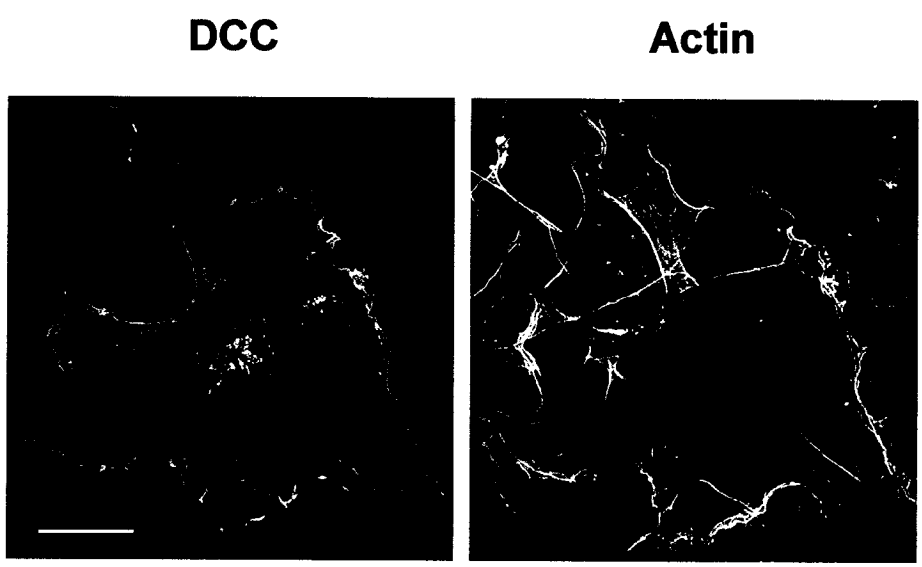

DCC
Actin

Figure 5.1. Ha-Ras is not required for the netrin-1 receptor DCC to induce lamellipodia in Swiss 3T3 fibroblasts.

Serum-starved Swiss 3T3 cells were co-injected with pRK5-DCC and pEXV encoding Myc-tagged RasN17. 5 h after microinjection, netrin-1 (500 ng/ml) was added to the medium for 30 min. F-actin was visualized with fluorescently tagged phalloidin, and DCC was revealed by co-staining with an anti-DCC antibody and by indirect immunofluorescence. The expression of Myc-tagged RasN17 was detected using anti-Myc antibodies and by indirect immunofluorescence. Scale bar, 10 μm.

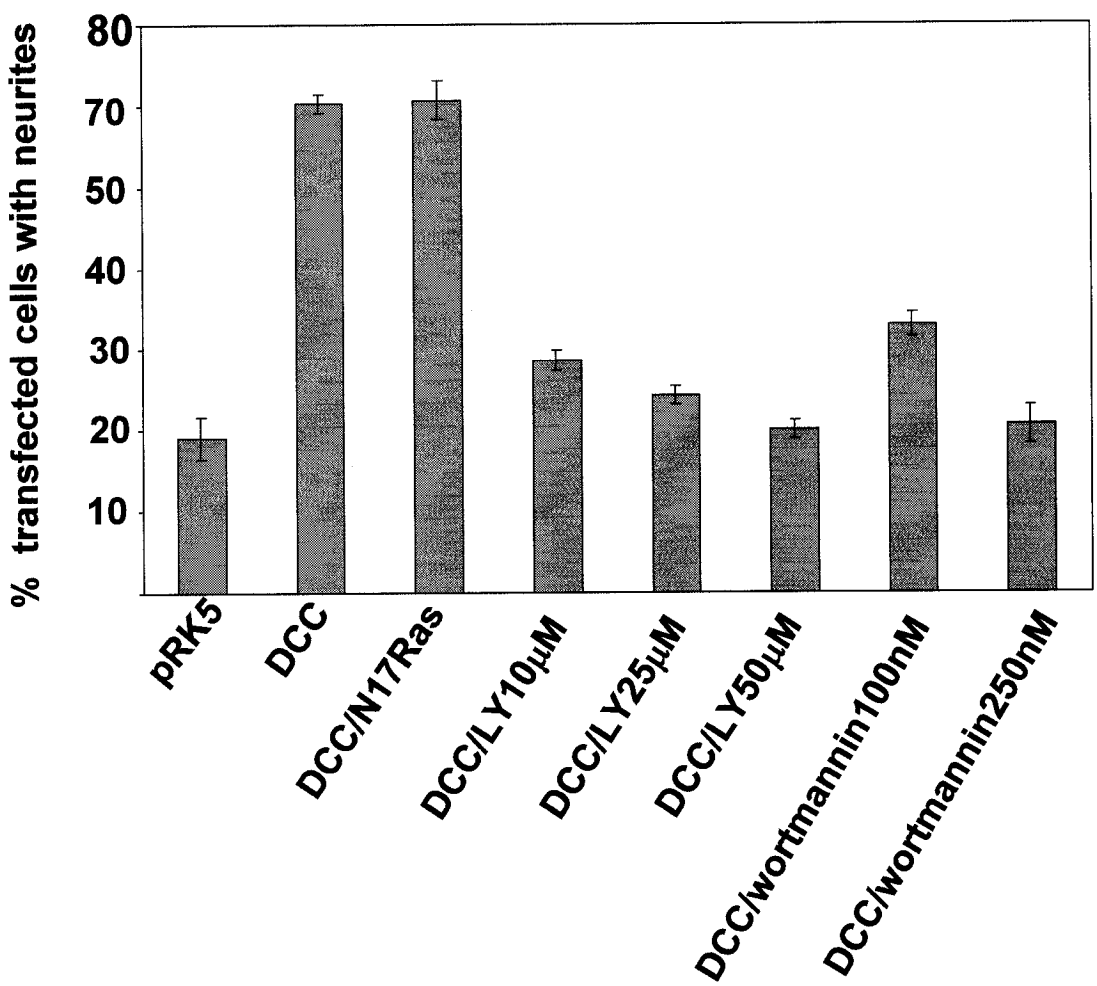

% transfected cells with neurites
80
70
50
40
30
20
10
pRK5
DCC
DCC/N17Ras
DCC/LY10μM
DCC/LY25μM
DCC/LY50μM
DCC/wortmannin100nM
DCC/wortmannin250nM

Figure 5.2. PI-3 kinase but not Ha-Ras is involved in DCC-induced neurite outgrowth.

N1E-115 neuroblastoma cells were either transfected with pRK5, pRK5-DCC alone or co-transfected with pEXV-RasN17. When indicated, the cells transfected with pRK5-DCC were treated either with 10 μM, 25 μM, 50 μM LY294002 or with 100 nM, 250 nM wortmannin for 2 h prior to fixation. Values indicate the percentage of transfected cells with neurite extension and corresponding to the average of at least three independent experiments.

N1E-115 cells. In supporting of this model, Wahl et al. (Wahl et al., 2000) showed that ephrin-A5 activates RhoA and inhibits Rac1 in cultured RGCs. Shamah et al. (Shamah et al., 2001) identified ephexin, a novel GEF, which interacts directly with the EphA4 receptor and acts as a direct link between Eph receptors and Rho GTPases. In the absence of stimulation, ephexin is constitutively bound to the cytoplasmic domain of EphA4 receptor and is in a position to activate Cdc42 and Rac1. In this state, growth cone extension is promoted. When EphA4 receptors are stimulated with ephrinA1, ephexin activates RhoA while reducing the activation of Cdc42 and Rac1. The net effect is to induce growth cone collapse. In *D. melanogaster*, plexinB mediates motor axon guidance by inhibiting active Rac1 and enhancing RhoA signaling (Hu et al., 2001). From our data, we propose that upon netrin-1 binding, DCC may activate Rac1 and inhibit RhoA activity leading to growth cone extension. The cross-talk we showed between Rac1 and RhoA in fibroblasts may be restricted to non-neuronal cells.

5.3 SIGNALING PATHWAYS UNDERLYING THE NETRIN-1 RECEPTOR DCC

5.3.1 RhoGEF Trio

The cytoplasmic domain of DCC did not interact physically with Rac1, suggesting an indirect link between DCC and Rac1. Trio was thought to be a good candidate protein that may link DCC to the activation of Rac1. Trio was the first RhoGEF extensively studied in the context of growth cone guidance. It contains two GEF domains. Genetic and biochemical data suggest that GEF1 activates RhoG and GEF2 activates RhoA (Bellanger et al., 1998; Debant et al., 1996; Newsome et al., 2000; Steven, 1998). The analysis of *C. elegans* unc73 mutants and *D. melanogaster* Trio mutants has indicated that Trio functions to regulate the outgrowth and guidance of axons (Awasaki et al., 2000; Newsome et al., 2000; Steven et al., 1998). The cytoplasmic domain of DCC contains several putative SH3-binding motifs, PxxP, which may interact with the two SH3 domains of Trio. We did GST pull-down assays using each of the two SH3 domains of Trio expressed as GST fusion proteins and lysates from COS-7 cells overexpressing DCC, no interaction was observed between DCC and Trio (data not shown). Therefore, in our assay, Trio did not interact with DCC via its SH3 domains. We found that Trio by itself induces neurite outgrowth in N1E-115 cells (data not shown). Consistent with this, Trio has been shown to induce neurite outgrowth in PC12 cells in a GEFD1-dependent manner (Estrach et al., 2002). Co-expression of DCC and Trio was found to induce lamellipodia formation, however, no increase of neurite outgrowth was observed (data not shown). These results may suggest that Trio acts in the DCC-induced signaling pathway leading to Rac1 activation and lamellipodia formation. GTPases cycle between active and inactive states. However, when Rac1 is kept in an active state, neurite outgrowth is blocked. Activation of Rac1 by either DCC or Trio may not be sufficient to induce lamellipodia formation. Therefore, co-expression of DCC and Trio synergizes to activate Rac1 leading to lamellipodia formation but not neurite outgrowth. Consistent with this, overexpression of constitutively active Rac1-Rac1V12 in N1E-115 cells induces lamellipodia formation not neurite outgrowth (Sarner et al., 2000). Expression of either constitutively active or dominant negative Rac1 in *D. melanogaster* embryonic sensory neurons results in selective defects in axonal outgrowth (Luo et al., 1994).

5.3.2 PI3-K is involved in DCC signaling pathways.

PI3-K mediates activation of Rac1 downstream of many tyrosine kinase receptors, and it has been shown to be required for the turning response to netrin-1 in the *Xenopus* spinal growth cone (Bar-Sagi, 2000; Ming et al., 2002). To determine whether PI3-K is involved in DCC-induced signaling pathways, we transfected DCC into N1E-115 cells and treated cells with PI3-K inhibitors LY294002 and wortmannin. As indicated in Fig. 5.2, both inhibitors inhibit DCC-induced neurite outgrowth, suggesting that PI3-K is involved in DCC-induced neurite outgrowth. PI3-K links Ras to the activation of Rac1 in integrin-dependent neurite outgrowth in N1E-115 cells (Sarner et al., 2000). However, in DCC-induced signaling pathways, PI3-K is not activated by Ras, since co-expression of DCC and dominant negative Ras in N1E-115 cells did not block the neurite outgrowth induced by DCC. These results suggest that PI3-K bot not Ras is involved in DCC-induced neurite outgrowth. However, whether PI3-K links DCC to the activation of Rac1 remains to be determined.

5.3.3 cAMP and PKA

Several studies indicate that PKA plays a key role in determining whether a neuronal growth cone is attracted or repelled by netrin-1. Elevating the intracellular concentration of cAMP causes PKA activation (Nairn et al., 1985). Utilizing dissociated neurons from embryonic *Xenopus* spinal cord, Ming et al. (Ming et al., 1997) demonstrated that PKA activation correlates with chemoattraction to netrin-1, while PKA inhibition switches the response to repulsion. It is known that PKA phosphorylates RhoA leading to the translocation of membrane-associated RhoA to the cytoplasm (Lang et al., 1996), thus PKA activation may influence growth cone guidance by inhibiting RhoA activity and having a chemoattractive response to netrin-1. In N1E-115 cells, upon netrin-1 binding, DCC may activate PKA through elevating cAMP levels, which inactivates RhoA leading to neurite outgrowth. Corset et al. (Corset et al., 2000) showed that A2B is a netrin-1 receptor and induces cAMP accumulation upon binding to netrin-1. It is possible that in N1E-115 cells, A2B induces cAMP accumulation upon netrin-1 binding, which inactivates RhoA leading to neurite outgrowth.

Netrin-1-induced turning response depends on Ca^{2+} influx through plasma membrane Ca^{2+} channels, as well as Ca^{2+}-induced Ca^{2+} release from cytoplasmic stores. Reduction of Ca^{2+} signals by blocking either of these two Ca^{2+} sources converts the netrin-1-induced response from attraction to repulsion (Hong et al., 2000). A potential target of Ca^{2+} is adenylyl cyclase (Xia and Storm, 1997). Type I and type III adenylyl cyclases are stimulated by Ca^{2+} and CaM to produce cAMP that activate PKA. DCC may increase cytosolic Ca^{2+} levels that activate adenylyl cyclases leading to PKA activation and RhoA inactivation in N1E-115 cells when it binds to netrin-1.

5.3.4 Tyrosine phosphorylation

Tyrosine phosphorylation plays important roles in signal transduction. The importance of tyrosine phosphorylation in axon guidance is underscored by the finding that RTKs and RPTPs are important in several aspects of neuronal development, ranging from cell fate determination to axon guidance and fasciculation (Holland et al., 1998; Van Vactor, 1998). It has been shown that the *C. elegans* homologue of DCC, UNC-40, is phosphorylated on tyrosine residues *in vivo* (Tong et al., 2001). There are several tyrosine residues in the cytoplasmic domain of DCC, and they are conserved across the species. Therefore, tyrosine phosphorylation in the cytoplasmic domain of DCC may be involved in the activation of Rac1 by interacting with a SH2 domain-containing protein. Indeed, the work in our lab (Meriane et al., submitted) has shown that tyrosine phosphorylation in the cytoplasmic tail of DCC is required for the activation of Rac1 and neurite outgowth upon netrin-1 binding.

5.3.5 MAPK pathway

The signaling pathways activated by Rac1 that mediate the response of netrin-1 are still unknown. It is well known that Rac1 regulates the c-Jun N-terminal or stress-activated protein kinase (JNK/SAPK) MAPK related cascade (Coso et al., 1995; Minden et al., 1995). Indeed, we have detected that DCC expressed in COS-7 cells induces a two-fold increase in JNK activation upon netrin-1 binding (Fig. 5.3). However, whether JNK activation contributes to DCC-induced neurite outgrowth remains to be determined.

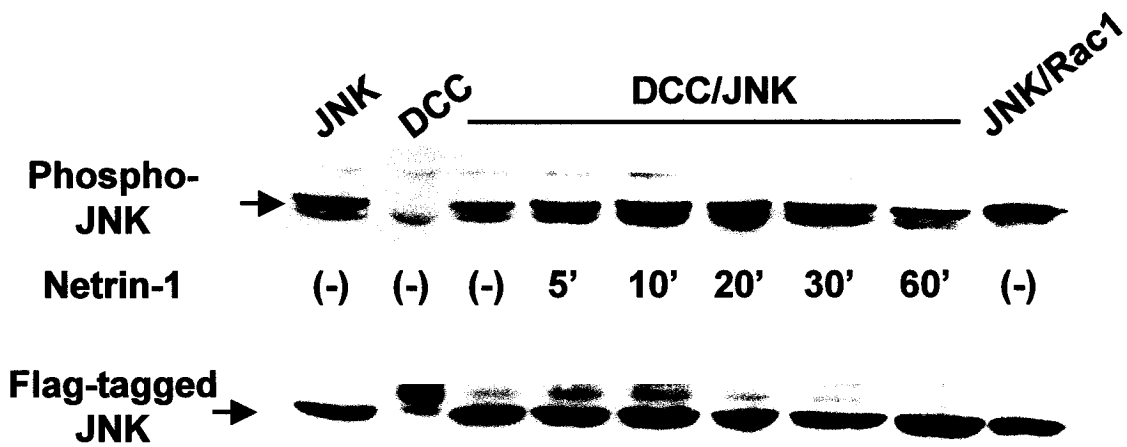

JNK
DCC
DCC/JNK
JNK/Rac1
Phospho-JNK
Netrin-1
(-) (-) (-) 5' 10' 20' 30' 60' (-)
Flag-tagged JNK

Figure 5.3. Netrin-1 receptor DCC activates JNK/SAPK signaling pathway in COS-7 cells.

COS-7 cells transfected with pCMVflag-JNK, pRK5-DCC alone, or co-transfected with pCMVflag-JNK and pRK5-DCC or pCMVflag-JNK and pRK5myc-RacL61 were serum-starved overnight and stimulated with 500 ng/ml netrin-1 for different period of time. Cell lysates were submitted to SDS-PAGE and transferred to nitrocellulose membrane. Activation of JNK was detected by western blotting using anti-phosphoJNK antibody (upper panel). The membrane was stripped and the level of JNK was detected by western blotting using anti-flag antibody (lower panel).

Recently, Forcet et al. (Forcet et al., 2002) demonstrated that netrin-1-mediated axon outgrowth requires DCC-dependent MAPK activation. They showed that DCC activates ERK-1 and ERK-2 in both transfected cells and commissural neurons in a netrin-1-dependent manner. This activation can cause transcriptional activation, as evidenced by examining transcription factor Elk-1 activation and SRE-dependent gene expression. Inhibition of ERK-1/2 blocks netrin-1-dependent axon outgrowth and guidance. In netrin-1 and DCC knockout animals, they observed diminished activated phospho-ERK-1/2. These results support a role for the MAPK pathway in response to the chemoattractant netrin-1. In addition, it has been observed that protein synthesis is stimulated by netrin-1 and required for netrin-1-mediated attraction of *Xenopus* retinal growth cones *in vitro* (Campbell and Holt, 2001). The main factors for translation initiation like eIF4E and eIF4E-BP1 are phosphorylated by an ERK-1/2-dependent pathway (Herbert et al., 2002), providing a potential mechanism linking netrin-1 to protein synthesis for axon growth and guidance. In our system, DCC may activate ERK-1/2 pathways leading to synthesis of the cytoskeleton proteins responsible for lamellipodia formation and neurite outgrowth.

5.4 THE ADAPTOR PROTEIN NCK

5.4.1 Different roles of Nck-1 in different cells

Using biochemical assays, we have identified the adaptor protein Nck-1 as a linker to couple DCC to the activation of Rac1. Overexpression of Nck-1 alone has no effect on actin re-organization in Swiss 3T3 cells (Fig. 5.4). However, Nck-1 induces neurite outgrowth in N1E-115 cells. Nck-1 is able to bind to many proteins via either the SH2 or SH3 domains, it may induce neurite outgrowth through these protein complexes via DCC-independent mechanism. Co-expression of DCC and Nck-1 in N1E-115 cells increases DCC-induced neurite outgrowth, suggesting that DCC and Nck-1 induce neurite outgrowth using two parallel pathways. It has been shown that Nck inhibits NGF-induced neurite outgrowth in PC12 cells (Rockow et al., 1996), which is different from our results in N1E-115 cells. The different effect of Nck on neurite outgrowth may be due to the different cell line used in these two studies.

5.4.2 SH3 domains of Nck-1 mediate the interaction with DCC

Studies in mammalian systems demonstrate that Nck-1 links cell surface tyrosine kinase receptors through the SH2 domain. However, the association of DCC with Nck-1 is mediated by both the first and the third SH3 domains. Consistent with this, Dock, the *D. melanogaster* homolog of Nck, has been shown to interact with DInR via both the SH2 and SH3 domains, and DInR functions as a guidance receptor upstream of Dock in the *D. melanogaster* visual system (Song et al., 2003). Dscam is another cell surface receptor for Dock in axon pathfinding in the embryonic CNS and Bolwig's nerve (Schmucker et al., 2000). Dscam binds to Dock through both the SH2 and SH3 domains and acts as an upstream activator of the Dock-Pak guidance pathway. KETTE, a member of HEM family in *D. melanogaster*, has been implicated in cytoskeletal organization during axon pathfinding (Hummel et al., 2000). The KETTE protein is homologous to the transmembrane protein HEM-2/Nap1 whose interaction with Nck has been well documented (Kitamura et al., 1997), whereas KETTE has been shown to interact with Dock genetically (Hummel et al., 2000). Nck/Nap1 interaction is mediated through the first SH3 domain of Nck.

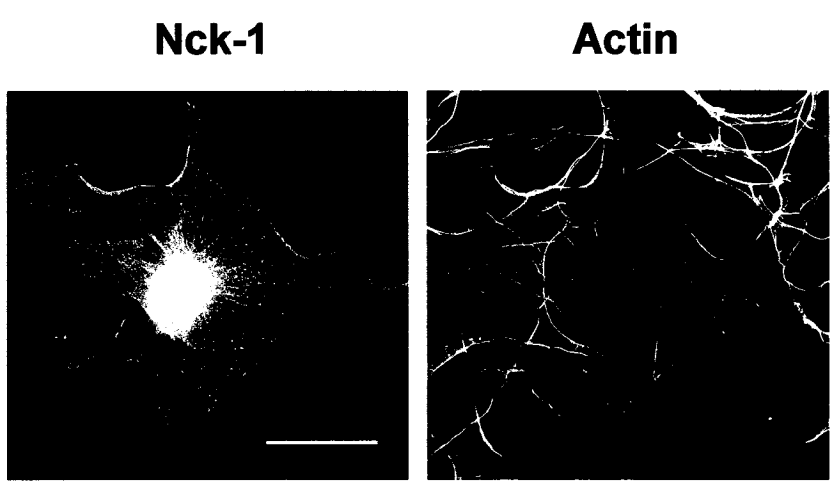

Nck-1
Actin

Figure 5.4. Nck-1 does not induce actin reorganization in Swiss 3T3 fibroblasts. Serum-starved Swiss 3T3 cells were microinjected with pRK5 encoding HA-tagged Nck-1 and fixed 5 h later. F-actin was visualized as in Figure 5.1. Nck-1 was determined by co-staining with an anti-HA antibody and by indirect immunofluorescence. Scale bar, 10 μm.

5.4.3 Nck family members

Mammals have two Nck family members (Nck-1/Nckα and Nck-2/Nckβ) (Braverman and Quilliam, 1999; Chen et al., 1998; Lehmann et al., 1990; Park, 1997; Tu et al., 1998). Nck-1 and Nck-2 exhibit 68% amino acid identity to each other, with most of the sequence variation being located in the linker regions between SH3 and SH2 domains. A variety of data have suggested that Nck-1 functions to couple phosphotyrosine signals to the regulation of the actin cytoskeleton. However, little is known concerning the binding properties and biological function of Nck-2, although these are in general similar to Nck-1 (Braverman and Quilliam, 1999). Nck-2 has been identified as selectively interacting through its third SH3 domain with the LIM4 domain of PINCH, a protein involved in integrin signaling (Tu et al., 1998). In addition, the SH2 domain of Nck-2 specifically binds to tyrosine phosphorylated B-type ephrins and links B-ephrin reverse signaling to the cytoskeletal regulatory proteins (Cowan and Henkemeyer, 2001). Nck-2 has also been proposed to have a unique role in linking the PDGF receptor to actin polymerization in NIH3T3 fibroblasts (Chen et al., 2000). We detected the interaction of Nck with DCC in new-born rat brains and in commissural neurons form E13 rat spinal cord as well as the colocalization of the two proteins in commissural neurons. Because the anti-Nck antibody we used can recognize both Nck-1 and Nck-2, and the SH2 domain and SH3 domains of Nck-1 and Nck-2 have high sequence identity, therefore Nck-2 may have similar function in DCC-induced neurite outgrowth and actin reorganization. Nck-1 and Nck-2 have highly overlapping expression patterns and are particularly prominent in the developing nervous system (Bladt et al., 2003), implicating a similar function in the developing nervous system. Indeed, the development of Nck-1$^{-/-}$ and Nck-2$^{-/-}$ knockout mice is apparently normal, suggesting the functional redundancies during development (Bladt et al., 2003).

5.4.4 Nck and other guidance receptors

Nck-1 has been shown to interact with other guidance receptors. EphB2 indirectly associates with and induces the tyrosine phosphorylation of Nck-1 following ligand stimulation (Holland et al., 1997), whereas EphB1 binds directly to Nck-1 following the ephrinB1 treatment (Stein et al., 1998). These implicate the role of Nck-1 in axon

guidance. Nck-2 may have the similar function in mediating axon pathfinding. Indeed, Nck-2 has been shown to transduce B-ephrin reverse signals through binding to the cytoplasmic domain of B ephrins in a phosphotyrosine-dependent manner (Cowan and Henkemeyer, 2001). The reverse signal transduced by B ephrins is thought to lead to axonal repulsion (Birgbauer et al., 2000; Henkemeyer et al., 1996). EphrinB1 reverse signaling leads to cell rounding, loss of stress fibers, a redistribution of paxillin away from focal adhesions, and an increased phosphorylation of Tyr397 in FAK. All of these changes are consistent with the repellent roles for B-ephrins reverse signaling. Thus, the interaction of Nck-2 and B-ephrins may have functional significance by regulating the cytoskeleton during axonal pathfinding and cell migration.

5.4.5 The coupling mechanism

As described in Chapter 3, Nck and DCC interact in new-born rat brains, in commissural neurons from E13 rat spinal cord, and in COS-7 cells overexpressing DCC and Nck-1. The *in vitro* interaction between Nck-1 and DCC is direct and independent of netrin-1. The interaction of SH2 domains with phosphotyrosine is usually induced by extracellular signals, whereas the interaction of SH3 domains with PxxP motif is mostly constitutive (Li et al., 2001). The expression of the three SH3 domains of Nck-1 blocked the ability of DCC to induce neurite outgrowth in N1E-115 cells or to induce lamellipodia in the presence of netrin-1 in fibroblasts, indicating that the SH2 domain of Nck-1 is essential in mediating DCC-induced neurite outgrowth in N1E-115 cells and the activation of Rac1 in Swiss 3T3 cells. The first and third SH3 domains of Nck-1 are required for binding to DCC, whereas the SH2 domain appears to be functionally essential to mediate the immediate downstream signaling pathway.

Many studies indicate that Nck-1 links cell surface tyrosine kinase receptors via SH2-Tyr(P) interaction to downstream effectors through the SH3-PxxP binding (Li et al., 2001). According to our findings, we propose an atypical "coupling mechanism". Nck-1 is constitutively bound to DCC via the first and third SH3 domains. Upon netrin-1 binding, a conformational change in Nck-1 promotes interaction between the SH2 domain and phosphotyrosine-containing protein(s), leading to activation of a RhoGEF followed

by Rac1 activation. The activation of Rac1 results in actin assembly at the cell membrane, leading to neurite outgrowth.

The phosphotyrosine-containing proteins still remain to be identified. Phosphoproteins known to associate directly with the SH2 domain of Nck are either receptor tyrosine kinases or their substrates. Among these proteins, Dok (downstream of tyrosine kinases), a 62-kDa RasGAP-associated phosphotyrosine protein, was purified and cloned from chronic myelogenous leukemia progenitor cells and from v-Abl-transformed B cells (Carpino et al., 1997; Yamanashi and Baltimore, 1997). Dok contains a PH domain, a PTB domain, proline-rich sequences and several potential tyrosine phosphorylation sites (Carpino et al., 1997; Grimm et al., 2001; Yamanashi and Baltimore, 1997). It is an important SH2-binding partner of Nck. Dok family of proteins includes five known members in mammalian cells (Dok1-5) (Grimm et al., 2001). Dok4 and Dok5 seem to promote neurite outgrowth in PC12 cells. In contrast to other Dok family members, they do not bind to RasGAP (Grimm et al., 2001). Thus, Dok4 and Dok5 might bind to the SH2 domain of Nck-1 via potential tyrosine phosphorylation sites and mediate the signaling pathways downstream of Nck-1 involved in DCC-induced neurite outgrowth.

5.5 ASSOCIATION OF DCC/NCK-1 WITH THE ACTIN CYTOSKELETON

5.5.1 Association of DCC with the actin cytoskeleton

DCC expressed in Swiss 3T3 fibroblasts in the presence of netrin-1 was found to cluster within lamellipodia and co-localize with regions enriched in polymerized actin. After treatment with a cytoskeleton buffer containing 0.5% Triton X-100, DCC was found to remain present in lamellipodia structures in netrin-1-stimulated Swiss 3T3 cells expressing DCC, suggesting that DCC associates with the actin cytoskeleton. This was confirmed by a biochemical assay in which 32% of DCC was resistant to extraction with 0.4-1% Triton X-100 in cells either treated or untreated with netrin-1. Thus, a significant fraction of DCC was associated with the underlying actin cytoskeleton independently of netrin-1. Using the cytoplasmic domain of DCC expressed as a GST fusion protein in an *in vitro* binding assay, no interaction was detected between DCC and F-actin (Fig. 5.5), suggesting that DCC interacts with the actin cytoskeleton indirectly. Consistent with our biochemical data, DCC was found to interact with actin in a yeast two-hybrid assay (Corset et al., 2000). We have shown that DCC induces actin reorganization through the activation of Rac1 in fibroblasts in Chapter 2. When DCC and constitutively active Rac1 were co-expressed in Swiss 3T3 cells, DCC was found to cluster within the regions enriched in polymerized actin in the absence of netrin-1 treatment, whereas constitutively active RhoA has no effect on DCC localization (Fig. 5.6). Here, we propose that DCC associates with the underlying actin cytoskeleton, and that activation of Rac1 by DCC brings actin filaments to the plasma membrane which is essential for growth cone motility.

5.5.2 DCC relocalizes Nck-1 to the Triton-insoluble fraction

In the absence of DCC, Nck-1 was found mainly in the detergent-soluble fraction. Interestingly, when Nck-1 is expressed together with DCC, 51% of Nck-1 proteins were found in the detergent-insoluble fraction in the presence or absence of netrin-1. Therefore, in the absence of ligand, DCC may be responsible for the association of Nck-1 with the underlying actin cytoskeleton. DCC binding to netrin-1 may result in a conformational change of Nck-1 that is now able to interact with specific effectors via the SH2 domain to promote neurite outgrowth and activation of Rac1. These results provide further evidence

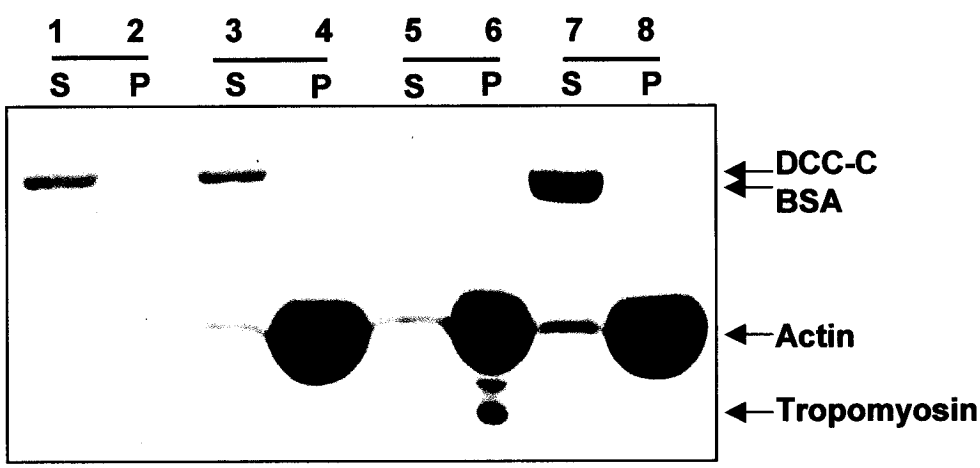

1 2 3 4 5 6 7 8
S P S P S P S P
DCC-C
BSA
Actin
Tropomyosin

Figure 5.5. DCC does not associate directly with the actin cytoskeleton.

A GST fusion protein of the carboxyl terminus of DCC (DCC-C) was incubated with buffer alone (lanes 1 and 2) or F-actin (lanes 3 and 4) for 30 min. Tropomyosin (lanes 5 and 6) and BSA (lanes 7 and 8) were used as positive and negative controls, respectively. The protein complexes were sedimented at 150,000 x g. Proteins recovered in either the supernatants (S) or pellets (P) were separated by SDS-PAGE and visualized by Coomassie brilliant blue staining.

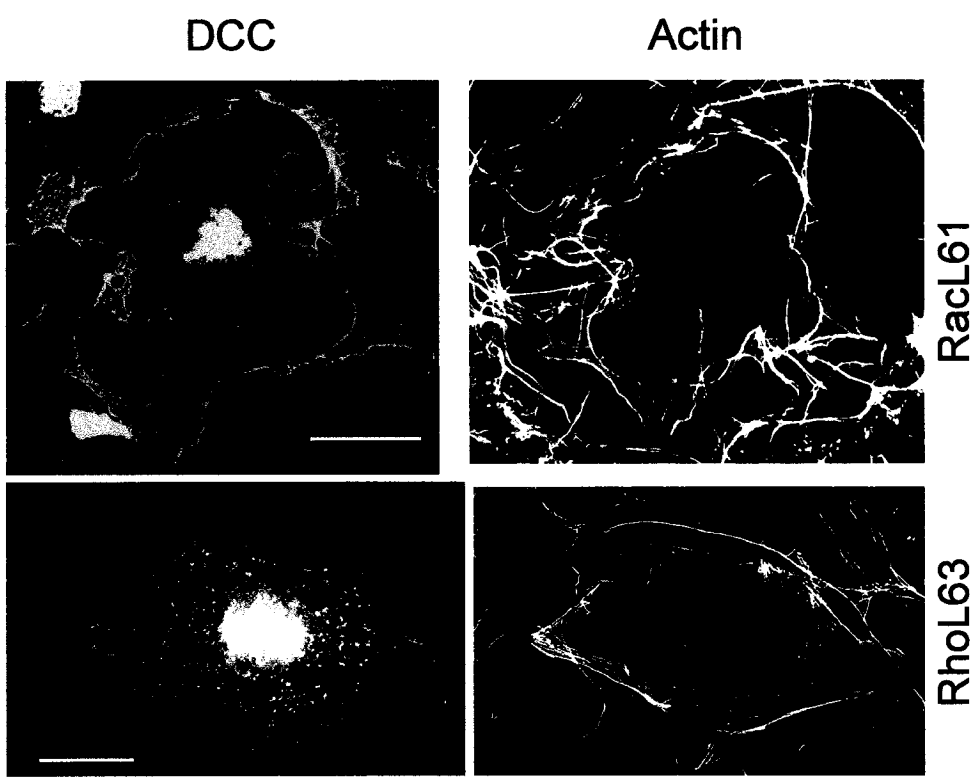

DCC
Actin
RacL61
RhoL63

Figure 5.6. Activated Rac1 relocalizes DCC to the membrane ruffles in Swiss 3T3 fibroblasts.

Serum-starved Swiss 3T3 fibroblasts were co-injected with pRK5-DCC and Myc-tagged RacL61 or RhoL63. Cells were fixed 5 h after microinjection. F-actin and DCC were visualized as in Figure 5.1. The expression of Myc-tagged RacL61 and RhoL63 was detected using anti-Myc antibodies and by indirect immunofluorescence. Scale bar, 10 μm.

that the netrin-1 receptor DCC interacts with Nck-1 in the same cellular compartment coincident with actin changes and axon outgrowth. The formation of a DCC/Nck-1 molecular complex associated with the underlying actin cytoskeleton is likely to be a major step involved in growth cone motility and guidance in response to a source of netrin-1.

When Nck-1 lacking the SH2 domain is co-expressed with DCC, DCC is not able to relocalize the mutant Nck-1 to the Triton-insoluble fraction upon netrin-1 treatment (Fig. 5.7), implicating the SH2 domain of Nck-1 as essential for relocalization to the Triton-insoluble fraction. Nck-1 lacking the SH2 domain does not associate with the actin cytoskeleton, therefore it cannot induce neurite outgrowth and actin reorganization.

In the Triton extraction assay, we observed a small mobility shift of Nck-1 (Fig. 3.7) in the insoluble fraction, suggesting a potential phosphorylation event of Nck-1 in the actin cytoskeleton compartment. Nck has been shown to undergo intensive phosphorylation on tyrosine, threonine, and serine residues upon growth factor stimulation (Li et al., 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992). Meisenhelder et al. has mapped the tyrosine and the majority of the serine sites of phosphorylation in the intervening sequence between the first and second SH3 domains of both Nck-1 and Nck-2. These sites are conserved in both isoforms but are phosphorylated to varying degrees in the two Nck molecules. Nck is also phosphorylated in response to forskolin and phorbol ester treatment, suggesting that it can be a substrate for PKA and PKC (Li et al., 1992; Park and Rhee, 1992). The functional relevance of these phosphorylation sites, however, is unknown. For instance, in response to the netrin-1 receptor DCC, phosphorylated Nck-1 may induce a protein complex in the actin cytoskeleton compartment.

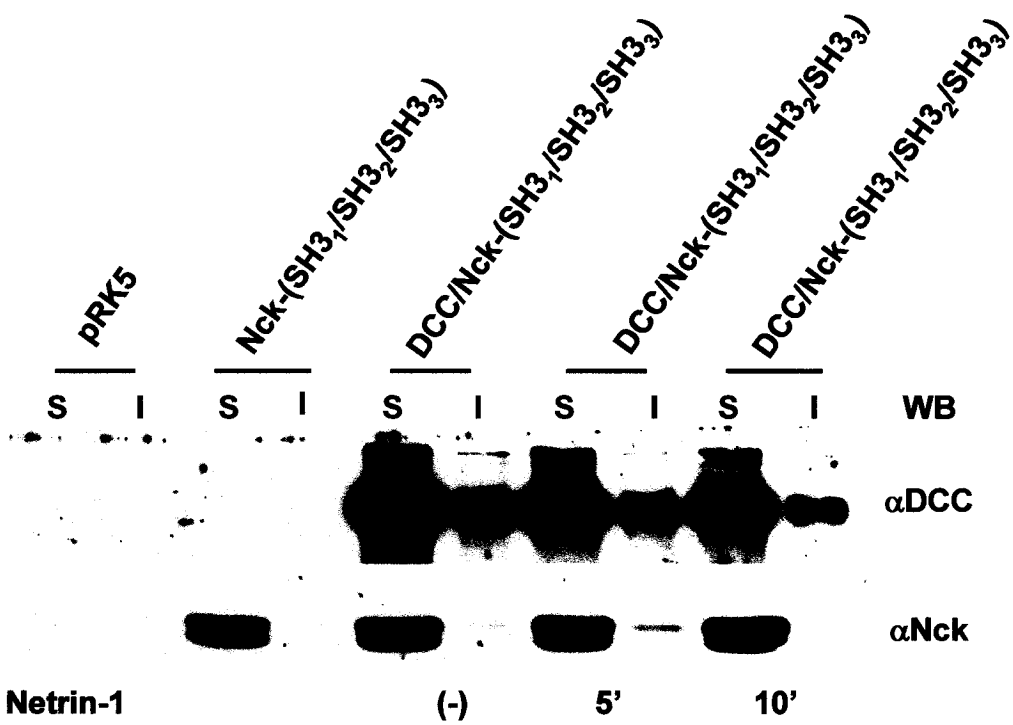

pRK5
Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
DCC/Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
DCC/Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
DCC/Nck-(SH3$_1$/SH3$_2$/SH3$_3$)
S I S I S I S I S I
WB
αDCC
αNck
Netrin-1
(-)
5'
10'

Figure 5.7. The SH2 domain of Nck-1 is required for relocalization of Nck-1 to the Triton-insoluble fraction.

COS-7 cells transfected with pRK5, pcDNAmyc-Nck-$(SH3_1/SH3_2/SH3_3)^{1-251}$ alone, or co-transfected with pRK5-DCC and pcDNAmyc-Nck-$(SH3_1/SH3_2/SH3_3)^{1-251}$ were serum-starved overnight and stimulated with 500 ng/ml netrin-1 for different period of time followed by incubation on ice with buffers containing 10% Triton X-100. Triton X-100 soluble (S) and insoluble (I) protein fractions were separated on SDS-PAGE and transferred to nitrocellulose membrane. The presence of DCC (upper panel) and Nck-$(SH3_1/SH3_2/SH3_3)^{1-251}$ (lower panel) were detected by Western blotting using anti-DCC and anti-Myc antibodies and ECL detection system.

5.6 ARE PXXP MOTIFS INVOLVED IN THE INTERACTION OF DCC WITH NCK-1?

In an effort to identify binding partners for SH3 domains, two proteins were cloned based on their abilities to bind to the Abl SH3 domain (Cicchetti et al., 1992). The binding sites on both proteins were mapped to a short 10 amino acid long proline-rich motif (Ren et al., 1993). In subsequent years, a large number of SH3 ligands have been identified that could play important roles in a variety of physiological contexts. Structural and mutagenic studies have revealed that the peptides associated with SH3 domains adopt a left-handed polyproline-type II helix (PPII), with the minimal consensus sequence PxxP (Pawson, 1995). In addition, basic amino acids are important for the orientation of the interaction of ligands with SH3 domains. Ligands that contain basic amino acids at the amino-terminal of the interacting sequence (class I ligand) usually bind to target SH3 domains with an amino-carboxyl orientation. In contrast, proline-rich peptides possessing basic amino acid at the carboxyl-terminal of the peptide (class II ligand) bind to SH3 domains with a carboxyl-amino orientation (Feng, 1994). However, the SH3-binding motifs have been extended to sequences with PxxDY (Mongiovi et al., 1999), RkxxYxxY (Hong et al., 2000), and Px(V/I)(D/N)RxxKP (Kato et al., 2000; Lewitzky et al., 2000) lacking the typical SH3-binding consensus. Another Nck family member Nck-2 interacts with PINCH containing only LIM domains, via the fourth LIM domain of PINCH and the third SH3 domain of Nck-2. DCC has 10 putative SH3-binding motifs, PxxP. However, we are not able to detect the interaction of Nck-SH3 domain with peptides containing the PxxP motifs. We are also not able to abolish the interaction of DCC mutant proteins lacking each of the PxxP motifs with Nck-1. One possibility is that more than one PxxP motif is involved in the interaction of DCC with Nck-1. Another possibility is that non-PxxP motifs mediate the interaction of DCC with Nck-1. By carefully analysing the sequence of the cytoplasmic domain of DCC, no consensus non-PxxP motif described above was found, implicating that a novel SH3-binding motif in the cytoplasmic domain of DCC may mediate the interaction with Nck-1.

5.7 CONCLUSIONS

In the first part of this study, we demonstrated that both Rac1 and Cdc42 activities are required for DCC-induced neurite outgrowth in N1E-115 cells, whereas down-regulation of RhoA and Rho kinase activities stimulates the ability of DCC to induce neurite outgrowth. These results implicate the small GTPases Rac1, Cdc42, and RhoA as essential components that participate in signaling the response of axons to netrin-1 during neural development. In the second part of the study, we identified the adaptor protein Nck-1 that couples DCC to the activation of the small GTPase Rac1 through an atypical mechanism, providing evidence for an important role of mammalian Nck-1 in a novel signaling pathway from an extracellular guidance cue to changes in the actin-based cytoskeleton responsible for axon guidance. Finally, we found that disruption of each of the PxxP motifs in the cytoplasmic domain of DCC is not able to block the interaction of DCC with Nck-1, suggesting that more than one PxxP motif or non-PxxP sequences may mediate the interaction of DCC with Nck-1.

Studies in mammalian systems demonstrate that Nck-1 links cell surface tyrosine kinase receptors through its SH2 domain to downstream effectors via the SH3 domains. We propose an atypical "coupling mechanism" in which Nck-1 links the growth cone guidance receptor DCC via SH3 domains to intracellular effectors through its SH2 domain, leading to activation of Rac1. This coupling mechanism may represent a general mechanism of action of Dock/Nck-1 coupled to axon guidance receptors including Frazzled/DCC, Dscam, and DInR in *D. melanogaster* and in mammalian systems.

Understanding the signaling pathways underlying netrin-1 receptor DCC will help us to understand how the guidance receptors are connected to Rho GTPases and in turn, how Rho GTPases regulate actin dynamics resulting in a coordinated and directed response of growth cone navigation. Molecules underlying the mechanisms of axon guidance will be good candidate drug targets in neurodegenerative diseases and spinal cord injuries.

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APPENDIX

Copyright waivers from the co-authors and the publisher for:

Xiaodong Li, Etienne Saint-Cyr-Proulx, Klaus Aktories, and Nathalie Lamarche-Vane. RAC1 AND CDC42 BUT NOT RHOA OR RHO KINASE ACTIVITIES ARE REQUIRED FOR NEURITE OUTGROWTH INDUCED BY THE NETRIN-1 RECEPTOR DCC (DELETED IN COLORECTAL CANCER) IN N1E-115 NEUROBLASTOMA CELLS. **Journal of Biological Chemistry 2002; 277: 15207-15214.**

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Xiaodong Li, Mayya Meriane, Ibtissem Triki, Masoud Shekarabi, Timothy E. Kennedy, Louise Larose, and Nathalie Lamarche-Vane. THE ADAPTOR PROTEIN NCK-1 COUPLES THE NETRIN-1 RECEPTOR DCC (DELETED IN COLORECTAL CANCER) TO THE ACTIVATION OF THE SMALL GTPASE RAC1 THROUGH AN ATYPICAL MECHANISM. **Journal of Biological Chemistry 2002; 277: 37788-37797.**

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Nathalie Lamarche-Vane TELEPHONE: 398-1784

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DEPARTMENT: Anatomy and Cell Biology

PROJECT TITLE: Intracellular mechanisms regulated by the axon guidance cue netrin-1

2. FUNDING SOURCE: MRC X NSERC NIH FCAR FRSQ

INTERNAL OTHER (specify) _____

Grant No.: MOP-14701 Beginning date 01/04/2002 End date 31/03/2007

3. Indicate if this is

Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.

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CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed