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**REGULATION OF PROCESS RETRACTION AND CELL MIGRATION BY
EPHA3 IS MEDIATED BY THE ADAPTOR PROTEIN NCK1**

By

TIANJING HU

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ABSTRACT OF THE DISSERTATION

REGULATION OF PROCESS RETRACTION AND CELL MIGRATION

BY EPHA3 IS MEDIATED BY THE ADAPTOR PROTEIN NCK1

By TIANJING HU

Dissertation director:

Dr. Renping Zhou

Members of the largest RTK (receptor tyrosine kinase) family, Eph receptors, and their ligands play pivotal roles in embryonic development. They have been shown to regulate axon guidance, cell migration, tissue boundary formation, synaptogenesis, and angiogenesis. In addition, mis-regulation of the Eph receptor function leads to tumorigenesis and possibly other diseases. However, little is known about signaling pathways downstream of Eph receptors. To shed light on signaling mechanisms of Eph receptors, we conducted a large-scale yeast two-hybrid screen to identify protein that interact with EphA receptors. In this screen, many promising candidates were identified, several of which warrant future investigation. A SH2/SH3 adaptor protein, Nck1, stands out as the strongest interactor with both EphA3 and EphA5. To better understand the signaling mechanisms of the EphAs and the role of Nck1, we performed in-depth studies on fibroblast cells and primary neurons. Here we present evidence that Nck1 interacts directly with activated EphA3 receptor through its SH2 domain and the juxtamembrane domain tyrosine residue 602 on EphA3. We find that the disruption of Nck1 binding by either SH2 domain or SH3 domains negatively regulates EphA3-mediated signaling

during cell migration and cell process extension in HEK293A cells. Intriguingly, we also find that expression of Nck1 mutants through adenoviruses is not able to prevent growth cone collapse in primary hippocampal explants. These observations suggest that Nck1 plays a key role in mediating some aspects of EphA receptor functions.

Key words: Eph, ephrin, Receptor tyrosine kinase, SH2 domain, Nck1, Yeast two-hybrid, Hippocampal neuron culture.

DEDICATIONS

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Without your encouragements,

I would not have been able to travel half across the globe,

and experience a completely different life.

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by working hard and treating others with sincerity, respect and kindness.

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TABLE OF CONTENTS

Abstract	ii
Dedications	iv
Acknowledgements	v
Table of contents	vi
List of tables	xi
List of illustrations	xii
<u>Chapter I. Background</u>	1
Part I. The Eph receptors and their ligands	3
1.1.1. Receptor tyrosine kinases	3
1.1.2 Molecular properties of Eph receptors and ephrins	5
1.1.3 Activation of Eph receptors	7
1.1.4 Signal transduction after Eph activation	9
1.1.5 Adhesion vs. repulsion	10
1.1.6 Eph/ephrin signaling is unique among RTKs	13
Part II. Functions of Eph and ephrin signaling	14
1.2.1 Axon guidance	14
1.2.2 Axon branching	17
1.2.3 Axon fasciculation and defasciculation	18
1.2.4 Spine formation	18
1.2.5 Cell proliferation and differentiation	20
1.2.6 Tissue patterning	20

1.2.7	Cell migration	21
1.2.8	Angiogenesis	22
1.2.9	Tumorigenesis	22
Part III. Signaling pathways downstream of activated Eph receptors and ephrins.		24
1.3.1	Binding proteins of Eph receptors	24
1.3.2	Eph signaling: Src family kinases and Nck1	25
1.3.3	Eph signaling: Ras/MEK/ERK pathway	28
1.3.4	Eph signaling: PIP ₂ related pathways	30
1.3.5	Eph signaling: small GTPases	31
1.3.6	Eph signaling: cell adhesion	33
1.3.7	Reverse signaling through ephrin-Bs	35
1.3.8	Reverse signaling through ephrin-As	38
Summary		39
<u>Chapter II. Screening for EphA receptor binding proteins using the Yeast two-hybrid method</u>		58
2. 1. Introduction		58
2. 2. Results		59
2.2.1	Construction of the bait plasmid and screening of a mouse brain cDNA library	59
2.2.2	Fifteen interactors were identified through the screen	61
2.2.3	Nck1 binds strongly with EphA5 receptor	62
2. 3. Discussion		63

2.3.1	The identified EphA interactors regulate different aspects of signal transduction	63
2.3.2	The binding of Nck1 and EphA receptors links activated EphA receptor and the cytoskeleton regulation.	65
<u>Chapter III. Characterization of EphA3-Nck1 binding and evaluation of Nck1 in EphA3 signaling</u>		
		81
3. 1. Introduction		81
3. 2. Results		82
3.2.1.	Interaction between Nck1 and EphA3 is mediated by the binding of Nck1 SH2 domain to the tyrosine 602 of EphA3	82
3.2.2	EphA3 and Nck1 form a protein complex in ephrin-A5-stimulated cells	85
3.2.3	Nck1 mutants down-modulate ephrin-A5-mediated inhibition of cell migration	86
3.2.4	Nck1 mutants inhibit ephrin-A5-induced cell process retraction	88
3. 3. Discussion		90
3.3.1	Nck1 interacts with activated EphA3	90
3.3.2	Nck1 mediates regulation of cell migration and process retraction by EphA3 activation	92
<u>Chapter IV. Analysis of EphA3-Nck1 binding in neurons and assessment of Nck1 in hippocampal neuron growth cone collapse</u>		
		116
4. 1. Introduction		116
4. 2. Results		117
4.2.1	The expression of Nck1 in primary neuron culture	117

4.2.2	EphA3 and Nck1 interact in hippocampal neurons and cortical neurons	118
4.2.3	Ephrin-A5 induces growth cone collapse in a hippocampal neuron culture	120
4.2.4	Construction of adenoviruses carrying wild type and dominant negative clones of Nck1	122
4.2.5	Nck1 DN mutants do not prevent growth cone collapse induced by ephrin-A5	123
4. 3.	Discussion	124
4.3.1	Nck1 binds to activated EphA3 receptor in neurons and they co-localize highly on the growth cones	124
4.3.2	Nck1 signaling may regulate aspects of EphA3 signaling but not growth cone collapse	126
<u>Chapter V. Assessment of the binding of EphA3 with Grb2 and RasGAP</u>		142
5. 1.	Introduction	142
5. 2.	Results	143
5.2.1	The SH2 domain of both Grb2 and RasGAP bind strongly to EphA3	143
5.2.2	Tyrosine 602 on EphA3 is a binding site for Grb2 SH2 domain	144
5.2.3	RasGAP binds to EphA3 partially through tyrosine 602	145
5. 3.	Discussion	145
5.3.1	EphA3 interacts with multiple SH2 domain containing proteins	145
5.3.2	Grb2 may compete with Nck1 for the binding of EphA3	147
5.3.3	RasGAP binds to EphA3 through multiples sites including tyrosine 602	148
5.3.4	Other SH2 domain proteins that interact with the juxtamembrane domain	149

<u>Chapter VI. Materials and Methods</u>	161
<u>Chapter VII. Conclusions</u>	171
7. 1. Novel binding proteins are implicated in EphA receptor signaling	171
7. 2. Nck1, Grb2 and RasGAP all bind to tyrosine 602 through their SH2 domains	171
7. 3. Nck1 is an integral part of EphA3 mediated signaling	172
<u>References</u>	176
<u>Curriculum Vitae</u>	198

LIST OF TABLES

Table 1-1. Signaling proteins that interact with Eph receptors.	49
Table 1-2. Signaling proteins downstream of ephrin ligands.	54
Table 2-1. A summary of fifteen identified binding proteins of EphA5 in the yeast two-hybrid screen.	79

LIST OF ILLUSTRATIONS

Figure 1-1. Domain structure of Eph receptors and ephrin ligands.	41
Figure 1-2. Phylogram of Eph family kinases and class-specific interactions between Eph receptors and ephrins.	43
Figure 1-3. Eph/ephrin activation, proteolysis, and endocytosis.	45
Figure 1-4. A schematic representation of the retinal-tectal projection in chick.	47
Figure 1-5. Signaling pathways implicated in Eph receptor mediated signal transduction.	52
Figure 1-6. Signaling pathways involved in reverse signaling through ephrin-Bs.	56
Figure 2-1. A schematic representation of the yeast two-hybrid screen.	67
Figure 2-2. A summary of the yeast two-hybrid screen procedure.	69
Figure 2-3. Structure and expression of the bait LexA-EphA5_{intra}.	71
Figure 2-4. Large-scale preliminary screens for EphA5 bait through LacZ reaction.	73
Figure 2-5. Yeast mating test of the binding proteins identified in earlier screens.	75
Figure 2-6. Nck1, a strong binding protein of EphA5.	77
Figure 3-1. Interaction of Nck1 with EphA3 through SH2 domain.	96
Figure 3-2. A series of EphA3 mutants containing single tyrosine mutations.	98
Figure 3-3. Identification of the Tyr602 as the Nck1 binding tyrosine residue on EphA3.	100
Figure 3-4. Precipitation of EphA3 by Nck1 antibody in 293A cells.	102
Figure 3-5. Co-localization of Nck1 and EphA3 in transfected HEK 293A cells.	104
Figure 3-6. EphA3-induced inhibition of cell migration mediated by Nck1.	106

Figure 3-7. Cell process retraction induced by activation of EphA3.	108
Figure 3-8. Disruption of EphA3-induced process retraction by Nck1 DN genes.	110
Figure 3-9. Quantification of Nck1 mutant effects on EphA3-induced process retraction.	112
Figure 3-10. A model of the effects of Nck1 DN genes in EphA3 signaling.	114
Figure 4-1. Expression of EphA3 and Nck1 in cortical and hippocampal neuron cultures.	130
Figure 4-2. Co-localization of EphA3 and Nck1 in hippocampal neuron growth cones.	132
Figure 4-3. Co-precipitation of EphA3 and Nck1 in cortical neurons.	134
Figure 4-4. Growth cone collapse induced by ephrin-A5 treatment in hippocampal neurons.	136
Figure 4-5. A schematic outline of the construction of adenoviral vectors carrying the gene of interest.	138
Figure 4-6. Growth cone collapse assay on neurons infected with adenoviruses expressing Nck1WT, Nck1R308K, and Nck1W38/143/229K.	140
Figure 5-1. Interaction of EphA3 with Grb2 SH2 domain and RasGAP N-terminal SH2 domain.	151
Figure 5-2. Identification of Tyr602 as one binding site of Grb2 protein.	153
Figure 5-3. Identification of Tyr602 as one of RasGAP binding sites on EphA3.	155
Figure 5-4. Domain structure of all SH2 domain containing proteins that bind to Eph receptors.	157

Figure 5-5. A schematic representation of the interactions of EphA3 receptor with Grb2 and RasGAP. **159**

Figure 7-1. A summary of EphA3 signal transduction pathway involving Nck1, Grb2 and RasGAP. **174**

Chapter I. Background

The first member of Eph receptors was identified in 1987 in hepatocellular carcinoma cells (Hirai et al. 1987). Up to this date, a total of 16 members have been discovered. They constitute the largest Receptor Tyrosine Kinase (RTK) family. The ligands for Eph receptors are called “ephrins”. A sum of 8 ephrins have been isolated so far in mammals (Beckmann et al. 1994; Davis et al. 1994). Eph and ephrin family members are expressed highly in a wide variety of tissues in both developing and adult organisms (Nakamoto 2000; Boyd and Lackmann 2001). Consistent with their wide expression, Eph receptors and ephrins are implicated in diverse biological processes, including but not limited to: axon projection, axon fasciculation, cell migration, cell sorting, tissue patterning, synaptogenesis, neuronal spine modulation, angiogenesis, and tumorigenesis (Boyd and Lackmann 2001; Wilkinson 2001; Kullander and Klein 2002; Pasquale 2005; Heroult et al. 2006).

Much progress on elucidating the mechanisms of Eph receptor signaling has been made in recent years. These signaling pathways become activated downstream of Eph receptors and regulate Ras-like small GTPase activity, actin cytoskeleton, integrin signaling, MAPK activity and more (Lawrenson et al. 2002; Huot 2004; Meyer et al. 2005; Sahin et al. 2005). However, detailed mechanisms of these pathways remain unclear. Thus, a study was undertaken to gain insights into the signal transduction mechanisms of Eph receptors.

We began our study with a large-scale screen for EphA interacting proteins. The screen was conducted with the intracellular domain of EphA5 (EphA5_{intra}) as the bait

against a mouse embryonic brain cDNA library, using the DupLEX yeast two-hybrid system. Through this screen, fifteen novel binding proteins were identified and confirmed later by yeast mating tests. Among these positives, Nck1, a SH2/SH3 domain protein, displays the strongest interaction with EphA5. Interestingly, Nck1 was also identified when a similar screen using EphA3 bait was performed. Previously, Nck1 has been shown to play critical roles in signaling events downstream of other RTK family members, including EphB receptors (Holland et al. 1997; Tang et al. 1997; Stein et al. 1998a; Rivera et al. 2006). However, its interaction with EphA receptors has never been established. The interaction of Nck1 and EphA3 therefore became the focus of the current study.

In our study, we confirmed the direct interaction of Nck1 and EphA3. The interaction sites on both proteins were identified, using GST fusion proteins and a group of EphA3 mutants. Nck1 dominant negative mutants were used to show Nck1 activity was necessary in EphA3 signaling in 293A cells. Furthermore, the expression of Nck1 in primary neuron culture was determined and its role in EphA3 mediated growth cone collapse assessed. Additionally, the binding of two other SH2 domain proteins Grb2 and RasGAP with EphA3 was evaluated.

Chapter I provides the background to the current study and consists of (1) a general introduction to the characteristics of Eph receptors and their ligands. (2) a discussion of various functions regulated by Eph and ephrin signaling. (3) a summary of signaling proteins for Eph receptors as well as ephrins and the signaling pathways they regulate.

Part I. The Eph receptors and their ligands

Receptor tyrosine kinases

Eph receptors belong to the Receptor Tyrosine Kinase (RTK) superfamily. RTKs are transmembrane receptors that have intrinsic kinase activity. To date, fifty-eight receptor tyrosine kinases have been found through a genome-wide search in human (Pawson 1995; Robinson et al. 2000). Based on sequence homology, these receptors are further categorized into 19 classes (Shurin et al. 1998; Mellitzer et al. 2000). Well-known examples of the RTK superfamily include epidermal growth factor receptors (EGFR, ErbB), insulin receptors (INSR), platelet-derived growth factor receptors (PDGFR), hepatocyte growth factor receptors (HGFR) and nerve growth factor receptors (NGFR or TRK).

In general, RTKs are composed of three parts, an extracellular ligand binding domain, a transmembrane domain, and an intracellular kinase domain. The extracellular domain is usually composed of multiple domain modules such as Immunoglobulin like domains, Fibronectin type III domains, Cysteine-rich repeats and Leucine-rich regions (Zwick et al. 2001). For example, TrkA, a receptor for NGF, consists of one Cysteine-rich domain and two Immunoglobulin like domains on the extracellular fragment (Holden et al. 1997).

The ligands of RTKs are usually secreted and they reach target cells through diffusion (Zwick et al. 2002; Barbieri et al. 2004). However, some RTKs such as Eph receptors, bind to membrane-bound ligands exclusively. Binding of ligands and receptors within a class is usually promiscuous: that is, ligands of one RTK class may bind to

multiple receptors in this class and vice versa (Zwick et al. 2001). The specificity of the receptor/ligand binding is largely dependent on the conformation of the extracellular ligand-binding domain of the RTKs.

Unstimulated receptor tyrosine kinases are un-clustered and inactive. Once the diffusible ligands reach the cell surface and engage the ligand-binding sites on the RTKs, the receptor molecules undergo immediate dimerization. Although most dimerizations occur between two identical receptors, hetero-dimerization occurs as well. (Zwick et al. 2001; Normanno et al. 2006). For example, two isoforms of PDGF receptors exist and they are capable of dimerizing with each other in response to PDGF activation (Eriksson et al. 1992).

Following receptor dimerization, conformational changes on the intracellular domain of the receptors take place (Zwick et al. 2001; Barbieri et al. 2004; Zhang and Hughes 2006). These changes are critical to RTK function because they directly lead to the activation of kinase activity on the receptors. The kinase active receptors trans-phosphorylate each other on key tyrosine residues. The addition of negatively charged phosphate groups to the intracellular segment further enhances the kinase activity. More importantly, the tyrosine phosphorylation on the receptors provides important docking sites for effector proteins.

Some signaling proteins serve as scaffolds and enable effector proteins to associate with the activated RTKs. Since some effectors are directly phosphorylated by RTKs, association of these effectors with RTKs activates them. Consequently, the activated effectors transduce signals from membrane bound receptors to the signaling cascade including the MAPK, PI3 kinase, PLC gamma and JNK pathways (Zwick et al. 2001;

Oda et al. 2005). Because they are capable of triggering the activation of diverse signaling pathways, RTKs play a pivotal role in the regulation of cellular functions such as cell survival, proliferation, differentiation, adhesion, and apoptosis. Moreover, the many functions of RTK, implicate them in the progression of diseases such as cancer (Avruch et al. 2001). Indeed, one of the focal points of RTK research now involves the application of RTK receptor inhibitors to cancer treatment (Shinkaruk et al. 2003; Carlomagno and Santoro 2005).

RTK superfamily members share many common characteristics, including the activation process and the downstream pathways. The understanding of the mechanisms of other RTK members helps to shed light on Eph signaling.

Molecular properties of Eph receptors and ephrins

Eph receptors were named after “erythropoietin producing human hepatocellular cell line”, from which the first Eph receptor was isolated (Hirai et al. 1987). The Eph family includes a total of 16 members in mammals, which comprise the largest class in the RTK superfamily (Flanagan and Vanderhaeghen 1998; Himanen and Nikolov 2003a; Sasaki et al. 2003; Aasheim et al. 2005). Eph family members have highly conserved structures. Starting from the N-terminus, they consist of a globular ligand binding domain, a Cysteine-rich domain, two Fibronectin type III motifs, a juxtamembrane domain, a tyrosine kinase domain, a SAM domain and a PDZ binding motif (Fig. 1-1 A). Analysis of the DNA sequence of Eph receptors reveals that they share a high degree of homology, especially in the kinase domain (Fig. 1-2 A).

The ligands for Ephs are named “ephrins”, the abbreviation of “Eph receptor interacting proteins”. Being membrane-bound through a GPI anchor or a transmembrane domain, they are also highly conserved (Nakamoto 2000; Himanen and Nikolov 2003b). Depending on the type of connection to the cell membrane, ephrins are divided into two subclasses: the A subclass, which are anchored through a GPI link and lack a cytoplasmic fragment, and the B subclass, which have a transmembrane domain and a cytoplasmic tail (Fig. 1-1 B, C). In mammals, five ephrin-As and three ephrin-Bs have been identified so far (Frisen et al. 1999; Blits-Huizinga et al. 2004).

Based on sequence homology and ligand binding, Eph receptors are also categorized into A and B subclasses. In general, one receptor has higher sequence homology with other receptors of the same subclass than with receptors of a different subclass (Zhou 1998). Moreover, receptors of one subclass interact only with the ligands in the corresponding ligand subclass; although, within each subclass, the ligands and receptors bind promiscuously (Gale et al. 1996b). There exist some exceptions to the subclass specificity of receptor-ligand binding (Fig. 1-2 B). For example, EphA4 interacts with both the A- and the B-ephrins while EphB2 also binds ephrin-A5 in addition to the B-ephrins (Gale et al. 1996b; Himanen et al. 2004; Pasquale 2004). It has been reported that ephrin-A1, -A3 and -A4 bind to EphB1 with low affinity (Beckmann et al. 1994); however, it is unclear whether these interactions lead to any significant biological functions.

Site mutagenesis and crystal structure studies reveal that the “H-I” loop, located on the ephrin binding domain on Eph receptors, is critical in determining the subclass specificity of Eph/ephrin interaction (Himanen and Nikolov 2003b; Pasquale 2004). The

H-I loop is a part of the “low affinity receptor-ligand binding interface”. Its length is conserved within each subclass, but different between the two subclasses. The significance of this loop was further supported by the preferential binding to A class ligands by EphB2 when its H-I loop was replaced by the H-I loop of Eph A3. (Himanen et al. 1998). Consequently, the H-I loop is termed the “class-specificity loop”.

Activation of Eph receptors

Eph receptors and ephrins are expressed at high levels in a wide variety of tissues such as kidney, lung, heart, and muscle. The highest expression, however, is found in the nervous system, especially during development (Zhou 1998). Usually, Eph receptors and ephrins are found on different populations of cells and the receptor-ligand interaction produces repulsive effects, which lead to repulsive effects such as tissue boundary formation or axon retraction (Flanagan and Vanderhaeghen 1998; Knoll and Drescher 2002). Attractive effects are also observed in some biological events (Magal et al. 1996; Gao et al. 2000; Zhou et al. 2001; Hindges et al. 2002a; Holmberg and Frisen 2002; Eberhart et al. 2004). In addition to their roles during development, Eph receptors have been shown to regulate adult functions such as learning and memory (Dalva et al. 2000; Grunwald et al. 2001b; Henderson et al. 2001; Takasu et al. 2002b), and pathological conditions such as tumorigenesis (Brantley-Sieders et al. 2004; Surawska et al. 2004). These functions will be discussed in more detail in Part II.

Before Eph/ephrin signaling starts, ephrin molecules form loosely bound dimers (Himanen et al. 2001). By contrast, Eph receptors usually remain monomers (Fig. 1-3 A).

On the unstimulated Eph monomers, the kinase activity is inhibited because the kinase domain is blocked by the juxtamembrane domain (Wybenga-Groot et al. 2001).

When Eph expressing cells and ephrin expressing cells encounter each other, the Eph monomers bind to ephrin dimers on the opposing cells through a “high affinity receptor-ligand binding interface”. The interaction involves the insertion of a hydrophobic ephrin “G-H loop” into the ephrin-binding domain of the receptor. When two Eph monomers are bound to one ligand pair, they also form low affinity interactions through the H-I loop with the second ephrin in the ligand pair (Himanen et al. 2001; Smith et al. 2004). These interactions result in the dissociation of the ligand pair and allow four molecules to reorient to form a tetramer complex consisting of two Ephs and two ephrins. The tetramer complex adopts a ring like structure (Fig. 1-3 B) in which both receptors bind to both ligands. The formation of the tetramer places the two Eph receptors in a position to phosphorylate each other on key tyrosine residues (Fig. 1-3 C). The phosphorylation of tyrosine residues causes major conformational changes and release the inhibition on the receptor kinase activity.

After receptor-ligand tetramers form, they sometimes further cluster into bigger aggregates ,which consequently amplifies activation. The aggregation process relies on both the extracellular and the juxtamembrane domains of Eph receptors and ephrins (Himanen et al. 2001; Himanen and Nikolov 2003a). The SAM domain of Eph receptors is also involved. Evidence shows that it is the high degree of clustering that explains the activation of Eph receptors that are not bound to ephrins (Wimmer-Kleikamp et al. 2004). We found in our experiments that the overexpression of EphA3 receptor alone in fibroblast cells leads to some level of phosphorylation and activation of the receptors

even in the absence of ligand stimulation, likely through a similar mechanisms (data not shown).

Signaling transduction after Eph activation

Following Eph activation, a complex signaling network comprised of multiple signaling cascades is turned on. Similar to other RTKs, Eph receptors bind to signaling proteins and form substantial receptor/effector protein complexes. To date, an assortment of signaling proteins for Eph receptors has been identified (Pasquale 1997; Klein 2004; Pasquale 2005). Among them, many are adaptor proteins that have unique roles in the signal transduction process. These adaptor proteins possess a number of protein docking sites and enable other effectors to dock near activated Eph receptors and the plasma membrane. They serve as the “scaffolds” and help to organize the receptor/effector complexes (Birge et al. 1996; Buday et al. 2002). Inside the protein complexes, enzymes are activated through their association with the receptor and are able to catalyze reactions on their substrates efficiently. Consequently, downstream signaling pathways are turned on and various cellular phenotypes are expressed. This important topic will be discussed in more detail in Part III.

The activation of Eph/ephrin not only affects the state of downstream signaling pathways, it also has a direct effect on the localization of the receptors and their associated proteins. Previously, it has been shown that both Eph and ephrin molecules interact with PDZ domain proteins and membrane-bound receptors after activation. These interactions help to localize the receptor/effector complexes to specialized membrane regions such as synapses or lipid rafts. Lipid rafts are small membrane domains where

certain lipids (e.g. sphingolipids and cholesterol) and cytoplasmic enzymes (e.g. Src family kinases) are concentrated (Simons and Toomre 2000). Both the aggregation of Eph/ephrin molecules and the localization to lipid rafts or larger lipid patches are important events in Eph mediated signaling (Davy et al. 1999).

Adhesion vs. repulsion

Eph and ephrin signaling regulates both repulsive signaling and attractive signaling (Flanagan and Vanderhaeghen 1998; Zhou 1998; Drescher 2002; Knoll and Drescher 2002). It is intriguing to understand how the switch between adhesion and repulsion is modulated and what factors determine the different cellular responses.

First and foremost, the expression level of different effector proteins in different cell populations directly influences the outcome of Eph receptor activation in these cells. In addition, individual Eph receptors have varying binding affinity with certain effectors and therefore activate different signaling cascades. For instance, in hippocampal neurons, EphAs and EphB2 induce opposite effects in spine formation (Wilkinson 2000; Hindges et al. 2002a). These effects are likely mediated through two distinct groups of RhoGEF proteins that preferentially up-regulate RhoA or Rac1 respectively after activation of Eph receptors.

A second possible explanation for the dual effects stems from the different cell surface ephrin densities, which influence the aggregation level of the receptors. The resulting “receptor to ligand” ratio in the aggregates controls the activation of distinct binding proteins. Consequently, different cellular responses are observed. For example, high ephrin-B1 density induces the recruitment of LMW-PTP and Nck1 to EphB1

receptors and up-regulate integrin signaling (Huynh-Do et al. 1999). It has been speculated that LMW-PTP down-regulates Eph activity because it dephosphorylates Eph receptors (Marte et al. 1997). A second line of evidence supports this hypothesis: EphA3 signaling at a low level of activation mediates attractive effects in retinal neurons but changes to repulsive effects as the receptors become further activated (Hansen et al. 2004).

Alternately, the expression of different Eph receptors on the same cells might affect how they collectively respond to ephrin stimulation. It is known that the truncated form of EphA7 dimerizes with wild type EphA7 and that dimerization is detrimental to normal signaling (Holmberg et al. 2000). A similar blocking effect was also noted on cells expressing Eph receptors and their cognate ligands (Dutting et al. 1999; Yin et al. 2004). For example, temporal retinal neurons express high level of EphA3 receptor and therefore project towards the anterior tectum where ephrin expression is low. The ectopic expression of ephrinA2 in these neurons down-regulates the repulsion induced by high level of ephrins on the tectum and allows them to project to a posterior location on the tectum. This finding suggests that the binding of receptors and ligands on the same cells takes place but lead to no activation of the receptors.

In addition, the interaction between Eph receptors or ephrins with non-effector proteins plays a role in determining cell responses. Research shows that RTKs such as PDGFR, FGFR or cytoplasmic enzymes (e.g. Src family kinases) have the ability to phosphorylate Ephs and ephrins (Bruckner et al. 1997; Chong et al. 2000). Thus, the activity level of these kinases has a significant impact on Eph receptor signaling in reaction to ephrin stimulation under different conditions. Furthermore, extracellular

matrix proteins (ECM proteins) constitute another group of molecules that are involved in Eph mediated signaling. Interaction between Eph receptors with ECM protein receptors explains why ephrin-A5 induces opposite effects on neurons growing over laminin versus fibronectin (Gu and Park 2001; Weinl et al. 2003).

Receptor-ligand cleavage and endocytosis are also possible mechanisms through which the switch from adhesion to repulsion takes place. After the interaction of Eph receptors and ephrin ligands on two cells takes place, the high affinity receptor-ligand binding is difficult to uncouple. Consequently, attractive responses such as cell adhesion or cell migration are likely to be observed. However, when ADAM proteases are present in the extracellular cleft, they recognize the receptor-ligand tetramers and catalyze the proteolysis of the receptors [Fig. 1-3 D, (Hattori et al. 2000; Mancia and Shapiro 2005)]. Interestingly, ephrins are also substrates of the serine protease, rhomboid, which releases the ligands from cell membrane after proteolysis (Pascall and Brown 2004). Similar to other RTKs, the signaling complex is endocytosed and degraded following receptor or ephrin cleavage, [Fig. 1-3 E, F, (Hattori et al. 2000; Marston et al. 2003; Zimmer et al. 2007)]. Evidence exists to show that the cleavage and endocytosis of the bound Eph receptors or ephrins are critical to repulsive phenotypes such as the dissociation of Eph and ephrin expressing cells, cell detachment, and cell sorting. For example, when ephrin-B expressing dorsal retinal neurons are stimulated with EphB2, endocytosis takes place in the reverse direction and the growth cones of these neurons collapse (Mann et al. 2003). The endocytosis of ephrins is necessary for the growth cone collapse to take place. The RhoGEF protein, Vav2 may be involved in this process since Vav knockout animals have major defects in Eph endocytosis and the neurons from these animals display less growth

cone collapse in response to ephrin treatment (Cowan et al. 2005). Another protein, C-Cbl seems to be also important in the internalization of at least some Eph receptors as well (Walker-Daniels et al. 2002; Sharfe et al. 2003). When EphA2 expressing tumor cells were stimulated by ephrin-A1, endocytosis and degradation followed in a C-Cbl dependent way. Similarly, EphA3 activation by ephrin-A1 induces C-Cbl association with Crk adaptors and its phosphorylation by Src kinase. These changes lead to EphA3 degradation at the end. It is necessary to point out that receptor proteolysis and endocytosis are also effective methods to terminate Eph signaling and probably take place at the end of attractive signaling as well.

Eph/ephrin signaling is unique among RTKs

Eph/ephrin signaling has several unique characteristics. First, Eph receptors are only activated by membrane-bound ligands but not soluble ligand monomers. When soluble forms of the ligands are used, artificial cross-linking is usually required to dimerize the ligands before application (Davis et al. 1994). This characteristic is different from how other RTK receptors interact with their ligands. Second, when Eph receptors and their cognate ligands are expressed on the same cells, sometimes they form receptor-ligand complexes that do not transmit signals effectively (Yin et al. 2004). This cis-interaction may be a mechanism to block functional Eph receptor signaling, as we pointed out earlier (Dutting et al. 1999). In addition, both A and B subclass ephrins are able to mediate downstream signaling in spite of their lack of kinase activity (Mann et al. 2003; Davy et al. 2004). Signal transduction through the ligands is termed “reverse signaling” and has not been found on other RTK ligands (Grunwald et al. 2001a; Davy and Soriano 2005).

Lastly, although most of the functions of Eph receptors depend on the kinase activity of the receptors, kinase-independent signaling also exists (Wang et al. 1999; Matsuoka et al. 2005; Miao et al. 2005). We will discuss some of these important topics in detail in another chapter.

Part II. Functions of Eph and ephrin signaling

The wide expression pattern of Eph receptor or ephrin signaling is in accordance with the wide range of functions modulated by them. To this date, Eph receptors have been implicated in cellular events such as axon guidance, topographic projection, tissue patterning, cell sorting, cell migration, synapse regulation, angiogenesis, tumorigenesis and more. In the following section, we discuss some representative examples of these functions, especially in the nervous system.

Axon guidance

The development of the nervous system requires accurate neuronal innervations of targets over a long distance in a complex environment. The precise connections of the neural network are necessary for the nervous system to function. However, the mechanisms for the connections to form remained elusive for many years until Dr. Sperry first proposed the “chemoaffinity theory” in the 1940s. Through extensive research in fish and frogs, Dr. Sperry discovered that the neural circuits were indeed hardwired early in development and the expression of specific molecular tags on the neural cells determined their fate. Therefore, it has been proposed that receptors on the navigating

cells, through recognizing specific molecular tags on target cells, regulates precise neural innervations (Sperry 1950; Sperry 1951). In later studies these molecular tags were further characterized based on their sub-cellular localization and effects: membrane-bound attractive agents, membrane-bound repulsive agents, diffusible attractive agents and diffusible repulsive agents (Tessier-Lavigne 1995; Tessier-Lavigne and Goodman 1996). The ligands for Eph receptors fall in the categories of membrane-bound attractive or repulsive agents. For instance, they have been implicated in guiding neuronal axons across the midline and modulating the formation of the hippocampal-septal projection (Cheng et al. 1995; Drescher et al. 1995; Flanagan and Vanderhaeghen 1998; Wilkinson 2001; Yue et al. 2002).

A well-characterized system in which Eph/ephrin signaling plays a pivotal role is the retinotectal projection (Fig. 1-4). Axonal projections from the retina to the superior colliculus (SC) in mammal or optic tectum in chicken, frog and fish are topographically established during development. An inspection of the expression pattern of various Eph receptors and ligands discloses that along both A/P axis and D/V axis, graded expression pattern exists [(Drescher 1997; Monschau et al. 1997), Fig. 1-4 A]. In the retina, a high temporal to low nasal gradient of EphA3 and EphA5 expression is noted, while a high posterior to low anterior gradient of ephrin-A2 and ephrin-A5 is found on the chick tectum. Temporal retinal neurons, with high expression of EphA receptors, are sensitive to ligand-induced repulsion. As a result, they are repelled from the posterior tectum and project towards the anterior tectum instead, where low ligand concentration is encountered. In comparison, the nasal axons with low EphA expression venture into the high ligand expressing posterior tectum as a low level of ephrin stimulation has been

found to be attractive (Hansen et al. 2004). In addition, there is a uniform expression of EphA4 and a high nasal, low temporal expression of ephrin-A ligands in the retina as well. The retinal expression of ephrin-As, in a gradient opposite to the EphA receptors is found to sharpen the EphA gradient by exerting a blocking effect on EphA signaling (Knoll and Drescher 2002; Yin et al. 2004). Similar to the receptor/ligand expression along the A/P axis, graded expression of EphB receptors and ephrin-Bs exists along the D/V axis. The interaction between EphBs and ephrin-Bs regulates the topographic establishment of projection between the retina and the tectum along this axis (Drescher et al. 1997; Mann et al. 2002).

Studies have shown that when EphA3 is ectopically expressed in retinal neurons, these neurons are inclined to project to an anterior position whereas other neurons shift posteriorly to fill in the space (Brown et al. 2000). Furthermore, in ephrin-A5 null mice, the temporal retinal axons overshoot into the posterior SC, confirming the importance of ephrin-induced repulsion in the formation of the projection. Interestingly, nasal axons shift anteriorly to fill the space left empty by the temporal retinal neurons in these mice (Frisen et al. 1998; Feldheim et al. 2000). A competition mechanism was therefore proposed to explain these findings: the temporal and nasal retinal neurons compete to innervate the SC and their ability to do so largely depends on their sensitivity to repulsion.

Ephrin-Bs are also able to mediate axon guidance through reverse signaling. For instance, EphB2 null mice were found to have severe defects in the projection of the posterior part of the anterior commissure of the cerebellum, the acP tract. The axons in the tract no longer extend across the midline like in wild type animals. Instead, they

project ventrally (Henkemeyer et al. 1996). An analysis of the expression pattern reveals that acP tract neurons express ephrin-B2 while tissues underlying the acP tract express EphB2. This expression pattern indicates that the reverse signaling through ephrin-B2 is critical in the axon guidance of the acP tract axons. In some studies, the expression of a truncated EphB2 gene without kinase activity was able to rescue the EphB2 null phenotype, further confirming the significance of ephrin-B reverse signaling (Cowan et al. 2004).

Axon branching

During the establishment of topographic mapping of retinal axons, the formation of collateral branches from axonal shafts is a critical event. It is observed that usually retinal axons grow into the posterior region of the tectum past their final destinations. Although these axons originally overshoot their target sites, collateral branches extend into their future termination zones, preferentially towards where future synaptic connections form (Fig. 1-4 A). Later, the growth cones retreat to their final termination zone and form synapses, while all ectopic branches disappear when the topographic projection is complete [(Pasquale 2005), Fig. 1-4 B].

Eph forward signaling is involved in the inhibition of branching beyond the final termination zone through repulsion induced by high ephrin-A2/-A5 in the posterior tectum (Yates et al. 2001; Sakurai et al. 2002). To further support this finding, *in vitro* experiments have shown that temporal neurons preferentially branch over anterior tectal tissues (Yates et al. 2001). However, when EphA3-Fc was applied to block ephrin function in the tectal tissues, the temporal neurons branched equally over anterior or

posterior tectal membranes. Moreover, the Eph/ephrin signaling is also prominent in determining the direction of collateral branch extension within the termination zone. Ephrin-B1 is expressed in a high medial to low lateral gradient in the SC (Hindges et al. 2002b). Similar to the dual effects ephrin-A5 has on retinal axons along the A/P axis (Gao et al. 1999), ephrin-B1 at a low concentration attracts branches in a medial direction but guides the branches in a lateral direction through repulsion at a high concentration.

Axon fasciculation and defasciculation

In general, when axons originate from the same areas and project to neighboring sites, they often form bundles and extend in the same direction (Yates et al. 2001). Eph/ephrin signaling plays a critical role in fasciculation. For example, *in vitro* assays have demonstrated that ephrin A5 promotes the fasciculation of EphA expressing cortical axons. Fasciculation allows most axons to follow the pioneer axons and navigate efficiently to their targets. Indeed, it has been reported that axons that follow usually possess smaller and less elaborate growth cones compared to the pioneer axons (Wilson and Easter 1991; Caras 1997). Near the destination sites, the axon bundles undergo defasciculation to allow individual axons to exit and reach their target neurons. EphB receptors play major parts in regulating the de-fasciculation of hippocampal neurons (Chen et al. 2004).

Spine formation

During development, neuronal dendrites extend filopodia towards axons of connecting neurons. Once contact is made, the dendritic filopodia transform into

dendritic spines, which contain post-synaptic termini of the synapses (Murai and Pasquale 2004). The dendritic spines have a distinct mushroom-like appearance and an elaborate internal structure. The enrichment of neurotransmitter receptors on spines allow stimulus relay from the pre-synaptic termini to post-synaptic termini to take place. Formation of dendritic spines is a process that requires both Eph and ephrin functions. As reported by Penzes et al, after activation of EphB receptors in hippocampal neurons, RhoGEF Kalirin activates Rac1 and downstream effector PAK. The up-regulation of Rac1 activity is found to be necessary to spine formation (Ethell et al. 2001; Penzes et al. 2003). In addition, the clustering of Eph receptors promotes the spine maturation process via the interaction with proteins such as Syndecan-2 and PDZ domain containing proteins like PICK1, GRIP, Syntenin (Torres et al. 1998; Bruckner et al. 1999; Knoll et al. 2001). Indeed, syndecan-2 phosphorylation after EphB2 activation has been found to be adequate in inducing spine formation (Ethell et al. 2001).

Furthermore, Eph receptors are also involved in synaptic plasticity. Grunwald et al observed that EphB2 interacted with NMDA type glutamate receptors and regulated synaptogenesis (Grunwald et al. 2001a). Indeed, EphB2 induces the Src kinase mediated phosphorylation of NMDA receptor and increases calcium influx mediated by NMDA receptors in response to glutamate stimulation (Takasu et al. 2002a). In EphB2 null animals, long-term potentiation (LTP) and synaptic depression are both severely compromised, confirming the involvement of Eph signaling in spine function and synaptic plasticity.

Cell proliferation and differentiation

Eph/ephrin signaling affects cell proliferation and cell fate. During development, the injection of ephrin-A-Fc and EphA-Fc into the sub-ventricular zone increases cell proliferation by inhibiting the endogenous Eph-ephrin signaling (Conover et al. 2000). It is therefore speculated that Eph/ephrin activation regulates the size of brain by inducing apoptosis (Depaepe et al. 2005). *In vitro* treatment of cultured cortical progenitors confirms the speculation since soluble ephrin-A ligands induce rapid cell death in a caspase-3 dependent way. Furthermore, ephrin-A2 knockout animals have a larger than usual olfactory bulb, suggesting that ephrin-A2 mediated signaling negatively regulates cell proliferation (Holmberg et al. 2005). Similarly, in EphA7 loss-of-function mutant animals, a two-time decrease of cell death and a larger size brain were observed (Depaepe et al. 2005).

Eph signaling is also implicated in the differentiation of a variety of cells including human hematopoietic cells, stem cells, and retinal cells (Suenobu et al. 2002; Moore et al. 2004). The role of Eph signaling in neural cell fate is just beginning to be understood. For example, the forward signaling of EphA receptors promotes cell differentiation of neural precursor cells in a MAPK dependent fashion (Aoki et al. 2004).

Tissue patterning

The developing vertebrate hindbrain is characterized by a series of individual segments called “rhombomeres”. Within each segment, distinct sets of genes are expressed. The segmentation of the hindbrain and the maintenance of the boundaries between two adjacent rhombomeres are important events in the hindbrain development.

Insights on how Eph signaling is involved in these processes were gained when it was discovered that loss of Eph signaling led to the disruption of restricted gene expression pattern in the segments (Xu et al. 1995). The expression of Eph receptors and ephrin in the zebrafish hindbrain accords with such a role: EphA4, EphB2 and EphB3 are expressed in rhombomeres r3 and r5 while ephrin-B1, -B2 and -B3 are expressed in r2, r4 and r6 (Henkemeyer et al. 1994; Gale et al. 1996a). When ectopic expression of either ephrin-B2 or EphA4 was induced in developing zebrafish embryos, it was found that ephrin expressing cells were sorted to the edges of Eph expressing segments and Eph expressing cells were excluded from ephrin expressing segments (Mellitzer et al. 1999; Xu et al. 1999). Thus, both forward signaling and reverse signaling are required for this cell sorting process to take place.

Cell migration

The migration of neural crest cells is an important event that gives rise to spinal and autonomic ganglia. This process requires forward signaling through EphB receptors and EphA4 receptor in mice (Smith et al. 1997; Wang and Anderson 1997). *In vitro* assays revealed that ephrin-B ligands had the ability to control the migration of neural crest cells (Krull et al. 1997; Wang and Anderson 1997). In ephrin-B2 null embryos, the migration of neural crest cells follows a more scattered route compared to in wild type mice (Smith et al. 1997). Since a truncated ephrin-B2 without cytoplasmic segment rescued the phenotype, it was proposed that the reverse signaling through ephrin-Bs was not required.

Angiogenesis

Angiogenesis is the formation of blood vessels through remodeling of a primitive endothelial network during embryogenesis. The functional significance of Eph and ephrin molecules in angiogenesis was first demonstrated when ephrin-B2 deficiency in embryos was found to lead to a disrupted vascular system (Wang et al. 1998). It has also been shown that the activation of EphB receptors induces the formation of a primitive endothelial network (Daniel et al. 1996; Stein et al. 1998b). Furthermore, EphB4 and ephrin-B2 are expressed in the veins and arteries respectively (Wang et al. 1998; Gerety et al. 1999; Adams and Klein 2000). Repulsive interactions induced by Eph-ephrin signaling may help to create the arterial-venous boundaries. In addition, downstream signaling of Eph receptors and ephrins also regulates cell adhesion through integrin signaling, which is important in helping the vascular network to settle in different tissues (Huynh-Do et al. 1999).

Tumorigenesis

The initial identification of high expression of Eph molecules in cancer cell lines intrigued researchers, and led to studies of their role in tumorigenesis. Today, almost all Eph receptors and ephrin ligands have been found to display unusual expression pattern in tumor tissues, strongly suggesting a direct involvement of these proteins in tumor development. For example, EphA2 receptor is up-regulated in prostate tumors, colorectal tumors, lung tumors and melanomas and the level of up-regulation corresponds to the stage of tumor progression and metastasis (Easty et al. 1999; Ogawa et al. 2000; Kinch and Carles-Kinch 2003). Furthermore, Zelinski et al reported that overexpression of

EphA2 was enough to induce malignant transformation in mammary epithelial cells. More importantly, though the kinase activity of the receptor is critical in some cases, receptor activation takes place in a ligand independent way due to the clustering of the receptors (Zelinski et al. 2001; Fang et al. 2005). These observations all suggest that Eph receptors directly promote tumorigenesis at least in some cases.

Surprisingly, studies on the role of Eph receptors in tumorigenesis have also led to conflicting results. For instance, the loss of EphB receptors in colorectal cancer is strongly correlated with the state of cancer progression (Batlle et al. 2005). Similarly, EphA1 was found to be significantly down-regulated in two kinds of non-melanoma skin cancers (Hafner et al. 2006). In addition, ephrin-B and ephrin-A activation have been shown to suppress cell proliferation induced by neurotrophins, probably through the activation of PI3K/PDK1/Akt pathway (Tang et al. 2000a; Tang et al. 2000b). These findings seem to contradict other studies we discussed earlier and reveal the complex nature of Eph-ephrin signaling in tumor development. The dual effects of Eph-ephrin signaling in tumor tissues is therefore analogous to the signal transduction in the nervous system, where both attractive and repulsive responses are triggered by the same stimulation.

In addition to these studies which demonstrate that unusual expression of Eph receptors and ligands directly leads to tumor growth (Zelinski et al. 2001), evidence exists to suggest Ephs and ephrins contribute to tumor progression by regulating cell attachment and migration. Through regulating actin cytoskeleton reorganization and integrin signaling, the activation of Eph receptors increases the metastatic potential of melanomas and mammary tumors (Andres et al. 1994; Vogt et al. 1998).

Based on these works, numerous research groups have attempted to reverse tumor progression through disrupting Eph signaling and have achieved significant success (Brantley et al. 2002; Brantley-Sieders et al. 2004; Duxbury et al. 2004; Fang et al. 2005). These works will help to pave the road towards using Eph inhibition in cancer treatment.

Part III. Signaling pathways downstream of activated Eph receptors and ephrins

Binding proteins of Eph receptors

After ephrin stimulation, the phosphorylation of key tyrosine residues on Eph receptors or ephrins enables signaling molecules to interact with them (Kalo and Pasquale 1999). Activated signaling molecules turn on diverse downstream pathways, which eventually regulate the cellular events that we previously discussed (Kullander and Klein 2002). To date, more than 20 proteins have been confirmed to be directly associated with Eph receptors [Table 1-1, (Himanen and Nikolov 2003a; Huot 2004; Pasquale 2005)] and the signaling cascades they regulate are summarized (Fig. 1-5).

A large percentage of the identified binding proteins contain Src homology-2 domains (SH2), such as Src, Abl/Arg, Crk, Grb2, Nck1, SHP-2, PLC gamma, RasGAP, P85-PI3K (Pandey et al. 1995; Holland et al. 1997; Stein et al. 1998a; Zisch et al. 1998; Kalo and Pasquale 1999; Kullander and Klein 2002). Research on the properties of the SH2 motifs reveal that SH2 domains have high affinity binding for phosphorylated tyrosine residues and interact with the cytoplasmic domains of phosphorylated receptor tyrosine kinases (Chattopadhyay et al. 1999). It is important to point out that the SH2

domains of different proteins exhibit distinct binding preferences, which largely depend on the three amino acids behind the phosphorylated tyrosine residue (Songyang et al. 1993). In addition, SH2 domain proteins often possess other protein binding domains or catalytic domains, allowing them to recruit other proteins into the receptor/effector complex and regulate a wide range of signaling events such as actin cytoskeleton restructuring, MAPK activity modulation, and integrin signaling.

Another major group of binding proteins contain the PDZ domain. This domain interacts with the PDZ binding motif at the C-terminus of Eph receptors. Proteins with PDZ domains include Syntenin, Afadin-6 (AF-6), protein interacting with C kinase-1 (Pick1) and Glutamate receptor interacting protein (Grip) (Hock et al. 1998b; Torres et al. 1998; Buchert et al. 1999). The function of these proteins remains relatively unclear, though some studies suggest that they might assist in localizing Eph receptors or ephrins to specialized membrane regions such as synapses or lipid rafts. For example, research has shown that Pick-1 mediates the interaction between Eph receptors and other synaptic receptors and activates the PKC kinase cascade (Bruckner et al. 1999; Knoll et al. 2001).

Some signaling proteins interact with Eph receptors through domains other than SH2 or PDZ domains. These proteins include Ephexin, LMW-PTP, FAK (Stein et al. 1998a; Miao et al. 2000; Sahin et al. 2005).

Eph signaling: Src family kinases and Nck1

Several binding proteins have been implicated in the process of cytoskeleton restructuring. In response to ephrin stimulation and Eph activation, they set the cytoskeleton machinery in motion and lead to either repulsive or attractive responses in a

context-dependent manner. Src family kinases such as Abl/Arg and adaptor protein Nck1 are good examples of these proteins.

Abl and Arg proteins are members of Src family kinases (SFK), a family of SH2-domain-containing cytoplasmic tyrosine kinases. Like other SFK members, they phosphorylate a wide array of substrates including RTKs and cytoskeletal regulatory proteins. They are different, however, from other SFK members because they possess a F-actin-binding motif at the C terminus (Robinson et al. 2000; Hernandez et al. 2004). Abl/Arg proteins are co-expressed with Eph receptors in the brain and are mainly localized on the growth cones, indicating that Abl/Arg proteins may play significant roles in Eph mediated pathfinding (Salgia et al. 1997). Indeed, the Abl/Arg kinases associate directly with the juxtamembrane domain of Eph receptors through their SH2 domain upon ephrin-B1 stimulation. Surprisingly, this binding leads to a decreased kinase activity of Abl/Arg (Yu et al. 2001). In addition to the juxtamembrane domain binding, Abl/Arg also interact indirectly with Eph receptors through Crk and other Src family kinases (Wang et al. 1996; Plattner et al. 1999). Since Abl/Arg have been found to have a promotional role in neurite outgrowth (Zukerberg et al. 2000), it is possible that the stabilized association of Abl/Arg to Eph receptors serves to sequester Abl/Arg kinases from their substrates and leads to the downregulation of Abl/Arg function. A second line of evidences shows the functional absence of Abl/Arg in the knockout animals result in serious defects of the nervous system, which bear striking resemblance to ephrin-A5 knockout animals (Koleske et al. 1998; Holmberg et al. 2000).

Other SFKs are involved in cytoskeleton regulation as well. Src kinase is known to phosphorylate a range of proteins involved in focal adhesion or cytoskeleton activity,

such as cortactin, tensin, paxillin, p130Cas and FAK (Burridge et al. 1988; Huang et al. 1997; Parsons and Parsons 1997). Through the phosphorylation of these important substrates, SFKs exert direct regulatory effects on cytoskeleton dynamics. For instance, the binding of Src kinases and FAK leads to the phosphorylation of both proteins and the activation of MAPK pathway (Guan 1997). Cortactin, a F-actin binding protein, causes F-actin bundles to form. Its phosphorylation by SFKs causes a reduction of its cross-linking ability.

Nck1 also plays a prominent role in the regulation of cytoskeleton restructuring. The majority of Nck1 binding proteins we know so far have been implicated in cytoskeleton regulation. Among them, p21 activated kinase (PAK), Wiskott Aldrich syndrome protein (WASp), Nck1 interacting protein (NIK) and Nck1 associated protein 1 (Nap-1) are best characterized (Bagrodia et al. 1995; Rivero-Lezcano et al. 1995; Ruan et al. 1999). PAK proteins, WASp/WAVE proteins and Nap-1 have all been found to bind to Rho family GTPases, which are directly involved in regulating actin-based cytoskeleton (Nobes and Hall 1995; Hummel et al. 2000). Furthermore, when PAK or NIK is each mutated, the resulting phenotype resembles the phenotype of Nck1 knockout animals (Ruan et al. 1999). The function of Nck1 was confirmed through studies on *Drosophila* Nck1 homolog Dreadlocks (Dock) (Garrity et al. 1996; Desai et al. 1999). Without the expression of Dock, it has been shown that axons of photoreceptor neurons no longer stop at the correct targets. Since Nck1 is the focus of our research, its role in Eph receptor signaling will be discussed later in more detail.

Eph signaling: Ras/MEK/ERK pathway

Ras is a small GTPase that was first found in tumor cells. It mediates important signal transduction from cell surface RTKs to downstream pathways that regulate cell proliferation, transcription, and cytoskeleton modulation. Normally, active Ras molecules bind and activate Raf family kinases. Active Rafs then stimulate the mitogen activated extracellular signal regulated kinase activating kinase (MAPKK or MEK) family members. As the name suggests, these kinases phosphorylate and activate MAPK members [ERK1 and ERK2, (p42/44)]. The active ERK proteins are capable of phosphorylating many cytoplasmic substrates and regulating gene transcription, cell cycle, cell adhesion and cell mobility (Howe et al. 2002; Roux and Blenis 2004; Viala and Pouyssegur 2004).

Ras regulated pathway is critical in signal transduction downstream of Eph receptors. Several identified binding proteins of Eph receptors have been implicated in this pathway and the best understood one is Ras small GTPase guanine nucleotide activating protein (RasGAP) (Holland et al. 1997; Elowe et al. 2001). A SH2 domain containing protein, RasGAP interacts directly through both the juxtamembrane domain and the kinase domain with EphB1 receptor in a kinase dependent way (Hock et al. 1998a; Zisch et al. 2000; Elowe et al. 2001). Alternately, RasGAP has been found to interact with EphB2 indirectly through the mediation of p62dok (Holland et al. 1997). RasGAP converts GTP bound to Ras to GDP and renders the Ras protein inactive. Therefore, when RasGAP is brought in close proximity of Ras molecules localized near the plasma membrane by binding with Eph receptors, ERK1/2 activity is down-regulated. The down-regulation of ERK1/2 following EphB activation has been confirmed to be important in neurite

retraction, growth cone collapse, and other repulsive signaling events (Tong et al. 2003). In addition, down-regulated ERK activities triggered by Eph activation explains why Eph receptors, compared to other RTKs, do not have a strong mitogenic effect on cell cultures (Brambilla and Klein 1995; Bruce et al. 1999).

Opposite to the function of RasGAP, multiple SH2 adaptor proteins such as Crk, Grb2, and Nck1 associate constitutively with Sos, a Ras-guanine nucleotide exchange factor (RasGEF). Once Eph receptors become activated, these adaptor proteins associate with phosphorylated Eph receptors through their SH2 domains and recruit Sos (Stein et al. 1996; Hock et al. 1998a; Stein et al. 1998a). By catalyzing the exchange of GDP to GTP on Ras, Sos is able to activate Ras and turn on the MAPK pathway (Hu et al. 1995; Byrne et al. 1996). This pathway might be the underlying mechanism of Eph mediated tumorigenesis observed in some studies.

Together, these observations show Eph activation either up-regulate or down-regulate Ras/MEK/ERK pathway, depending on the signaling proteins present. It would be of great interest to understand how the interactions of RasGAP, Grb2, Nck1 and Crk with Eph receptors are differentially regulated, and if these differences account for different ephrin induced phenotypes.

The PDZ domain protein AF-6 is a Ras binding agent (Hock et al. 1998b). Through binding with Eph PDZ motif at the C-terminus, AF-6 might play a role in regulating Ras-activated MAPK pathway. Additionally, activated Ras/Raf/MAPK pathway is linked to the PI3 kinase pathway, important in cytoskeleton regulation, cell migration and cell adhesion (Hamada et al. 2003; Maekawa et al. 2003). Due to its importance in RTK signaling, PI3 kinase pathway will be described in the following section.

Eph signaling: PIP₂ related pathways

PI3 Kinase associates with Ras family members and becomes activated when they are translocated in close proximity of the plasma membrane (Navolanic et al. 2003). The association of PI3K p85 subunit with EphA2 and EphA8 through the SH2 domain assists in this translocation and activation process (Pandey et al. 1994; Gu and Park 2001). Active PI3 kinase catalyzes the phosphorylation of phosphoinositide and the phosphorylated products bind and activate phosphoinositide-dependent protein kinase 1 (PDK1) and anti-apoptotic factor Akt kinases. The activation of PI3K/PDK1/Akt pathway has been implicated not only in the regulation of apoptosis and gene transcription (Navolanic et al. 2003) but also in integrin activity regulation, cell adhesion, and vesicle trafficking (Wennstrom et al. 1994; Shimizu et al. 1995; Carpenter and Cantley 1996). It has been shown PI3K plays a direct role on cell adhesion through direct modification of lipid molecules or alternately, PI(4,5)P₂, a substrate of PI3K regulates actin cytoskeleton through acting-binding proteins (Carpenter and Cantley 1996).

Phospholipase C gamma (PLC γ) is a primary effector that associates with EphB2 and EphA4 receptors via the juxtamembrane domain (Holland et al. 1997; Zisch et al. 1998). After associating with Eph receptors and being translocated to the plasma membrane, PLC γ binds to PI(4,5)P₂ and converts PI(4,5)P₂ to diacylglycerol (Hall et al. 1996). Diacylglycerol regulates cytoskeleton restructuring through the activation of calcium channels and PKC (Clark and Brugge 1995). PKC in turn phosphorylates Raf kinase and contributes to the regulation of MAPK pathway (Navolanic et al. 2003).

Eph signaling: small GTPases

As discussed in an earlier section, cytoskeletal rearrangement is an integral part of Eph mediated cell responses such as cell migration, neurite outgrowth, and growth cone collapse. Other than the proteins we discussed before, small Ras-like GTPases, Rac, cdc42 and RhoA, are also known to be key players (Dickson 2001). The activities of these three GTPases are major determining factors in actin cytoskeleton dynamics. Activated RhoA through its effector ROCK, leads to growth cone collapse and increased contraction (Dergham et al. 2002; Fournier et al. 2003). The inhibition of Rho activity, in comparison, reduces growth cone collapse rate (Wahl et al. 2000). Rac and cdc42, on the other hand, transduce signaling through p21 cdc42/Rac activated kinases (PAKs) and promote axonal outgrowth (Nikolic 2002; Sahin et al. 2005). They are also implicated in the formation of lamellapodia and filopodia, respectively (Nobes and Hall 1995). The activity of Rac and cdc42 is down-regulated by RhoA, possibly through competition for binding partners, since Rac inhibition induces effects that are similar to those induced by RhoA activation (Dickson 2001).

The PAK proteins are directly activated by Rac and cdc42. Eph receptor activation, however, plays a role in the activation of PAKs through adaptor protein Nck1. PAKs bind to Nck1 through the SH3 domains (Rivero-Lezcano et al. 1995; Quilliam et al. 1996). As Nck1 binds to activated EphB1 receptor through the juxtamembrane domain tyrosines, it helps to translocate PAKs from cytosol to the plasma membrane and brings the PAK proteins in close proximity of Rac and cdc42 (Bokoch et al. 1996). Consequently, PAKs are activated and able to phosphorylate their substrate proteins.

Ephexin, a Dbl family GEF protein, was first identified through a yeast two-hybrid screen with the intracellular domain of EphA4 as the bait (Shamah et al. 2001). It interacts with the kinase domain of EphA receptors through its Dbl-homology-pleckstrin-homology domain (DH-PH) in a kinase independent way. As a Rho guanine nucleotide exchange factor (RhoGEF), ephexin activates Rho family GTPases. However, once EphA4 receptor becomes activated by ephrin-A1, ephexin is phosphorylated on the Tyr87 residue and the specificity of ephexin activity dramatically changes (Sahin et al. 2005). Instead of activating RhoA, cdc42 and Rac equally, ephexin preferentially activates RhoA activity, resulting in local growth cone collapse and morphological changes, phenotypes characteristic of RhoA up-regulation.

Another RhoGEF protein, Vav2 was identified recently as an EphA4 binding protein. Unlike ephexin, Vav2 is a Vav family guanine nucleotide exchange factor and contains a SH2 domain. After binding to phosphotyrosine residues on EphA4, Vav2 activates Rho small GTPases and induces growth cone collapse. Furthermore, as mentioned briefly earlier, endocytosis of EphA4, critical to growth cone collapse, may be regulated by Vav2 (Cowan et al. 2005).

Tiam1, a novel RhoGEF, interacts with both EphA2 and ephrin-B1. It up-regulates Rac activity preferentially. Dominant negative Tiam1 inhibits neurite outgrowth strongly, similar to the phenotype induced by overexpression of truncated forms of EphA2 or ephrin-B1 (Tanaka et al. 2004).

Other RhoGEFs, though not bound directly to Eph receptors, have been also implicated in the regulation of small GTPases downstream of Eph activation. Kalirin, for example, is a RhoGEF that specifically activates Rac1 after the activation of EphB2

receptor (Penzes et al. 2001; Penzes et al. 2003). An immediate result of this activation is enhanced spine formation in hippocampal neuron cultures.

In addition, RasGAP regulates RhoGAP activity through direct binding at least in some cases (McGlade et al. 1993). RhoGAP inactivates RhoGTPases by activating the hydrolysis of GTP on Rho GTPases. Thus, the interaction between RasGAP and RhoGAP links Ras/MEK/ERK pathway to small GTPases.

Eph signaling: cell adhesion

Integrin signaling is an important signaling pathway that directly regulates the response of cells to extracellular matrix proteins. It is closely related to the MAPK pathway, as neuronal cell attachment to fibronectin activates MAPK through integrin signaling. PLC γ activity may be responsible for this activation (Finkelstein and Shimizu 2000). EphB2 activation, on the other hand, causes a reduction of MAPK activity (Schlaepfer and Hunter 1998; Elowe et al. 2001) and has a regulatory effect on integrin signaling (Stein et al. 1998b).

Eph receptors exert their regulatory effects on integrin function through the modulation of R-Ras activity. A member of Ras small GTPase family, R-Ras activates PI3K but is not activated by Sos. Consequently, R-Ras does not activate Raf or the MAPK pathway (Marte et al. 1997). Instead, R-Ras is involved in the regulation of cell adhesion through integrin signaling. EphB2 receptors as well as Src family kinases phosphorylate R-Ras molecules on Tyr66 residue (Zou et al. 1999) and inhibit its binding with Raf kinase (Zou et al. 2002). RasGAP, on the other hand, regulates R-Ras activity by catalyzing the conversion of GTP bound to R-Ras to GDP. When RasGAP becomes

associated with Eph receptors, it suppresses cell adhesion through R-Ras inactivation (Dail et al. 2006). As shown by Dail et al, regulation of R-Ras activity through Src kinase mediated phosphorylation or through RasGAP mediated inactivation are both important to ephrin-mediated responses. Whereas RasGAP is required to induce EphB2 expressing Cos-1 cells to retract their peripheries after ephrin-B1 treatment, both RasGAP and Src kinase are critical to Eph mediated inhibition on cell migration. Moreover, growth cone collapse of primary cortical neurons also seems to require both mechanisms to down-regulate the R-Ras activity. In addition to these regulations on R-Ras, SHEP-1 protein is involved in the R-Ras activity regulation as well (Dodelet et al. 1999). SHEP-1, a protein consisting of a SH2 domain and a RasGEF domain, interacts with R-Ras but does not activate it. Instead, it recruits R-Ras to the vicinity of activated Eph receptors and leads to R-Ras phosphorylation by Src family kinases.

Focal adhesion kinase (FAK) is another key player in integrin signaling regulation. Integrin-mediated focal adhesions are highly enriched with FAK and FAK is directly activated by integrins (Nobes and Hall 1995). FAK is found to be expressed in the brain and involved in the regulation of cell adhesion and cell migration (Carter et al. 2002; Beggs et al. 2003). Some studies suggest that FAK is able to negatively regulate axonal extension and dendritic spine in hippocampal neurons (Karanian et al. 2005; Moeller et al. 2006). FAK activity is regulated by Ephs and ephrins in several ways. EphA2 binds to FAK constitutively and Eph-associated Src kinases phosphorylate and activate FAK (Parsons and Parsons 1997). Paradoxically, FAK is dephosphorylated by SHP-2, a phosphatase that associates with the activated EphA2 receptors (Miao et al. 2000). Following dephosphorylation, inactive FAK dissociates from EphA2. Indeed, it has been

noted that EphA2 activation in carcinoma cells results in the suppression of both FAK activity and integrin-mediated cell adhesion, indicating that FAK is dephosphorylated.

Eph receptor activation induces cell adhesion through integrin signaling (Stein et al. 1998b; Kikawa et al. 2002). In transfected human kidney cells, EphB1 activation elicits recruitment of LMW-PTP to the SAM domain and Nck1 to the juxtamembrane domain and leads to enhanced integrin $\alpha 5\beta 1$ mediated cell adhesion. Another line of evidence suggests that the recruitment of LMW-PTP and Nck1 is an event that occurs in the presence of high density of ephrin ligands (Stein et al. 1998b; Huynh-Do et al. 1999; Kikawa et al. 2002). In addition, Nck1 interacting kinase (NIK) links Nck1 recruitment and integrin signaling, as the activation of NIK following association with Nck1 results in the activation of both c-Jun N-terminal kinase (JNK) and integrin (Becker et al. 2000).

Lastly, active PI3K was found to be required for integrin signal transduction in addition to regulating cell apoptosis and gene transcription. As Gu and Park reported, EphA8 regulates integrin signaling by interacting with PI3K in a kinase-independent way and helps localize PI3K to the plasma membrane near the integrin signaling complex (Gu and Park 2001).

Reverse signaling through the ephrin-Bs

As briefly discussed before, ephrin ligands have the ability to transduce signaling (Davy et al. 1999; Knoll et al. 2001; Mann et al. 2002; Davy and Soriano 2005). The phenomenon was first discovered when research showed that EphB2 receptors activated ephrin-B1 and ephrin-B2 and led to the phosphorylation of their tyrosine residues (Holland et al. 1996). The phosphorylated ephrin-Bs are capable of recruiting effector

proteins and activating signaling cascades, similar to phosphorylated Eph receptors. This novel signaling was named “reverse signaling” as opposed to “forward signaling”, which takes place through the receptors. In order to understand the effects of reverse signaling, new strategies are devised to discern reverse signaling from forward signaling. In these new strategies, wild type ephrins or Ephs are not eliminated from the system. Instead, they are replaced by mutated forms that lack the functional cytoplasmic region. These mutated ephrins or receptors are incapable of inducing downstream signaling events, and therefore enable the experimenters to dissect out and analyze reverse and forward signaling as two separate events.

Through these new approaches, it was found that reverse signaling by ephrin-Bs is implicated in many functions including axon guidance, cell sorting, cell migration (Henkemeyer et al. 1996; Wang et al. 1999; Xu et al. 1999). Similar to forward signaling, reverse signaling leads to either repulsive or attractive responses (Henkemeyer et al. 1996; Knoll et al. 2001; Kullander et al. 2001). The details of the mechanism are just beginning to be understood.

The intracellular domain of ephrin-Bs contains a PDZ motif at the N-terminus and is highly conserved. After being stimulated by Eph receptors, the ephrinBs undergo phosphorylation by cytoplasmic kinases such as Src family kinases, or activated RTKs such as PDGFR or FGFR (Holland et al. 1996; Bruckner et al. 1997; Chong et al. 2000; Palmer et al. 2002). The phosphorylated ephrin-Bs recruit effectors and adaptor proteins. The signaling proteins downstream of ephrins are summarized in Table 1-2 and the signaling cascades regulated by ephrins are shown in Fig. 1-6.

Research shows five tyrosine residues of ephrin-B2, particularly tyrosine 304, are strongly phosphorylated upon Eph stimulation. The phosphorylation of ephrin-Bs mediates binding with downstream effectors directly, and also enables PDZ-RGS protein to dock on the PDZ motif (Su et al. 2004).

Nck2 has been found to associate ephrin-B1 *in vitro* through its SH2 domain (Cowan and Henkemeyer 2001). A member of Nck adaptor family, Nck2, is well-known in bridging effectors proteins to phosphorylated RTKs. Through its three SH3 domains, Nck2 interacts with several proteins including Abl-interacting protein-1 (Abi-1), a scaffold protein (Axin), and c-Cbl-associated protein (CAP). Interestingly, previous studies implicate all three of these proteins in actin cytoskeleton regulation, suggesting that Nck2 mediates ephrin-B signaling through regulating cytoskeletal rearrangements (Dai and Pendergast 1995; Shi et al. 1995; Ribon et al. 1998).

Though the mechanisms are not completely clear, it has been determined that the binding through the PDZ sequence is required in reverse signaling (Davy et al. 2004). For instance, PDZ-RGS, a PDZ binding protein and a regulator of heterotrimeric G protein signaling, links G protein coupled signaling to ephrin signaling (Lu et al. 2001; Lu et al. 2004). Other PDZ binding proteins including Syntenin, Pick1, Grip, Grip2, PHIP, and PTP-BL have been shown to interact with ephrin-Bs as well (Torres et al. 1998; Bruckner et al. 1999; Lin et al. 1999; Palmer et al. 2002). Some of these proteins such as Pick1, Grip1/2, and PHIP mediate synaptic localization of ephrins, while PTP-BL is involved in deactivating ephrin-Bs through dephosphorylation.

Recently, new progress has been made in identifying binding proteins for ephrin-Bs. Tiam1, a Rac guanine nucleotide exchange factor was found to associate with both

ephrin-B1 and EphA3 and mediate neurite outgrowth (Tanaka et al. 2004). E14 cortical neurons expressing ephrin-B1 extend neurites on surfaces, which are coated with EphB2 and the activation of ephrin-B1 leads to Tiam1 activation. Expression of a dominant negative form of Tiam1 results in disrupted neurite outgrowth. Another newly discovered ephrin-B binding protein is *Xenopus* Dishevelled (Xdsh), a protein that up-regulates RhoA and Rho kinase (ROCK) activity (Tanaka et al. 2003). Xdsh interacts with ephrin-B1 following its activation and mediates cell sorting through repulsion in the animal cap.

Reverse signaling through ephrin-As

The observation of reverse signaling through ephrin-As surprised researchers in the field because of the absence of cytoplasmic tails on A class ephrins. Convincing evidence, however, was presented with the discovery of four GPI-linked ephrin homologs and one Eph homolog (VAB-1) in *C. elegans* (Wang et al. 1999). Furthermore, it has been found the expression of VAB-1 in a kinase inactive form does not lead to a complete loss of function in mutant animals, suggesting that reverse signaling through the GPI linked ephrins must be important (Chin-Sang et al. 1999). This hypothesis is later confirmed by another study, in which mouse vermonasal neurons expressing high levels of ephrin-A5 project towards EphA6 expressing territory, suggesting an attractive effect of ephrin-A5 signaling (Knoll et al. 2001). The downstream signaling of ephrin-As remains unclear, though some studies suggest that Fyn tyrosine kinase is involved. It is likely that the interaction with other receptors through the extracellular domain is critical to ephrin-A mediated signaling. Indeed, integrin signaling is regulated by ephrin-A

activation, possibly through direct coupling of the integrin molecules to ephrins (Davy et al. 1999; Wang et al. 1999).

Summary

Receptor tyrosine kinases turn on many cytoplasmic pathways after their activation. As members of the RTK superfamily, Eph receptor family and ephrins are highly expressed in a wide variety of tissues in developing and adult organisms. They have been found to play important roles in many developmental processes, including tissue patterning, angiogenesis, neural crest cell migration, axon fasciculation, axon guidance, and topographic neural map formation. In addition to the roles during development, Eph receptors have been shown to regulate adult functions ranging from learning and memory, to inducing pathological conditions such as tumorigenesis. Eph receptors interact with their membrane bound ligands and form 2 receptors:2 ligands tetramer complexes. Furthermore, these complexes are known to achieve a high level of clustering and become localized to specific regions such as lipid rafts or synapses. Depending on the receptors/ligands involved and the cellular environment, Ephrin-Eph interaction produces either repulsive effects or attractive effects. Although most of the functions depend on the kinase activity of the receptors, both kinase independent signaling through receptors and reverse signaling via ephrin ligands exist.

Some insights have been gained on how Eph receptors and ephrin ligands mediate different signaling events. A group of signaling proteins has been identified, many of which possess SH2 domains or PDZ domains. They function to link activated Eph

receptors and ligands to major downstream signaling pathways such as Ras/MEK/ERK pathway, small GTPases, cytoskeleton, integrin signaling, PIP₂ pathway.

However, the complexity of Eph and ephrin signaling remains elusive. Intriguing questions remain, in particular about how the same receptors induce opposite effects in different populations of cells or under different circumstances. The switch between repulsion and adhesion has not yet been elucidated, even though many hypotheses exist. Furthermore, the diversity of functions and the multitude of signal transduction pathways suggest that more binding proteins in the signaling cascades have yet to be discovered. It is imperative to explore how signaling proteins induce different outcomes in response to ligand stimulation. In addition, Eph/ephrin signaling has been implicated in tumorigenesis, especially in metastasis. Our understanding of these functions will therefore enable us to use Eph/ephrin targeted therapies in treating cancer and other diseases.

We started the project with a large-scale screen for strong interacting proteins with Eph receptors. Several novel binding proteins were identified, among which Nck1 was the strongest interactor. Because Nck1 is recognized as a key player in RTK signaling but has never been implicated in EphA mediated signaling, we set out on an in-depth study of the role of Nck1. Both fibroblast cell line and primary neuron cultures are used in this study.

Figure 1-1. Domain structure of Eph receptors and ephrin ligands

(A) Domain structure of Eph receptors. They contain a globular domain, a Cysteine-rich domain, two Fibronectin III domains, the juxtamembrane domain, a tyrosine kinase domain, a SAM domain, and a PDZ binding motif. (B) Domain structure of ephrin-As. They connect to the cell surface through a GPI link. (C) Domain structure of ephrin-Bs. They contain an extracellular domain, a transmembrane domain, and a cytoplasmic tail.

A.

B.

Figure 1-2. Phylogram of Eph family kinases and class-specific interactions between Eph receptors and ephrins.

(A) A phylogram of Eph receptors, modified on the basis of *The protein tyrosine kinase family of human genome* (Robinson et al, Oncogene, 2000). The evolutionary distance was calculated based on the sequence homologies of the kinase domain. (B) Receptor-ligand interaction is usually subclass-specific but binding across subclasses sometimes occurs.

Figure 1-3. Eph/ephrin activation, proteolysis, and endocytosis.

(A) When Eph expressing cells and ephrin-B expressing cells are in close proximity, the ligand dimer binds to two separate Eph molecules through high affinity interfaces. (B) After the two Eph receptors bind to one ligand pair, they dimerize and form a tight tetramer. (C) The dimerization of Eph receptors leads to trans-phosphorylation of the tyrosine residues and activates the kinase activity. The B class ligands are also phosphorylated by RTKs or cytoplasmic kinases. Both the activated Eph receptors and ephrin ligands are fully capable of binding to downstream mediators and turning on signaling cascades. (D) One mechanism to terminate signaling involves the dissociation of the Eph-ephrin complexes by proteolysis. (E)-(F) The lysed Eph and ephrin molecules sometimes are recycled through endocytosis.

Figure 1-4. A schematic representation of the retinal-tectal projection in chick.

(A) During development, the nasal retinal neurons express a low level of EphA receptors and extend to the posterior tectum where ephrin-A expression is high. Conversely, temporal neurons expressing high levels of EphAs project to the anterior tectum where ligand expression is low. The growth cones usually travel past their termination zone (as indicated by small circles). Collateral branches form on the axon shafts within the termination zone, laterally on the nasal neurons and medially on the temporal neurons. The graded expression of Eph receptors and ephrins are indicated below the graph. (B) The topographic projection between the retina and the tectum is complete. As shown, the growth cones retract to their final destination and form synaptic connects with their targets. In addition, all ectopic branches have disappeared.

Signaling proteins that bind to Eph receptors

	Binding Eph	Binding site (protein/receptor)	Possible function	Reference
Fyn	EphA4, EphA3 EphB3, EphA8 EphB2	SH2 / JM	A member of Src family kinase, phosphorylates R-Ras and other substrates. Is involved in cytoskeleton regulation	Hock et al. 1998 Choi et al. 1999 Zisch et al. 1998
Src	EphA3, EphA4 EphB2, EphB5	SH2 / JM	A member of Src family kinase, phosphorylates R-Ras and other substrates. Is involved in cytoskeleton regulation	Zisch et al. 1998 Moeller et al. 2006
Yes	EphA3, EphA4 EphB2, EphB5	SH2 / JM	A member of Src family kinase, phosphorylates R-Ras and other substrates. Is involved in cytoskeleton regulation.	Zisch et al. 1998
Abl/Arg	EphB2, EphA4	SH2 / JM	A member of Src family kinase, phosphorylates R-Ras and other substrates, also has actin binding domain.	Zisch et al. 1998 Yu et al. 2001
Crk	EphA3, EphB3 EphB2	SH2 / JM	An adaptor protein, binds effector proteins. Binding to Sos, is involved in Ras/ERK pathway. Also regulates Rho activity and causes cell detachment.	Lawrenson et al. 2002 Okada et al. 1996 Hock et al. 1998
Nck1	EphB1, EphB2	SH2 / JM	An adaptor protein, binds effector proteins. Activates JNK, PAK and WASP proteins. Is involved in actin cytoskeleton regulation.	Holland et al. 1997 Stein et al. 1998
SHC	EphA2	SH2 / JM	An adaptor protein, binds effector proteins. Is involved in the activation of Vav, a Rho GEF.	Pratt et al. 2002 Ramos-Morales et al. 1994
SLAP	EphA2, EphB2	SH2 / JM	An adaptor protein, binds effector proteins.	Pandey et al. 1995
Grb2	EphA2, EphB1 EphB6	SH2 / Kinase	An adaptor protein, binds effector proteins. Binding to Sos, is involved in the activation of Ras and ERK pathway. Is also involved in cytoskeleton regulation.	Moeller et al. 2006 Stein et al. 1996 Pratt et al. 2002
Grb10	EphB1	SH2 / SAM	An adaptor protein, helps other effectors to bind. Is involved in cell migration and cytoskeleton regulation.	Stein et al. 1996.
PLCgamma	EphB2, EphA4	SH2 / JM	A phospholipase, activates PKC. Also up-regulates the Ras/MEK/ERK pathway.	Holland et al. 1997 Zisch et al. 1998
SHEP-1	EphB2	SH2 / JM	Containing a Ras GEF domain, is involved in ERK pathway.	Dodelet et al. 1999
p85-PI3K	EphA8, EphA2	SH2 / Kinase	Induces the activation of anti-apoptotic protein Akt. Also regulates the actin cytoskeleton.	Gu and Park, 2001 Pandey et al. 1994
SHP-2	EphA2	SH2 / unknown	A tyrosine phosphatase, dephosphorylates FAK.	Miao et al. 2000
RasGAP	EphB2, EphB3	SH2 / JM	A Ras activating protein, inactivates Ras proteins and down-regulates the Ras/MEK/ERK pathway. Binds to RhoGAP and Nck1. Is involved in cytoskeleton regulation.	Holland et al. 1997 Hock et al. 1998
Vav2	EphA4	SH2/ JM	A Vav family RhoGEF, activates small Rho GTPases and induces endocytosis of Eph receptors.	Cowan et al. 2005

Signaling proteins that bind to Eph receptors (Continued)

	Binding Eph	Binding site (protein/receptor)	Possible function	Reference
PICK1	EphB2, EphA7	PDZ / PDZ motif	Interacts with synaptic receptors. Is involved in PKC activation.	Torres et al. 1998
GRIP	EphB2, EphA7	PDZ / PDZ motif	Interacts with synaptic receptors and serves as a scaffold protein	Torres et al. 1998 Bruckner et al. 1999
Syntenin	EphB2, EphA7	PDZ / PDZ motif	A syndecan binding protein, is involved in actin dynamics and synaptic functions.	Torres et al. 1998 Koroll et al. 2001
AF6	EphB2, EphB3 EphB5, EphA7	PDZ / PDZ motif	A Ras binding protein, recruits Ras proteins.	Hock et al. 1998
Tiam1	EphA2	PDZ / PDZ motif	A Rac GEF, binds to ephrinB1, up-regulates Rac activity and induces neurite outgrowth	Tanaka et al. 2004
Dishevelled	EphB2	PDZ/ PDZ motif	A PDZ domain containing protein, regulates signal transduction in Wnt pathway. A Rho GEF, induces Rho up-regulation and cell repulsion	Tanaka et al. 2003
Ephexin	EphA4	DH-PH/ kinase	A neuronal RhoGEF, preferentially activates Rho small GTPases and leads to growth cone collapse	Shamah et al. 2001
LMW-PTP	EphB1, EphB2	? / SAM	A phosphotyrosine phosphatase, promotes cell adhesion	Stein et al. 1998
FAK	EphA2	? / ?	Is directly involved in integrin signaling. Is phosphorylated by EphA2 and dephosphorylated by SHP-2.	Miao et al. 2000
Syndecan-2	EphB2	? / ?	A transmembrane heparin sulfate proteoglycan, induces spine formation by clustering	Ethell et al. 2001
NR1	EphB2	? / ?	NMDA type glutamate receptor, is involved in Calcium influx regulation. Is phosphorylated by Eph receptors.	Grunwald et al. 2001 Takasu et al. 2002
ADAM17	EphB2	? / ?	A metalloprotease in the extracellular space, cleaves Eph receptors and leads to the termination of signaling.	Mancia et al. 2005 Hattori et al. 2000

Table 1-1. Signaling proteins that interact with Eph receptors.

Signaling proteins that bind to Eph receptors are summarized in the table. Also summarized in the table are the binding sites that mediate the interactions and the possible functions regulated by these signaling protein.

Figure 1-5. Signaling pathways implicated in Eph receptor mediated signal transduction.

Eph receptor signaling regulates cell adhesion, cell migration, cytoskeleton dynamics, cell proliferation, and synaptic activity via the many pathways that are turned on after receptor activation and phosphorylation. SH2 adaptor proteins including Grb2, SHC and Crk are responsible for organizing the assembly of Eph/effector protein complex. Eph receptors are able to inhibit Ras/MEK/ERK pathway, which disrupts integrin signaling and promotes cell proliferation. Ephexin and other RhoGEFs regulate the activity of small Rho GTPases and lead to cytoskeletal rearrangements. In addition, Eph receptors can dephosphorylate FAK through Src family kinases and reduce adhesion. The C-terminus of Eph receptors are capable of recruiting proteins containing PDZ binding domains, which help to localize Eph receptors to specific locations such as synapses or growth cones. In the extracellular space, ADAM proteins associate with Eph receptors and catalyze the proteolysis of the receptor/ligand pair. In this figure, the arrows show the direction in which the multi-step signaling cascade occurs. The black arrows indicate a positive effect, whereas red lines show inhibitory effects.

Signaling proteins that bind to ephrins

	Binding ephrin	Binding site (protein/receptor)	Possible function	Reference
PICK1	ephrin-B1	PDZ / PDZ motif	Interacts with synaptic receptors. Is involved in PKC activation.	Torres et al. 1998
GRIP	ephrin-B1	PDZ / PDZ motif	Interacts with glutamate receptors. Serves as a scaffold protein to localize ephrin to the synapse.	Torres et al. 1998
GRIP2	ephrin-B1	PDZ / PDZ motif	Interacts with glutamate receptors. Serves as a scaffold protein to localize ephrin to the synapse.	Lin et al. 1999
Syntenin	ephrin-B1 ephrin-B2	PDZ / PDZ motif	A syndecan binding protein, is involved in actin dynamics and synaptic functions.	Torres et al. 1998 Lin et al. 1999
PTP-BL	ephrin-B1	PDZ / PDZ motif	A PDZ domain containing tyrosine phosphatase, regulates negatively the phosphorylation of ephrin and Src kinases.	Palmer et al. 2002 Lin et al. 1999
PDZ-RGS3	ephrin-B1	PDZ / PDZ motif	A regulator of heterotrimeric G protein, regulates G protein coupled chemoattraction signaling.	Lu et al. 2001, 2004
PHIP	ephrin-B1	PDZ / PDZ motif	Pleckstrin homology domain interacting protein, is implicated in Insulin receptor signaling.	Lin et al. 1999
Tiam1	ephrin-B1	PDZ / PDZ motif	A Rac GEF, binds to ephrinB1, up-regulates Rac activity and induces neurite outgrowth	Tanaka et al. 2004
Dishevelled	ephrin-B1 ephrin-B2	PDZ/ PDZ motif	A PDZ domain containing protein, regulates signal transduction in Wnt pathway. Also a Rho GEF, induces Rho up-regulation and cell repulsion	Tanaka et al. 2003
Nck2	ephrin-B1 ephrin-B2	SH2 / ?	Dock homolog, a SH2/SH3 adaptor protein, binds to effectors like Abi-1, CAP, axin, regulates cytoskeleton.	Cowan et al. 2001
Src	ephrin-B1	SH2 / ?	A Src family kinase, phosphorylates ephrins as well as other substrates.	Palmer et al. 2002 Holland et al. 1996
Fyn	ephrin-B1	SH2 / ?	A Src family kinase, phosphorylates ephrins as well as other substrates. Is important for ephrin induced cell adhesion.	Davy et al. 1999
Yes	ephrin-B1	SH2 / ?	A Src family kinase, phosphorylate ephrins as well as other substrates.	Palmer et al. 2002
ADAM10	ephrin-A2	? / ?	A metalloprotease in the extracellular space, cleaves Eph receptors and leads to cell detachment and the termination of signaling.	Hattori et al. 2000

Table 1-2. Signaling proteins downstream of ephrin ligands.

The binding proteins of ephrin ligands are listed. Similar to Table 1-1, the information about their binding sites and their functions is also included.

Figure 1-6. Signaling pathways involved in reverse signaling through ephrin-Bs.

The aggregation of Eph receptors and their ligands potentially ephrins, especially the ephrin-Bs. Through phosphorylation by Src kinases and other RTKs, the ephrin-Bs are activated. The signaling through ephrins mediates modulation of cell migration, cell adhesion, cytoskeletal dynamics, neurite extension, and synaptic activity. Src family kinases regulate cell adhesion directly through phosphorylating FAK. Nck2, bound to activated ephrins, regulates cytoskeletal dynamics through Axin, CAP and Abi-1. Similar to Eph receptors, ephrins also recruit a great number of PDZ domain proteins. Among them, Tiam1 and Dishevelled modulate neurite outgrowth and growth cone collapse by affecting the balance of small Rho GTPases. PTP-BL antagonizes the activity of cytoplasmic kinases and dephosphorylates ephrins. Other PDZ domain proteins mediate the interaction between ephrins and receptors, especially at the synapses. Similar to Figure. 1-5, the black arrows mark positive effects and red arrows are indicative of inhibitory effects.

Chapter II. Screening for EphA receptor binding proteins

using the yeast two-hybrid method

Introduction

The yeast two-hybrid screen technique has been established as a powerful method to identify protein-protein interactions. Originally, this method made use of the yeast transcriptional activator GAL4 protein (Keegan et al. 1986; Fields and Song 1989). GAL4 protein regulates the transcription of genes involved in galactose metabolism. It consists of a N-terminal DNA binding domain and a C-terminal transcriptional activation domain. These two domains are functionally separate, but are both required for GAL4 protein to induce transcription activation (Brent and Ptashne 1985). In the initial experimental design, two fusion proteins were generated: DNA binding domain fused to protein X and activation domain fused to protein Y. These fusion proteins were co-expressed in yeast cells that also carried a reporter plasmid [(Chien et al. 1991), Fig. 2-1 A]. The association of protein X and protein Y brings the GAL4 DNA binding domain and GAL4 activation domain together, and leads to the expression of the reporter gene LacZ. As a result, the interaction of X-Y is detected by examining the LacZ activity in these yeast cells.

The yeast two-hybrid method we used is a modified system based on similar principles. Instead of GAL4 DNA binding domain, the *E. coli* LexA protein is fused to protein X (usually the molecule of interest), while the acid blob domain B42 replaces the GAL4 activation domain in the Y fusion protein (contained in a cDNA expression library). When the LexA protein and B42 domain are brought together through direct X-

Y interaction, the protein complex associates with the UAS and turns on LacZ gene transcription. Therefore, by testing for LacZ gene activity, we are able to screen for strong binding proteins in the cDNA library on large scale with confidence (Fig. 2-1 B).

Compared to the original yeast two-hybrid system, the modified method has several advantages. First, since yeast strains and reporter vectors of varying sensitivity are available, we are able to tailor the screen to suit our needs and select only for the strongest binding proteins. Second, the bait plasmid contains a nuclear localization sequence (NLS), which ensures that the bait fusion protein enters the nucleus and binds to the UAS. Thirdly, LexA proteins dimerize constitutively, allowing activation of the Eph receptor kinase domain and leading to phosphorylation on key tyrosine residues. In addition, since prokaryotic proteins are used in an eukaryotic system, the protein-protein interactions we identify through this system are highly specific and the occurrence of false positives is greatly reduced. Lastly, several tests are incorporated into the experimental design to confirm the validity of the binding proteins isolated in the preliminary screen.

Results

Construction of the bait plasmid and screening of a mouse brain cDNA library

A flow chart shows the overall procedure of the yeast two-hybrid (Fig. 2-2). The cytoplasmic domain of EphA5 (amino acid 564-1016) was first cloned and inserted into the bait plasmid pEG202-NLS behind LexA sequence. This plasmid has a nuclear localization sequence and ensures the nuclear delivery of the expressed bait protein. With LexA on the N-terminal, the complete bait (LexA-EphA5_{intra}) contains the

juxtamembrane domain, the tyrosine kinase domain, and the SAM domain of EphA5 receptor (Fig. 2-3 A).

The bait plasmid pEG202-NLS-EphA5intra was first transformed into strain EGY188 yeast cells, in which the preliminary screen was performed. EGY188 strain was chosen because of its low sensitivity, which ensured that only strong interactors would be isolated. The inserted gene was confirmed to be in the correct reading frame by Western blot with anti-EphA5 antibody and anti-lexA antibody. The size of the bait fusion protein was estimated to be about 72 KD (Fig. 2-3 B). Furthermore, it was confirmed that the resulted bait protein was delivered into the nucleus but was unable to turn on the reporter gene transcription on its own (data not shown). Later, EGY188 cells carrying pEG202-NLS-EphA5intra were co-transformed with pJK103, a reporter plasmid of medium sensitivity and a mouse embryonic cDNA expression library cloned in target plasmid pJG4-5.

The transformants carrying all three plasmids were diluted and plated on culture medium plates until individual colonies grew. The colonies were replicated onto a pair of culture plates, which contained glucose or galactose, respectively. Two days later, the colonies on the plates were lifted off onto nitrocellulose filter paper and the following test for LacZ expression was conducted. The colonies on filter papers were frozen and thawed repeatedly to release cell contents. Then the filters were submerged in a solution containing X-Gal for two hours to allow the LacZ reaction to take place. The design of the system dictated that a colony was positive only if it had LacZ expression on the plate containing galactose but not on the other plate. Since LacZ converts X-Gal to a blue

precipitate, we identified all positive colonies by means of comparing the intensity of the blue color on two plates (Fig. 2-4).

Plasmid DNA was then extracted from the positive colonies and sequenced. The identities of these genes were searched in public databases through BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>). In order to confirm the validity of the preliminary screen, we tested the positive cDNA sequences through individual mating tests. The extracted target plasmid and the reporter plasmid were co-transformed into EGY188 yeast strain, while the bait plasmid pEG202-NLS-EphA5intra, an empty bait plasmid pBait or a negative control plasmid pRHFm were each transformed into EGY40 strain. To conduct the mating test (Fig. 2-5 A), EGY188 carrying both the positive target plasmid and the reporter plasmid was streaked first across the culture plate horizontally. Then, the transformed EGY40 were streaked perpendicularly across the first streak. As a result, the two parental strains mixed and produced offspring cells, which inherited all three plasmids from the parental cells. When LacZ filter assay was performed on these offspring cells, proteins with real binding with EphA5 turned on the LacZ reaction in the presence of EphA5 bait expression but not the control proteins (Fig. 2-5 B). By contrast, false positives were unable to catalyze the conversion of X-gal in any of the three streaks (Fig. 2-5 B).

Fifteen interactors were identified through the screen

In our study, a total of 10^7 clones were screened and about 300 clones were sequenced. Despite the power of yeast two-hybrid, it has been shown in the past that some clones isolated through this method are mere artifacts and they appear in screens

regardless of the bait used. Luckily, Dr. Erica Golemis and Dr. Ilya Serbriiskii of the Fox Chase Cancer Center consolidated data from more than 100 yeast two-hybrid screens and posted the false positives on their website (<http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>). These proteins include heat shock proteins, ribosomal proteins, ubiquitin, proteasome subunits, mitochondrial proteins, transcription elongation factors and so on. Using this information, we were able to eliminate common false positive genes from our results. A large portion of the remaining positive genes was unknown cDNA sequences, which had been documented but never identified. Consequently, we were not able to analyze them further at that time. After individual mating tests were performed on the rest of the positive clones, fifteen genes tested positive and were considered real positives. These proteins were studied through literature search and their possible roles in Eph receptor signaling were evaluated. Importantly, many of these positive genes were encountered multiple times in our screen, indicating the consistency of the method.

Nck1 binds strongly with EphA5 receptor

Among the 15 binding proteins, one protein with the strongest binding with EphA5 is Nck1, a SH2/SH3 domain adaptor protein. The cDNA fragment of Nck1 isolated in the screen, when compared to full length Nck1 sequence, consisted of the SH2 domain, the second and third SH3 domains (Fig. 2-6 A). As shown through the mating test, this cDNA product binds to the bait LexA-EphA5_{intra} and turns on LacZ gene transcription (Fig. 2-6 B). The association between Nck1 and EphA5 was highly specific since no

LacZ activity was detected when either control plasmid was used in place of the bait plasmid.

Intriguingly, when a similar yeast two-hybrid screen was conducted using EphA3 intracellular domain as the bait in our lab, Nck1 was also one strong interactor (Dr. Guanfang Shi, unpublished data). Therefore, we hypothesize that the adaptor protein Nck1 binds to multiple EphA receptors and play critical roles in their signaling.

Discussion

The identified EphA interactors regulate different aspects of signal transduction

Fifteen binding interactors of EphA5 were identified in the yeast two-hybrid screen and they are summarized in Table 2-1. According to their known functions, they are grouped into 4 categories (1) membrane trafficking related genes, (2) cytoskeleton regulation related genes, (3) tumor regulation related genes and (4) enzymes.

Five of the identified binding proteins are implicated in the regulation of membrane trafficking. They are Arl3 (ADP-ribosylation-like 3), CDCrel-1 (cell division control related protein-1), OSBP (oxysterol-binding protein), Rab2 (Ras-like small GTPase Rab2) and Rab11 (Ras-like small GTPase Rab11) (Beites et al. 1999; Cheung et al. 2002; Kahn et al. 2005; Savina et al. 2005; Olkkonen et al. 2006; Perry and Ridgway 2006). This group of proteins is possibly important in the recycling of the Eph receptors, which determines the duration of Eph signaling. For example, Rab2 and Rab11 are both members of Ras-like small GTPase Rab family. They have been recognized as key mediators during endocytosis and the transport of the resulting vesicles in cell cytosol (Stein et al. 2003; Ceresa 2006).

An important event during EphA receptor signaling is cytoskeletal restructuring (Bruckner and Klein 1998; Murai and Pasquale 2005; Sahin et al. 2005). Usually, upon EphA activation, activated effector proteins modulate cytoskeleton dynamics. These modulations lead to cellular events such as cell migration or process retraction. Therefore, it was unsurprising to find several positive clones encoded for proteins involved in cytoskeleton dynamics. These proteins include Arl3 (ADP-ribosylation-like 3 protein), cdc42 (small Rho GTPase), Mayven, Mpp3 (MAGUK family member 3) and Nck1 (non-catalytic region of tyrosine kinase adaptor protein 1) (Bladt et al. 2003; Sahin et al. 2005; Williams et al. 2005; Kantardzhieva et al. 2006; Zhou et al. 2006).

Another group of interacting proteins include Lgi1 (Leucine-rich, glioma inactivated 1), Mayven, NOV (Nephroblastoma overexpressed gene), PELOTA, SPARC (secreted acidic cysterine rich glycoprotein) and TCTP (translationally-controlled tumor protein) (Jendraschak and Sage 1996; Kunapuli et al. 2004; Kyurkchiev et al. 2004; Tuynder et al. 2004; Bu et al. 2005; Xi et al. 2005). All proteins in this group play roles in tumorigenesis. Since Eph receptors are also implicated in tumor progression (Nakamoto and Bergemann 2002; Surawska et al. 2004), the identification of these proteins reveals possible pathways downstream of Eph receptors in tumor development. Furthermore, the disruption of these pathways may be effective in treating cancer.

In our study, we also found that non-receptor tyrosine phosphatase Ptpn5 (Barr and Knapp 2006) bound to the cytoplasmic domain of EphA5. Previously, it has been reported that protein tyrosine phosphatase Shp2 (Miao et al. 2000), low molecular weight PTPs (LMW-PTPs) (Kikawa et al. 2002; Parri et al. 2005) and the receptor-type phosphatase PTPO (Shintani et al. 2006) associate with Eph receptors and modulate the

phosphorylation level of substrates. Therefore, the identification of Ptpn5 indicates that Ptpn5 may play a similar role.

The results we obtained are probably far from being comprehensive. Proteins found to bind to EphA5 in the past, for example, were not identified in this screen, indicating that the downstream signaling of EphA receptors is complex and there are likely to be more unidentified interactors. Therefore, it would be rewarding to continue the yeast two-hybrid screen and isolate more binding proteins.

The binding of Nck1 and EphA receptors links activated EphA receptor and the cytoskeleton regulation.

Nck1 (also known as Nck, Nck α) stands for non-catalytic region of tyrosine kinase (McCarty 1998). Nck1 is a SH2/SH3 domain-containing adaptor protein (Buday et al. 2002; Rivera et al. 2006). SH2/SH3 adaptor proteins consist of various numbers of SH2 and SH3 domains. They lack have intrinsic enzymatic activities, but function as scaffold proteins. SH2/SH3 adaptor proteins bind to phosphorylated tyrosines on receptor tyrosine kinases through SH2 domains. Through SH3 domains, they bind to proline-rich domains of effector proteins and bring them in the proximity of the phosphorylated receptors (Birge et al. 1996; Mayer and Gupta 1998; McCarty 1998). Other than Nck1, SH2/SH3 containing adaptor proteins include Nck2, Grb2 and Crk (Pawson 1994; McCarty 1998; Buday et al. 2002).

In mammalian cells, there are two members in Nck family, Nck1 and Nck2 (a.k.a. Nck β). They both possess three SH3 domains in the N-terminus and one SH2 domain in C-terminus. Nck1 and Nck2 share high homology (60%) with each other, especially in

the functional domains. Nck family members bind to receptor tyrosine kinases such as EGFR, PDGFR and VEGFR and receptors such as DCC and Robo (Roche et al. 1996; Tang et al. 1997; Li et al. 2002; Stoletov et al. 2004). It has been shown that Nck1 plays a role in the signaling of both EphB1 and EphB2 (Holland et al. 1997; Stein et al. 1998). However, the association of Nck1 with EphA receptors has never been established before.

So far, 30 effectors have been identified for Nck1 and interestingly, many of these proteins were found to be associated with cytoskeleton regulation (McCarty 1998; Buday et al. 2002). For example, WASP family members and PAK proteins (Zhao et al. 2000; Rohatgi et al. 2001; Zalevsky et al. 2001) have been both implicated in actin restructuring. Therefore, our identification of Nck1 in the screen suggests that the function of Nck1 may be involved in the signaling of some EphA receptors by linking active receptors with the cytoskeletal machinery.

A.

B.

Figure 2-1. A schematic representation of the yeast two-hybrid screen.

(A) The original experimental design. Protein X and Y are fused to a Gal4 DNA binding domain and activation domain respectively. Strong binding between protein X and Y brings the two functional domains of Gal4 together, leading to transcription and expression of the reporter gene. (B) The current DupLEX system. This system employs a similar design in which protein X and Y are fused to *E. coli* proteins LexA protein and B42 protein instead.

Figure 2-2. A summary of the yeast two-hybrid screen procedure.

First, the bait plasmid pEG202-NLS-EphA5intra was constructed and tested. Using this bait, a preliminary screen was performed in EGY188 and about 10^7 clones from an embryonic mouse brain cDNA library were screened. Three hundred positives from the preliminary screen were then sequenced and identified. The positive clones were further confirmed through mating tests.

A.

B.

Figure 2-3. Structure and expression of the bait LexA-EphA5_{intra}.

(A) The structure of the expressed bait protein. LexA fragment is fused to the N-terminal of the EphA5 intracellular fragment. (B) By Western blot, the expression of the bait in transformed EGY188 strain was confirmed using both anti-lexA antibody and anti-EphA5 antibody.

Plate B Galatose

Plate A Glucose

Figure 2-4. Large-scale preliminary screens for EphA5 bait through LacZ assay.

Two otherwise identical plates containing either glucose or galactose were used to culture the transformant colonies. Only colonies with LacZ expression on the galactose plate but not on the glucose plate are identified as initial positive clones, as marked by blue underline.

A.

B.

Figure 2-5. Yeast mating tests of the binding proteins identified in earlier screens.

(A) A schematic representation of the mating test. Individual strains of transformed yeast cells are inoculated in the described order and mixed well. (B) Representative negative and positive test results.

A.

B.

Figure 2-6. Nck1, a strong binding protein of EphA5.

(A) The Nck1 cDNA fragment isolated from the yeast two-hybrid screen, as compared to the full-length mouse Nck1 gene. (B) Positive results for Nck1 in the yeast mating test.

Membrane trafficking

Arl3	ADP-ribosylation-like 3, ARF like GTPase	Involved in vesicular transport and cytoskeleton assembly. Important to photoreceptor cell development.
CDCrel-1	cell division control related protein-1, septin family member	Degraded by Parkin, a gene linked to juvenile Parkinsonism. Regulates exocytosis, highly expressed in presynaptic nerve terminals.
OSBP	oxysterol-binding protein	Binds to Rab7, involved in lipid metabolism and membrane trafficking. Also involved in ERK1/2 activation.
Rab11	Ras-like small GTPase RAB11	Members of Rab family. Regulate vesicular trafficking.
Rab2	Ras-like small GTPase RAB2	

Cytoskeleton regulation

Arl3	ADP-ribosylation-like 3, ARF like GTPase	Involved in vesicular transport and cytoskeleton assembly. Important to photoreceptor cell development.
cdc42	Cell division cycle 42	Small GTPase involved in cytoskeleton modulation and cell behavior regulation.
Mayven	Kelch like protein 2	Overexpressed in breast cancer and involved in oligodendrocyte process formation. Binds to actin.
Mpp3	membrane protein, palmitoylated 3, MAGUK family member	Contains a SH3 domain. Involved in cytoskeleton regulation and signal transduction. Localized in the retinal outer membrane.
Nck1	non-catalytic region of tyrosine kinase adaptor protein 1	Found to bind EGFR, PDGFR, DCC, Robo. A scaffold protein.

Tumor regulation

Lgi1	Leucine-rich, glioma inactivated 1	A secreted protein. Involved in neuronal cell survival and tumorigenesis suppression.
Mayven	Kelch like protein 2	Overexpressed in breast cancer and involved in oligodendrocyte process formation. Binds to actin.
NOV	nephroblastoma overexpressed gene, CCN3, a CCN family member	Involved in cancer progression. Interacts with integrins, Notch1 and other receptors and ligands.
PELOT A		A nuclear protein involved in cell cycle regulation. Also necessary to eye patterning in <i>Drosophila</i>
SPARC	secreted acidic cysterine rich glycoprotein, osteonectin	A secreted protein involved in ECM regulation. Also exists in the cytoplasm. Involved in tumor growth. Important for lens epithelial cell function.
TCTP	translationally-controlled tumor protein	Regulates cell proliferation and cell survival, involved in tumor progression

Enzymes

Mpp3	membrane protein, palmitoylated 3, MAGUK family member	Contains a SH3 domain. Involved in cytoskeleton regulation and signal transduction. Localized in the retinal outer membrane.
Ptpn5	protein tyrosine phosphatase, non-receptor type 5, STEP	Inactivates MAPK and enriched in striatum. A regulator of NMDA receptors.

Table 2-1. A summary of fifteen identified binding proteins of EphA5 in the yeast two-hybrid screen.

The positive proteins are categorized into four groups based on their functions. Their individual names, full description of their functions are listed.

Chapter III. Characterization of EphA3-Nck1 binding and evaluation of Nck1 in EphA3 signaling

Introduction

The strong binding observed between Nck1 and EphA receptors (EphA3 and EphA5) in the yeast two-hybrid screens indicates that Nck1 may be pivotal to EphA receptor function. Thus, we set out to investigate the roles of Nck1 in the signal transduction of EphA receptor. We chose to focus on EphA3 because this particular receptor has been implicated in important functions such as topographic map formation (Ciossek et al. 1998; Otal et al. 2006). In addition, the availability of specific anti-EphA3 antibodies and EphA3 tyrosine mutants would allow the mapping of the Nck1 interaction.

To validate the binding between Nck1 and EphA3, a series of GST fusion protein pulldown experiments using fusion proteins containing full length or parts of mouse Nck1 protein were conducted. Furthermore, multiple single tyrosine mutants of EphA3 were used to map the domains through which the interaction occurs.

To demonstrate the interaction of EphA3 and Nck1, HEK293A cells transfected with both genes were used. Immunostaining showed that EphA3 receptor co-localized with Nck1 after ephrin-A5 treatment. In addition, the EphA3-Nck1 interaction was confirmed by co-immunoprecipitation.

Since EphA3 binds Nck1 strongly, we hypothesize that Nck1 is a major effector downstream of activated EphA3. This hypothesis was evaluated by assaying for cell process retraction and cell migration. In order to determine if cells with high expression

of EphA3 responded to ligand stimulation in a Nck1 dependent way, Nck1 dominant negative proteins were introduced into EphA expressing cells.

Results

Interaction between Nck1 and EphA3 is mediated by the binding of Nck1 SH2 domain to the tyrosine 602 of EphA3.

Glutathione S-Transferase (GST) fusion proteins are commonly used in pulldown assays to test the physical interactions of two proteins (Li et al. 2002). A protein of interest, protein A is fused to GST fragment and overexpressed in bacteria in large quantity. After purification, the fusion protein is conjugated to Glutathione sepharose beads. Beads conjugated with desired fusion proteins are then incubated with cell lysates containing a high level of protein B. If proteins A and B exhibit strong binding, protein B will be detected in the precipitates with fusion protein GST-A through Western blot.

To map the binding site of Nck1, wild type EphA3 (EphA3WT) was transfected into HEK293A cells, and forty-eight hours after the transfection, the cells were stimulated with cross-linked ephrin-A5 for 30 minutes. The lysates of the treated cells were incubated with GST fusion proteins that contained either full length (GST-Nck1), the SH2 domain (GST-Nck1SH2) or the SH3 domains (GST-Nck1SH3) of mouse Nck1 (provided by Dr. L. Larose, Fig. 3-1 A). Both GST-Nck1 and GST-Nck1SH2 proteins precipitated EphA3 while GST control and GST-Nck1SH3 fusion protein did not (Fig. 3-1 B). An experiment performed with cell lysates of unstimulated EphA3 expressing 293A cells showed similar results except that the amount of precipitated EphA3 by GST-Nck1 and GST-Nck1SH2 was reduced (data not shown). This was probably because of the

auto-activation of EphA3 receptors in the cell lysates due to over-expression. The pulldown studies indicate that the EphA3-Nck1 binding we identified in the yeast two-hybrid screen is specific. Additionally, the SH2 domain in Nck1 is found to be responsible for EphA3 binding, since GST-Nck1SH2 precipitates EphA3 protein while GST-Nck1SH3 fails to do so (Fig. 3-1 B, lane 3 and 4). This discovery is consistent with our observation that the extracted Nck1 cDNA fragment in the yeast two-hybrid screen contains the complete SH2 domain. Indeed, when the SH2 domain was deleted from the cDNA fragment, binding with EphA no longer tested positive in the yeast mating test (data not shown).

Interestingly, Western blot analysis also shows that an unknown protein of about 100 KD was pulled down by both GST-Nck1SH3 and GST-Nck1 protein (Fig. 3-1 B, lane 4 and 5). The absence of this protein in lane 3 indicates that it was not a degradation product of EphA3, suggesting that its interaction with the anti-EphA3 antibody resulted from non-specific binding. Previous studies on Nck1 function suggest that this protein may be dynamin, a protein of about 100 KD. Dynamin is involved in endocytosis regulation and binds to Nck1 SH3 domains (Wunderlich et al. 1999; Cowan and Henkemeyer 2001).

The SH2 domains typically bind to phosphorylated tyrosine residues (Tanaka et al. 1995; Wunderlich et al. 1999; Frese et al. 2006). An unbiased screen against a degenerate phosphopeptide library further reveals that SH2 domain binding largely depends on the three amino acids behind the phosphotyrosine residue. For example, Nck1 SH2 domain binds preferentially to “pTyr-hydrophilic-hydrophilic-Pro/Ile” sequence (Songyang et al. 1993; Zvelebil et al. 1995).

To identify the tyrosine residues on EphA3 to which Nck1 SH2 domain binds, several important cytoplasmic domain tyrosine residues of EphA3 known to be phosphorylated upon activation (Y596, Y602, Y736, Y779, Y937) (G Shi and R Zhou, unpublished data) were mutated to phenylalanine by *in vitro* mutagenesis (Fig. 3-2 A, B). In addition, a lysine to arginine mutation at amino acid position 653 generated a mutant lacking any phosphorylation or kinase activity and therefore was included as a negative control (K653R). These EphA3 mutants and wild type EphA3 were each transiently expressed in HEK293A cells, and the cell lysates were incubated with sepharose beads conjugated with GST-Nck1SH2 protein. The kinase dead mutant EphA3-K653R failed to bind Nck1 SH2 domain (Fig. 3-3 B, lane 6), indicating that phosphorylation of the cytoplasmic tyrosine residues is necessary for binding (Fig. 3-3 A, lane 6). Among the tyrosine mutants, both Y596F and Y602F showed no binding to Nck1 SH2 domain similar to the K653R, while the others displayed normal binding (Figure 3-3 B, lane 1 to 5). Since Y596 and Y602 may regulate EphA3 kinase activity (Fig. 3-3 A, Lane 1-2), the loss of binding we observed could be due to either a lack of receptor phosphorylation, or the loss of the key tyrosine residues. To test these two possibilities, two additional mutants (Y596E and Y602E) were used, each with one tyrosine residue replaced by glutamic acid (Fig. 3-2 A, B). The glutamic acid replacement has been shown to mimic both the size and charge of a phosphorylated tyrosine and restore kinase activity on similar mutants [(Zisch et al. 2000), Fig. 3-3 A, lane 8-10]. Pulldown studies using these mutants showed that only Y602E failed to bind GST-Nck1SH2 protein although it exhibited normal levels of tyrosine phosphorylation (Fig. 3-3 B, lane 8-10). This

observation indicates that Y602 is the key tyrosine residue that mediates direct Nck1 binding.

EphA3 and Nck1 form a protein complex in ephrin-A5-stimulated cells.

To examine whether Nck1 interacts with EphA3 in mammalian cells, EphA3 and HA tagged Nck1 were transiently expressed in HEK293A cells. The expression of both proteins was confirmed by Western blot analysis using anti-EphA3 antibody and anti-HA monoclonal antibody (Fig. 3-4). To conduct co-immunoprecipitation, the transfected cells were stimulated either with 2 μ g/ml cross-linked ephrin-A5 or control IgG for 15 minutes, and lysed with lysis buffer. Nck1 protein was precipitated from the cell lysates with a mouse monoclonal anti-HA antibody. The immunoprecipitated proteins were analyzed by Western blot to determine if EphA3 was co-precipitated. The analysis revealed that from cell lysates with ephrin-A5 treatment, but not with control stimulation, a significant amount of EphA3 receptor co-precipitated with Nck1 (Fig. 3-4, lane 4 and 5). This observation indicates that EphA3 activation is required for EphA3-Nck1 binding in living cells. It is necessary to point out we did note a small amount of precipitated EphA3 from lysates of cells only transfected with EphA3 (Fig. 3-4, lane 3). This was probably due to the endogenous Nck1 expression in the HEK293A cell line (data not shown).

To further study interactions between Nck1 and EphA3, the sub-cellular localization of these two proteins was also examined. Cells transfected with EphA3 and HA-Nck1 were treated with cross-linked ephrin-A5 for 30 minutes and then fixed with 4% paraformaldehyde in PBS. Fixed cells were stained with a rabbit anti-EphA3 antibody

followed by a FITC-conjugated anti-rabbit secondary antibody to label EphA3 receptor and a monoclonal anti-HA antibody followed by a Cy3-conjugated secondary antibody to mark Nck1 localization. It was found that both EphA3 and Nck1 were highly expressed in the transfected cells and there was considerable co-localization of two proteins in transfected 293 cells (Fig 3-5; yellow areas in A'' and B''). Intriguingly, we also found there were some protein aggregates in the cytosol, which stained strongly of both EphA3 and Nck1 (Fig. 3-5, A-A''). We speculate that these aggregates were endocytosed EphA3/Nck1 protein complex (Barbieri et al. 2004; Irie et al. 2005; Ceresa 2006). Indeed, previous studies have shown that at the end of Eph receptor signaling, the ligand-receptor dimers are cleaved and endocytosed for recycling (Mancia and Shapiro 2005).

Nck1 mutants down-modulate ephrin-A5-mediated inhibition of cell migration.

To examine whether Nck1 is important for EphA3 mediated functions, we tested the effects of EphA3 activation on cell migration and Nck1's role in this process. Two Nck1 dominant negative mutants and Nck1 wild type clone (generously provided by Dr. B. Mayer) were used in our study. The two Nck1 dominant negative mutants: Nck1R308K and Nck1W38/143/229K each contain mutations on key amino acid residues (Tanaka et al. 1995; Gupta and Mayer 1998). The R308K mutation disrupts the ability of the SH2 domain to bind to phosphotyrosine residues, while the W38/143/229K mutations abolish the ability of all three SH3 domains to interact with downstream targets containing proline-rich sequences. It was previously reported that both mutants inhibit functions of wild type Nck1 (Tanaka et al. 1995).

HEK293A cells were transfected with EphA3WT alone or together with Nck1WT, Nck1R308K, or Nck1W38/143/229K. Twenty-four hours after transfection, the cells were plated on Transwell inserts (8 μ m pore size) in serum free medium in the upper chamber. The lower compartment of the transwell apparatus was filled with complete culture medium with or without cross-linked ephrin-A5 added (Fig. 3-6 A). After overnight culture, quantification of the migration assay was performed with the aid of ImagePro program. Ten random pictures were taken of each insert on a microscope. The number of all cells that migrated through the membrane (T2) and the number of the transfected cells that migrated (T1) in each picture were both quantified. The efficiency of each individual transfection was determined separately: $R = \text{transfected cell} / \text{all cells}$. The migration ability (M) of cells expressing different genes was then expressed using this formula: $M = T1 / (T2 \times R)$. The M values of cells expressing different genes were normalized with EGFP control as 1 and plotted in Fig. 3-6 B.

Quantification showed that transient expression of EphA3WT caused a sharp decline in cell migration, compared to EGFP controls or EphA3KD. The decline of cell migration was found to be statistically very significant (student t-test, $p < 0.001$). Thus, EphA3 has a negative effect on cell migration and the kinase activity is indispensable to this regulation.

To examine whether Nck1 expression alone influences cell migration, we tested effects of expression of Nck1WT, Nck1R308K or Nck1W34/143/229K individually. The migration of cells expressing any Nck1 clone alone was similar to the EGFP control (Fig. 3-6 B), indicating that Nck1 expression alone did not affect cell migration. However, when Nck1R308K or Nck1W34/143/229K mutants were co-expressed with EphA3WT,

they were able to partially block the inhibition of cell migration induced by ephrin-A5 treatment (Fig. 3-6 B). In contrast, Nck1WT had no such effect. The differences were found to be also very statistically significant (student t-test, $p < 0.001$). Collectively, these observations indicate that the interaction through SH2 and SH3 domains of Nck1 are both necessary for regulating cell migration of EphA3-expressing cells.

Nck1 mutants inhibit ephrin-A5-induced cell process retraction.

Ephrin-A5 stimulation in 293 cells has been shown to lead to the retraction of cellular processes when EphA3 receptor is expressed [(Lawrenson et al. 2002), Fig. 3-7, C-C']. This provides the basis of a convenient assay to examine whether Nck1 functions downstream of EphA3, and if dominant-negative Nck1 mutants affect EphA3 signaling. To conduct this assay, HEK293A cells were plated sparsely on 35 mm tissue culture dishes and transfected either with EphA3 alone, or EphA3 with wild type Nck1 (Nck1WT), Nck1R308K, or Nck1W38/143/229K. Forty-eight hours after transfection, cells were treated with 2 μ g/ml cross-linked ephrin-A5 for one hour. Parallel cultures were treated with no ephrin-A5 as controls. In each culture dish, select cells with processes were photographed before and after treatment (Fig. 3-7 and Fig. 3-8). Using ImagePro program, we analyzed the images of the same groups of cells to determine how much process retraction occurred when cells were expressing different Nck1 genes. The total process length of a cell was measured before and after treatment: L1 and L2. Using the formula $E = (L2-L1)/L1$, we calculated the process length change index E as a percentage of the initial total process length. The E values were then plotted in Fig. 3-9.

In the graph, a positive number indicates process extension and a negative number indicates retraction.

Ephrin-A5 treatment of cells transfected with an empty EGFP vector did not cause any retraction of the cellular processes (Fig. 3-7, A and A'). On the contrary, on average, the branches extended about 4.7% ($\pm$ 3.9%). Ephrin treatment to cells transfected with the kinase-dead EphA3 also did not lead to any deleterious effects on the length of the processes (Fig. 3-7, B and B'). However, when the cells were transfected with EphA3WT, ephrin-A5 treatment induced significant process retractions (Fig. 3-7, C and C'). Quantification of the process length of treated cells showed that EphA3WT expressing cells retracted their processes by about 24.3% ($\pm$ 5.0%). In comparison, cells expressing EphA3KD extended about 0.4% ($\pm$ 3.3%) during the same period of time. These statistically significant differences (student t-test, $p < 0.05$) suggest kinase activity of EphA3 is necessary to the ephrin-induced inhibition of process extension.

Co-transfection of Nck1WT with EphA3WT did not inhibit process retraction of EphA3WT-expressing cells in response to ephrin-A5 challenge (Fig. 3-8, A and A'). The process length was shortened by about 22% ($\pm$ 3.0%) similar to cells expressing only EphA3WT. However, when either Nck1R308K, or Nck1W38/143/229K, was co-expressed with EphA3WT, the process retraction induced by ephrin-A5 was significantly less (Fig. 3-8, B-C'). In the presence of Nck1R308K, or Nck1W38/143/229K, the decrease of process length of cells expressing EphA3WT was only 9.2% ($\pm$ 3.9%) and 6.5% ($\pm$ 2.1%), respectively. These results suggest that Nck1 protein is important in EphA3 signal transduction and the interaction mediated by both SH2 and SH3 domains are critical.

Discussion

The present study confirms the hypothesis that Nck1 is a strong binding partner of the EphA3 receptor, and demonstrates that the binding is mediated by a specific interaction between the Nck1 SH2 domain and the phosphotyrosine at 602 on EphA3. Inhibition of Nck1 function using dominant-negative mutants tempers inhibitory effects of EphA3 on cell migration and process extension, suggesting that Nck1 is a key downstream mediator of EphA3 functions.

Nck1 interacts with activated EphA3.

As described earlier, Nck1 has been found to interact with both EphA3 and EphA5 receptors. An early study by Stein et al. has showed that Nck1 binds to the intracellular domain of EphB1 (Stein et al. 1998). It was also reported that EphB2 bound to p62 dok after activation and formed a protein complex that included both RasGAP and Nck1 (Holland et al. 1997). These data together lend support to the notion that Nck1 function downstream of both EphA and EphB receptors and mediate key functions.

Consistent with the observation in the yeast two-hybrid experiments, we found that Nck1 protein was co-precipitated with EphA3 from transfected cells, and determined the interaction was mediated by Nck1 SH2 domain. These findings indicate that the interaction of Nck1 with EphA3 is similar to its interaction with EGF receptor and Crk-associated substrate (p130^{Cas}) in PDGF-stimulated cells (Roche et al. 1996; Tang et al. 1997; Rivera et al. 2006), but different from its binding to DCC and Robo, which occurs through the SH3 domains (Li et al. 2002). Furthermore, mutagenesis coupled with *in*

vitro pulldown experiments conducted in this study allowed us to identify key interaction sites on EphA3 for Nck1. It was found that neither EphA3-Y602F nor EphA3-Y602E bound Nck1, although both mutants exhibited normal kinase activity as measured by autophosphorylation and phosphorylation of exogenous substrate (Fig. 3-3 A and G. Shi and R. Zhou, unpublished observations). Thus, tyrosine residue 602 of EphA3 is indispensable for Nck1 binding and likely to be responsible for most, if not all, of the interaction. In addition, Nck1 SH2 domain has been shown to bind strongly to the sequence of “pTyr-hydrophilic-hydrophilic-Pro/Ile” through an unbiased screen in a degenerate phosphopeptide library (Songyang et al. 1993). The EphA3 amino acid sequence behind tyrosine 602 (pYEDP) fits this description. Interestingly, earlier studies showed that Nck1 interacts with EphB1 through the SH2 domain and Y594 (the equivalent of Y596 of EphA3) (Stein et al. 1998), indicating that there are variations in interaction mechanisms among different Eph receptors.

We also showed that activation of EphA3 by ephrin-A5 treatment induced the EphA3-Nck1 interaction. In the absence of ephrin-A5 stimulation, no EphA3 was co-immunoprecipitated by anti-Nck1 antibody from transfected 293A cells. Protein subcellular co-localization studies were consistent with the results from the co-immunoprecipitation experiments. Considerable amount of Nck1, although not all, was co-localized with EphA3 receptors in transfected cells, especially on the plasma membrane.

Similar to EphA3, EphA5 was found to bind Nck1 through the yeast two-hybrid screen. Since EphA3 and EphA5 share high homology with each other (Zhou 1998), we speculate that the binding between Nck1 and EphA5 is also through the SH2 domain and

tyrosine 656 (the equivalent of 602 of EphA3). It is likely that other EphA receptors also bind to Nck1 through this conserved tyrosine residue in the juxtamembrane domain. However, more research has to be conducted to confirm these speculations.

Nck1 mediates regulation of cell migration and process retraction by EphA3 activation.

In both process retraction and migration assays, we have shown that the over-expression of either dominant negative Nck1 mutant is able to inhibit effects of EphA3 activation. In contrast, Nck1 wild type has no such effects. Since the dominant negative clones are deficient in either SH2-mediated binding or SH3-mediated binding (Tanaka et al. 1995; Gupta and Mayer 1998), our observations suggest that both the recruitment of Nck1 to activated EphA3 through the SH2 domain and the interaction with downstream effectors through the SH3 domains are important steps in the signal transduction of EphA3 (Fig. 3-10). A potential concern in using dominant negative mutants is that the effects observed from these studies were due to non-specific inhibition of binding of other SH2 or SH3 domain-containing proteins by the mutants. However, if the effects of the mutants were non-specific, and Nck1 had no role in EphA3 signaling, it is expected that the expression of wild type Nck1 would also block EphA3 function. Our observation that wild type Nck1 did not block EphA3 function indicates that the mutants specifically inhibit Nck1 function and Nck1 is an important signaling intermediary downstream of the Eph receptor signaling.

Up to this date, Nck1 has been shown to interact with more than 30 proteins through the SH3 domains, most of which have been implicated in actin cytoskeleton re-

organization (Li et al. 2001). These interactions strongly support for a critical role of Nck1 in regulating Eph regulated cell migration and process outgrowth. Indeed, clustering of Nck1 SH3 domain alone was found to be sufficient to induce localized actin polymerization (Rivera et al. 2006), suggesting that the SH3 domains mediate actin cytoskeleton regulation through effectors.

Nck1 interacts with the p21-activated kinase (PAK1) via its second SH3 domain (Galisteo et al. 1996; Lu et al. 1997; Zhao et al. 2000) and translocate PAK1 from the cytosol to plasma membrane. As a result, PAK1 is brought in close proximity of Rac1 and cdc42 and activated (Bokoch 2003). Active PAK1 phosphorylates substrates such as LIM (Lin-11, Isl-1 and Mec-3 domain) kinase, which in turn inactivates actin depolymerizing factor (ADF) family proteins. Members of ADF family such as cofilin and myosin light chain kinase (MLCK) are important regulators of actin/myosin organization (Edwards et al. 1999; Sanders et al. 1999). As a result, inactivation of PAK1 leads to cofilin dephosphorylation and actin depolymerization. Indeed, PAK1 activity is reduced in ephrin-A1 treated cortical neurons (Shamah et al. 2001), although it is not known if ADF/cofilin activity is increased. The existence of a Nck1-PAK1 pathway is further supported by genetic data from *Drosophila*: the guidance and targeting of photoreceptor growth cones requires activity of both Nck1 and PAK homologs (Garrity et al. 1996; Hing et al. 1999). It would be interesting to examine whether ephrin-induced inhibition of cell migration and process outgrowth is mediated through the Nck1-PAK1 pathway.

Another important group of Nck1 binding proteins are the WASP family proteins such as WASP and N-WASP (Rohatgi et al. 2001; Rivera et al. 2004; Samarin 2005;

Takenawa and Suetsugu 2007). WASP mutations were first discovered in studies on an X chromosome linked immuno-deficient disease (Derry et al. 1994), which was characterized by cell abnormalities and actin cytoskeleton defects. Highly enriched in the brain, WASP proteins activate actin filament restructuring (Zalevsky et al. 2001) and regulate neurite growth (Takenawa and Suetsugu 2007). The WASP family proteins possess a C-terminal motif that binds to Arp2/3, a protein that mediates actin polymerization (Machesky et al. 1999; Rohatgi et al. 2001; Ibarra et al. 2005). When Nck1 SH3 domain was over-expressed in cells with N-WASP and Arp2/3, an unusually high rate of actin nucleation was observed (Rohatgi et al. 2001). In addition, WIP, a WASP interacting protein, was also found to bind to Nck1 through a site different from WASP (Anton et al. 1998). WIP has been shown to associate with profilin, an actin cytoskeleton regulatory protein. Thus, Nck1 may function through the WASP-WIP pathway to regulate cell morphology and migration following EphA receptor activation.

A recent study reported that EphB stimulation led to the formation of a protein complex that included Nck1, NIK, dok1, and RasGAP. Consequently, active NIK turned on the c-Jun amino-terminal kinase (JNK) pathway (Becker et al. 2000). Interestingly, when the *Drosophila* NIK homolog, Misshapen was mutated, the photoreceptor axon targeting was disrupted (Su et al. 1998), in a manner similar to the phenotype of Dock (Nck1 homolog in *Drosophila*) knockout animals (Ruan et al. 1999). It was therefore proposed that NIK was directly involved in Nck1 mediated axon guidance downstream of Eph receptors.

However, in the present study, neither Nck1 mutant was able to completely inhibit EphA3 effects in the assays we used. It is possible that complete inhibition requires high

levels of expression of the mutant proteins, which were not achieved in our studies. Alternately, EphA3 might exert its effects in part through Nck1-independent pathways, likely through other SH2/SH3 domain proteins like Grb2, RasGAP, and Crk (Stein et al. 1996; Holland et al. 1997; Lawrenson et al. 2002). Another possibility is that Nck2, the other member in the Nck family plays a redundant role in EphA3 signaling since it shares high homology with Nck1 (Chen et al. 2000; Buday et al. 2002; Bladt et al. 2003). It has been reported that Nck2 binds to Abi-1, Axin, and CAP, all of which are involved in cytoskeleton regulation. In addition, Nck2 also regulates ILK (integrin-linked kinase) (Tu et al. 1998; Buday et al. 2002; Xu et al. 2005), providing a link from EphA receptors to integrin signaling.

Figure 3-1. Interaction of Nck1 with EphA3 through SH2 domain.

(A) Domain structure of wild type Nck1 protein and Nck1 fusion proteins used in GST pulldown assay. (B) Identification of the EphA3 binding domain of Nck1. The GST fusion proteins were incubated with the lysates of HEK293A cells expressing EphA3 protein. The precipitated protein complex was analyzed by Western blot with anti-EphA3 antibody. “Input” shows the total EphA3 level in the whole cell lysate of transfected HEK 293A cells.

A.

B.

Location	Domain	Tyr -> Phe	Tyr->Glu	Lys->Arg
Tyr 596	Juxtamembrane domain	Y596F	Y596E	
Tyr 602	Juxtamembrane domain	Y602F	Y602E	
Tyr 736	Kinase domain	Y736F		
Tyr 779	Kinase domain	Y779F		
Tyr 937	SAM domain	Y937F		
Lys 653	Kinase domain			K653R

Figure 3-2. A series of EphA3 mutants containing single tyrosine mutations.

(A) Map of the mutated residues on the EphA3 receptor. (B) Table summarizing all of the mutants used in this study.

Figure 3-3. Identification of Tyr602 as the Nck1 binding tyrosine residue on EphA3.

(A) Auto-phosphorylation of EphA3 mutants. Five tyrosine-to-phenylalanine mutants (Y596F, Y602F, Y736F, Y779F, Y937F), two tyrosine-to-glutamic acid mutants (Y596E, Y602E), a kinase inactive mutant (K653R) and wild type EphA3 (WT) were expressed individually in 293A cells. EphA3 proteins were immunoprecipitated and analyzed by Western blot with anti-phosphotyrosine antibody and anti-EphA3 antibody. (B) Y602 mutation inactivates Nck1 binding as shown by the GST pulldown assays using GST-NckSH2 fusion protein.

Figure 3-4. Precipitation of EphA3 by Nck1 antibody in 293A cells.

EphA3 and Nck1 were co-transfected into 293A cells and Nck1 protein complex was precipitated with a monoclonal anti-Nck1 antibody. The immunoprecipitates were analyzed by Western blot. EphA3 was precipitated by Nck1 antibody only when the co-transfected cells were stimulated with ephrin-A5.

Figure 3-5. Co-localization of Nck1 and EphA3 in transfected HEK 293A cells.

Two groups of cells, shown in panel A-A'' and B-B'', were stained with FITC-anti-EphA3 (Panels A and B) and Cy3-anti-Nck1 (Panels A' and B'). Superimposition of the two single channel pictures (Panels A'' and B'') shows the co-localization of EphA3 and Nck1 protein, especially on the plasma membrane, as indicated by white arrows.

Figure 3-6. EphA3-induced inhibition of cell migration mediated by Nck1.

(A) The transwell apparatus used in migration assay. HEK293A cells were transiently transfected with EGFP, EphA3KD, Nck1WT, Nck1R308K, Nck1W38/143/229K, EphA3WT, EphA3WT+Nck1WT, EphA3WT+Nck1R308K and EphA3WT+Nck1W38/143/229K. Twenty-four hours later, cells were plated in transwells and a migration assay was performed as shown in the graph. (B) Quantification data is summarized in the graph. Each bar in the graph represents the migratory ability (M) of transfected cells, normalized to EGFP expressing cells. Asterisks indicate a statistically significant difference (student t-test, $p < 0.001$), when compared to proper controls (EphA3KD for EphA3WT, EphA3WT+Nck1 for EphA3WT+Nck1R308K and EphA3WT+Nck1W38/143/229K).

Figure 3-7. Cell process retraction induced by activation of EphA3.

HEK293A cells were transfected with EGFP (A-A'), or EphA3KD (B-B') or EphA3WT (C-C'). Forty-eight hours after transfection, the cells were treated with cross-linked ephrin-A5 for 60 minutes. Select cells were photographed before and after the treatment. The length of all identifiable processes on each cell was measured. Arrows indicate the end positions of the processes in each picture.

Figure 3-8. Disruption of EphA3-induced process retraction by Nck1 DN genes.

Nck1WT (A-A'), Nck1R308K (B-B'), or Nck1W38/143/229K (C-C') were co-transfected with EphA3WT in order to examine the effects of Nck1 clones in process retraction induced by ephrin-A5 stimulation. Arrows show the end positions of cellular processes in each picture.

Figure 3-9. Quantification of Nck1 mutant effects on EphA3-induced process retraction.

Plotted are changes of process length after ephrin-A5 treatment, normalized to EGFP control cells. A positive number indicates extension during treatment, while a negative value shows retraction of the processes. Asterisks indicate statistical significant differences when compared to proper controls (EphA3KD for EphA3WT, EphA3WT+Nck1WT for EphA3WT+Nck1R308K and EphA3WT+Nck1W38/143/229K; student t-test, $p < 0.05$).

Figure 3-10. A model of the effects of Nck1 DN genes in EphA3 signaling.

Nck1R308K and Nck1W34/143/229K are Nck1 clones that show disrupted binding through the SH2 domain or all three SH3 domains, respectively. The overexpression of either protein overrides the normal function of endogenous Nck1 and leads to the disruption of EphA3 mediated functions such as process retraction and inhibition of cell migration.

Chapter IV. Analysis of EphA3-Nck1 binding in neurons and assessment of Nck1 in hippocampal neuron growth cone collapse

Introduction

Thus far, all the research we have conducted on Nck1 provides convincing evidence on the significant role Nck1 plays in EphA mediated signaling. In fibroblast cells expressing both proteins, Nck1 molecules in the cytosol respond to EphA3 stimulation by binding to phosphorylated tyrosine 602 in the juxtamembrane domain and modulating downstream pathways. Dominant negative forms of Nck1, when overexpressed, disrupt this process and consequently disrupt EphA3 regulated cell responses such as process retraction and cell migration inhibition.

This study, however, has only tested systems in which EphA3 and Nck1 were over-expressed artificially. In order to show the functional significance of binding between endogenous EphA3 and Nck1, we set out to investigate the possible role of Nck1 in EphA3 mediated signaling in the developing nervous system. EphA3 is known to be highly expressed in late embryonic hippocampus and cortex (Yue et al. 2002), a time when the formation of critical neural circuits occurs. EphA3 regulates various important neural events such as axon guidance and axon fasciculation (Winslow et al. 1995; Friedman and O'Leary 1996). It is likely that Nck1 is involved in the regulation of these functions. Indeed, the initial yeast two-hybrid screen was performed in an embryonic mouse brain cDNA library. Taken together, these data prompted us to hypothesize that

Nck1 adaptor protein plays a critical role in neurons, linking extracellular signal through Eph receptors to downstream functional pathways such as the PAK pathway, the Sos/Ras pathway and effectors such as WASP and NIK. More specifically, we speculate that the disruption of Nck1 association with either activated EphA receptor or downstream effectors would render the signaling cascade dysfunctional and cause major defects in EphA3 signaling in the developing nervous system.

E18 rat embryonic brains were dissected and cortical and hippocampal neurons were isolated for this study. The endogenous expression of both EphA3 and Nck1 was evaluated by Western blot. Immunoprecipitation and immunostaining methods were applied to assess whether Nck1 directly associates with activated EphA3 receptor.

After studying EphA3-Nck1 binding in the neuron cultures, a growth cone collapse assay was employed to test in neurons if dominant negative Nck1 mutants were able to disrupt cellular events that are regulated by EphA3 receptor, similar to what occurs in transfected HEK293A cells.

Results

The expression of Nck1 in primary neuron culture

In the embryonic rat, EphA3 expression emerges at E12 at high levels in the sub-ventricular zone and persists through adulthood (Kilpatrick et al. 1996; Ernst et al. 1998). In addition, motor neurons and retinal neurons also express EphA3 (Sajjadi and Pasquale 1993; Marin et al. 2001; Otal et al. 2006). Nck1 is expressed in a large variety of tissues including heart, brain, testis, liver. Most important to this study, EphA3 is expressed at a stable level in the developing rat brain (Li et al. 1992; Park and Rhee 1992). The question

remains, however, if Nck1 is co-expressed with EphA3 in the same group of developing neuronal cells.

To answer this question, the cerebral cortices and the hippocampi were dissected from E18 rat embryonic brain. The dissociated cortical and hippocampal neurons were cultured on Poly-D-lysine coated dishes (Fig. 4-1 A). Two days after initial plating, the neuron cultures were lysed and the total cell lysates were separated on SDS-PAGE gel and analyzed by Western blot. Both anti-EphA3 antibody and anti-Nck1 antibody were used to assess the expression level of the two proteins at E18 (Fig. 4-1 B).

The Western blot data revealed that EphA3 receptor was expressed in both cortical neurons and hippocampal neurons of rats at E18, consistent with previous reports (Martinez and Soriano 2005; Otal et al. 2006). In addition, we observed that there was substantial expression of Nck1 in both cortical neurons and hippocampal neurons as well. These findings confirm that Nck1 and EphA3 are co-expressed in developing cortical and hippocampal neurons.

EphA3 and Nck1 interact in hippocampal neurons and cortical neurons

In Chapter III, we showed by immunostaining and immunoprecipitation methods, that ephrin-A5 stimulation promotes the binding of Nck1 with activated EphA3 receptor. Similarly, we applied these two techniques to analyze Nck1-EphA3 binding in primary neuron cultures.

E18 embryonic rat hippocampal neurons were cultured for two days before they were treated with cross-linked ephrin-A5 and fixed. Anti-EphA3 antibody, followed by FITC conjugated secondary antibody, was first used to label EphA3 localization. Anti-Nck1

antibody and Cy3 conjugated secondary antibody were then used to mark Nck1 protein. The stained neurons were photographed under a fluorescent microscope. The staining revealed that both proteins were expressed highly and they displayed a high degree of co-localization on the plasma membrane, especially on the growth cones as marked by the yellow areas (Fig. 4-2).

Immunoprecipitation was also used to ascertain the putative association of endogenous EphA3 and Nck1 in E18 neurons. One limitation of this method is a large quantity of total cell lysates is usually required to perform this assay. To circumvent this limitation, embryonic cortical neurons instead of hippocampal neurons were used, since the cortical neurons exist in greater abundance in rats. The cortical neurons were cultured for two days on Poly-D-lysine coated dishes and then treated with or without 1 μ g/ml dimerized ephrin-A5 for 30 minutes before being lysed. Cell lysates were then precipitated with a mouse anti-Nck1 antibody. As a negative control, ephrin-A5 stimulated cortical neuron lysates were incubated with control mouse IgG instead of the Nck1 specific antibody. The precipitated proteins were separated by SDS-PAGE and analyzed by Western blot (Fig. 4-3). Total neuron lysates were included to show expression of both EphA3 and Nck1.

In this experiment, Nck1 antibody precipitated high level of EphA3 from stimulated cell lysates (Fig. 4-3, lane 2). By contrast, neither precipitating from untreated cell lysates with Nck1 antibody nor precipitating from stimulated cell lysates with control IgG brought down EphA3 (Fig. 4-3, lane 1 and 3). These observations collectively show that Nck1 binds to activated EphA3 and ephrin-A5 stimulation is a prerequisite to this

binding. Furthermore, the confirmation of EphA3-Nck1 in neuron lysates suggests that Nck1 is an important factor in EphA3 regulated signaling cascades in neurons.

Ephrin-A5 induces growth cone collapse in a hippocampal neuron culture

Dissociated hippocampal neurons in culture adopt the characteristic neuronal morphology very quickly upon plating. This is characterized by the extension of long axons on one side of the cell body and the growth of multiple short dendrites on the other side as visualized by staining the cells with Texas-red Phalloidin (Fig. 4-4 A). The overall morphology of the neurons is best visualized by staining with Texas-red Phalloidin, a dye specific for actin filaments. Growth cones, the hand-like structures located at the end of neurites, stain heavily with Texas-red because of the high actin content. (Fig. 4-4 B). Because growth cones are responsible for guiding the axons through the environment in response to guidance cues, they are pivotal to axon guidance. A typical growth cone consists of two parts: the central domain and the peripheral domain (Fig. 4-4 C). The central domain, which connects the neurite and the growth cone, is enriched with microtubules and organelles. The peripheral domain, with its high actin cytoskeleton content, is the functional part of the growth cone that detects guidance cues and undergoes rapid changes. Upon close inspection, the peripheral domain is composed of relatively stable sheet-like expansions called “lamellapodia” and fine processes called “filopodia” that extend and retract constantly (Goldberg and Burmeister 1986; Forscher et al. 1987; Orioli and Klein 1997; Wilkinson 2000). Growth cones are known to contain an abundance of receptors and signaling molecules. Working together closely, these proteins orchestrate rapid responses of to extracellular stimuli.

Expression of EphA3 receptor in both hippocampal neurons and cortical neurons enables these neurons to respond to ephrin-A ligand stimulation efficiently. The receptor, as shown earlier, is highly localized to the growth cone and along the neurites. Thus, ephrin-A treatment triggers an immediate response on the growth cone and continues on the axon and soma (Meima et al. 1997; Meima et al. 1997). This immediate response is best exemplified by a growth cone collapse assay.

E18 hippocampal neuron explants were cultured in serum-free neurobasal medium for two days. Prior to ephrin-A5 treatment, cultured hippocampal neurons usually possessed characteristic growth cones, exhibiting an active exploration of the surroundings (Fig. 4-4 E). However, within as little as 15 minutes of ephrin stimulation, dramatic morphological changes were observed on a majority of the growth cones (Fig. 4-4 F). Instead of maintaining the hand-like morphology, the growth cones collapsed into a lean tapered shape (Fig. 4-4 D). The initially recognizable lamellapodia and filopodia were no longer obvious.

We also tested the responses of cortical neurons to ephrin-A5 incubation in a culture setting. Similarly, the elaborate growth cones of the cortical neurons also collapsed shortly after ephrin stimulation (data not shown).

Since immunoprecipitation and immunostaining methods both indicated that Nck1 interacts with phosphorylated EphA3 receptor in cultured neurons, we were interested in determining if Nck1 was critical to the process of EphA3 mediated growth cone collapse. By expressing dominant negative Nck1 clones in hippocampal neurons, we examined whether the disruption of endogenous Nck1 functions blocked ephrin-A5 induced growth collapse.

Construction of adenoviruses carrying wild type and dominant negative clones of Nck1

Neurons are difficult to transfect because they are sensitive to harsh experimental procedures. Regular lipid-based transfection reagents such as Lipofectamine do not produce high efficiency (<5%) and neurons are sensitive to the toxicity of these reagents. Calcium phosphate method does not have high toxicity but the efficiency is also very low (<10%). Nucleofection, a method based on electroporation principles, has decent efficiency (>20%) but requires a large number of cells (>10⁶ cells) for each experiment. After evaluating these methods, we recognized that adenovirus-mediated gene transfer was the ideal approach. Adenovirus is known to infect neurons with high efficiency and the expression of desired genes is not dependent on cell division. The latter attribute is especially meaningful since cultured neurons do not proliferate. In addition, adenoviruses are not cytotoxic to neurons so the infected neurons stay relatively healthy.

The procedure of adenovirus construct generation was summarized [Fig. 4-5, (He et al. 1998)] and described in detail in Chapter VI. Recombinant adenoviral vectors, pAdEasy-CMV, pAdEasy-CMV-Nck1WT, pAdEasy-CMV-Nck1R308K and pAdEasy-CMV-Nck1W38/143/229K were each digested and transfected into HEK293A cells to generate viruses. On these plasmids, the inserted gene of interest and an EGFP gene were located behind separate CMV promoters, allowing easy recognition of the infected cells. The transfected 293A cells were harvested seven days later and the adenoviruses were collected. Several rounds of amplification were carried out to obtain high-titer viral stocks. The final adenovirus stocks were used to infect 293A cells and the expression of

Nck1 mutants in infected cells was confirmed by Western blot (Fig. 4-6 A). In general, for neuronal infection, recombinant adenoviruses with a titer of 10^9 to 10^{10} /ml of recombinant adenovirus were used.

Nck1 DN mutants do not prevent growth cone collapse induced by ephrin-A5

To examine effects of Nck1 DN mutants on the growth cone collapse, E18 rat embryos were sacrificed and the hippocampi were collected. In a glass dish, the hippocampi were cut into small explants of uniform size. These hippocampal explants were plated in serum free neurobasal medium on glass culture dishes that had been coated with Poly-D-lysine and Laminin.

Adenovirus stocks were diluted in neurobasal medium and filtered with a .25um filter to remove all residual cell debris. The adenovirus solution was added to the hippocampal explant cultures. After overnight incubation, the adenoviruses were removed and fresh culture medium was added.

On day 4, the cultured explants were treated with or without cross-linked ephrin-A5 for 30 minutes before being fixed with fresh fixative. The explants were permeablized with TritonX-100 and then stained with Texas-red phalloidin to visualize the actin cytoskeleton, allowing easy observation of growth cones.

The infected neurons were identified by monitoring EGFP expression. On each explant, every green growth cone that was on the periphery of the explant was examined and marked as either “collapsed” or “uncollapsed”. The number of all infected growth cones (G1) and the number of all infected growth cones that had collapsed (G2) were counted and the growth cone collapse index was expressed as $I=G2/G1$. For each

experiment, at least 5 explants were quantified and the average I value was calculated. The results were found to be consistent in three experiments and one representative experiment is shown in Figure 4-6 B.

Before ligand stimulation, about 33% (+/-4%) of wild type hippocampal growth cones expressing only EGFP were collapsed. After 30 minutes of ephrin-A5 treatment, about 77% (+/-2%) of all EGFP expressing growth cones were collapsed. When explants expressing various Nck1 mutants were inspected, we found that the expression of any Nck1 mutant did not alter the response of hippocampal neurons to ephrin-A5 stimulation. Despite the expression of dominant negative Nck1 clones, the majority of infected growth cones still collapsed in reaction to ephrin-A5, similar to the EGFP control. It was therefore concluded that the expression of Nck1 mutants through adenoviruses was not able to disrupt EphA3 mediated growth cone collapse induced by ephrin-A5 treatment.

Discussion

Nck1 binds to activated EphA3 receptor in neurons and they co-localize highly on the growth cones

During development, many neural circuits form and many signaling factors are involved in this process, among which is the EphA3 receptor. For instance, interactions of EphA3 with the ephrin-A2 and -A5 ligands on the tectum is the shaping force for a precise retinotectal topographic projection (Cheng et al. 1995; Drescher et al. 1995). In the past two decades, much effort has been invested in understanding how the downstream signaling pathways are regulated.

We isolated Nck1 in a screen for EphA3 binding proteins in an embryonic brain cDNA library. In HEK293A cells, we have found that Nck1 SH2 domain binds directly to the tyrosine residue 602 of EphA3. Additionally, in 293A cells, the expression of dysfunctional Nck1 proteins is able to lead to disrupted EphA3 signaling. Furthermore, at E18, when EphA3 expression is present in cortical and hippocampal neurons, Nck1 is also co-expressed. These data together strongly suggest Nck1 plays a major part in EphA3 signal transduction in neurons.

In contrast to control IgG, Nck1 specific antibody successfully precipitates EphA3 molecules. This co-precipitation of EphA3 and Nck1 was dependent on the activation of EphA3 receptor by ephrin-A5 treatment. The necessity of ligand treatment for EphA3-Nck1 binding is in accordance with previous research and indicates that phosphorylation and activation of EphA3 receptor are paramount to Nck1 binding.

Immunostaining, on the other hand, helped us to determine the sub-cellular localization of EphA3 receptor and Nck1 molecule. It was found that EphA3 molecules were localized on the plasma membrane of both the cell body and the neurites, especially on the growth cones. This finding was consistent with the fact that growth cones guide axons in a complex environment towards their appropriate targets. Immunostaining also revealed that after ephrin-A5 treatment, Nck1 molecules translocated from the cytosol to interact with activated EphA3 receptors. Consequently, both proteins were found to have a high degree of co-localization on the plasma membrane.

The data from neuron cultures and from transfected HEK293A cells support our hypothesis that Nck1 plays a significant role in EphA signal transduction.

Nck1 signaling may regulate aspects of EphA3 signaling but not growth cone collapse

EphA3 is expressed in the hippocampi in a graded manner (medial high, lateral low) along with EphA4, -A5 and -A7. The corresponding ligands ephrin-A2, -A3, -A5 are expressed in the septum in a ventral high, dorsal low gradient (Gao et al. 1996; Zhang et al. 1996). During the formation of this projection, the growth cones of medial hippocampal neurons are turned away from the ventral lateral septum due to the high expression of EphA3 receptors, which results in strong repulsion (Gao et al. 1996; Zhou 1997; Otal et al. 2006). Consequently, the medial axons retract and move towards the dorsal lateral part of the septum, where ephrin-A ligands are expressed at a lower level. The lateral hippocampal neurons, on the other hand, express low level of EphA receptors and are able to project into the ventral septum. Therefore, the complementary gradients of receptor and ligands enable hippocampal neurons to innervate appropriate targets on the septum and form a precise topographic projection.

Similarly, neurons in culture exhibit growth cone collapse and axon retraction, in reaction to ligand addition to the culture medium. This observation provides the foundation of growth cone collapse assay. However, as we noted, some growth cones remained uncollapsed after ephrin treatment probably because a heterogeneous population of hippocampal neurons was tested. Since the lateral hippocampal neurons express low levels of Eph receptors, they are less responsive to ephrin-A5 treatment.

Using adenoviruses expressing different genes, we tested our hypothesis that Nck1 is involved in EphA3 signaling in hippocampal growth cone collapse. Surprisingly, no alteration to the growth cone collapse rate of was detected in infected hippocampal

neurons, in which the function of endogenous Nck1 was blocked by dominant negative mutants.

Although we tested the infection of various mutants in 293A cells by Western blot, we did not confirm the expression of these genes in neurons. In addition, we relied on EGFP expression to help us identify infected neurons, but the presence of green fluorescence in the cells does not necessarily reflect the expression of the desired genes at a high level or alternately, the infection efficiency and expression level in neurons were not high enough in our assay. It is expected that a significant amount of dysfunctional Nck1 molecules is required to override the function of endogenous Nck1, which was very abundant in neurons (Fig. 4-1 B). Therefore, other methods of blocking Nck1 function should be tested before we conclude that Nck1 is not involved in the process of growth cone collapse triggered by ephrin-A5 stimulation. For instance, siRNA is a commonly used technique for knocking down the endogenous expression of the protein of interest. By using Nck1 specific siRNA in neurons to effectively disrupt Nck1 translation, we would be able to evaluate Nck1's role through a different approach.

We use hippocampal explants in our assay because the hippocampal neurons express both EphA3 and Nck1 and are well characterized. However, the graded expression of multiple Eph receptors across the hippocampus suggests a high degree of redundancy (Gao et al. 1999). When dysfunctional Nck1 genes were expressed in these neurons, it is probable that the signaling through Eph receptors was not completely silenced, therefore leading to no detectable alteration to growth cone collapse.

When the only Nck homolog in *Drosophila* Dock was eliminated, severe disruption of the correct innervations of photoreceptor neurons was observed (Garrity et al. 1996;

Rao and Zipursky 1998). These neurons, instead of stopping at their proper targets, tend to overshoot. Furthermore, the expression of Ste20-like kinase Misshapen (Msn, a homolog of mammalian NIK) was found to co-localize with Dock in the growth cones of these neurons ((Ruan et al. 1999; Su et al. 2000). Loss of Msn resulted in a similar phenotype to the R cells as in Dock knockout. These combined observations suggest that Nck proteins, which are conserved in mammals are likely to regulate growth cone collapse as well. Since the two mammalian Nck members, Nck1 and Nck2 show high homology (60%) and their binding preferences seem to be indistinguishable from each other (Frese et al. 2006), it is very likely that the endogenous expression of Nck2 compensates for the loss of normal Nck1 function in hippocampal neurons. Even though we did not test the expression of Nck2 in neurons, it is very likely that Nck2 is also present in hippocampal and cortical neurons. We suspect that this redundancy explains why the expression of dominant negative Nck1 was not able to lead to a reduced rate of growth cone collapse, similar to how the Nck1 DN genes were not able to completely reverse ephrin-A5 induced phenotypes in 293A cells expressing EphA3. Previously, when Nck1, Nck2 double knockout mice were generated, it was found out that the double knockout animals died during embryonic development. An inspection of fibroblasts of these animals reveals that these cells become defective in cell motility and actin restructuring (Bladt et al. 2003). An analysis of the hippocampal axon guidance in Nck1, Nck2 double knockout animals would therefore shed light on the possible redundant role of Nck2 in the EphA3-Nck1 signaling pathway.

Another possibility is that though our hypothesis about Nck1's involvement in EphA3 mediated signaling still holds true, Nck1 does not directly regulate growth cone

collapse, one aspect of EphA3 signaling in neurons. As we discussed earlier, ephrin-A5 treatment of EphA3 expressing neurons causes an immediate collapse of growth cones in the majority of the neurons. This is followed by a period during which neurite growth is generally stalled. Later, the neurites may exhibit either retraction or turning as they are further repelled by the ligands (Muller et al. 1990; Weinl et al. 2003; Huot 2004). These observations show that the response to ephrin treatment takes place in multiple stages and probably requires the coordination of many signaling pathways, especially the cytoskeleton machinery. For instance, the actin network in the growth cone and the microtubule filaments in the neurite shaft both have to be mobilized during this process. Nck1 has been known to link receptor tyrosine kinase to downstream signaling pathways such as the p21 cdc42/Rac activated kinase (PAKs), the WASP mediated actin restructuring and Sos/Ras/MAPK pathways (Tang et al. 1997). Through its many effectors, Nck1 is likely to be an integral part of EphA3 signaling in developing nervous system. Since our assay focused on detecting a difference in growth cone collapse on the hippocampal neurons but not other changes, more experiments need to be designed to assess if Nck1 plays a part in other aspects of ephrin-A5 induced responses. For instance, neurite retraction assay is an assay that has been used in some labs to test the signaling of Eph receptors in neurons. When young dissociated hippocampal neuron culture are treated with ephrin-A5 overnight, a ~50% of average neurite retraction was noted (Dr. S. Hsu and Dr. S. Wang, personal communication). It will be intriguing to find out when the function of Nck1 is compromised, whether the hippocampal neurons still retract the axons at such a rate.

Figure 4-1. Expression of EphA3 and Nck1 in cortical and hippocampal neuron cultures.

(A) Bright field image of E18 rat embryonic hippocampal neuron culture. (B) Bright field image of E18 rat embryonic cortical neuron culture. (C) The endogenous expression of EphA3 and Nck1 in neuron cultures, as assessed by Western blot.

A.

A'.

A''.

B.

B'.

B''.

Figure 4-2. Co-localization of EphA3 and Nck1 in hippocampal neuron growth cones.

E18 rat embryonic hippocampal neurons were cultured for two days before being treated, fixed, and stained. Anti-EphA3 antibody followed by FITC secondary antibody was used to stain for EphA3. Anti-Nck1 antibody with Cy3 secondary antibody was used to stain Nck1. Two groups of cells are shown in A-A'' and B-B'' respectively. Panel A'' and B'' are superimposed pictures of two single channels and the white arrows indicate co-localization of EphA3 and Nck1.

Figure 4-3. Co-precipitation of EphA3 and Nck1 in cortical neurons.

EphA3 molecules were precipitated from E18 rat hippocampal neuron lysates by Nck1 specific antibody in a stimulation-dependent manner. Total expression of EphA3 and Nck1 in the neuron lysates is also shown.

Figure 4-4. Growth cone collapse induced by ephrin-A5 treatment in hippocampal neurons.

(A). Fluorescent image of cultured hippocampal neurons after staining with Texas-red Phalloidin. (B) Enlarged box in panel A. (C) A schematic representation of a representative growth cone. “N” denotes the neurite; “C” indicates the central domain of a growth cone and “P” marks the peripheral domain that includes the lamellapodia and filopodia. (D). A collapsed growth cone after ephrin-A5 treatment. (E). Hippocampal explant cultures displaying many growth cones after two days. (F). A brief treatment with ephrin-A5 resulting in the collapse of growth cones in the explant culture.

Figure 4-5. A schematic outline of the construction of adenoviral vectors carrying the gene of interest.

The figure is modified from the paper *A simplified system for generating recombinant adenoviruses* (He et al. PNAS, 1998). The gene of interest was cloned into pAdTrack-CMV vector and the resulted plasmids were recombined with pAdEasy vectors in the *E. coli* strain BJ5183 to obtain desired recombinant plasmids. These plasmids were then digested and transfected into 293 cells to produce adenoviruses. In the plasmid maps, the "left arm" and "right arm" are the regions mediating homologous recombination between pAdTrack-CMV-GOI and pAdEasy. Abbreviations: An, polyadenylation site; Bm, *Bam*HI; RI, *Eco*RI; LITR, left-hand ITR and packaging signal; RITR, right-hand ITR; Sp, *Spe*I.

A.

B.

Figure 4-6. Growth cone collapse assay on neurons infected with adenoviruses expressing Nck1WT, Nck1R308K, and Nck1W38/143/229K.

(A) The expression of various Nck1 mutants through adenovirus infection was confirmed by Western blot. (B) When the amplified adenoviruses were used to infect hippocampal neuron explants, no significant differences in growth cone collapse response was observed in neurons treated with ephrin-A5.

Chapter V. Assessment of the binding of EphA3 with Grb2 and RasGAP

Introduction

We have shown in earlier chapters that Nck1, consistent with the yeast two-hybrid screen results, is able to regulate key aspects of EphA3 signaling. Furthermore, we showed that the interaction between Nck1 and Eph3 occurs through the SH2 domain and tyrosine 602, respectively. Tyrosine residue 602 is located in the juxtamembrane domain of EphA3 and is highly conserved throughout the Eph receptor family.

Even though Nck1 is the only SH2 domain containing protein identified in the yeast two-hybrid screen, it is well-known that other SH2 domain containing proteins also initiate direct interactions with the Eph receptors ((Birge et al. 1996; Mellitzer et al. 2000; Nakamoto 2000; Buday et al. 2002). Intriguingly, many of these proteins have been found to interact with the juxtamembrane domain of the Eph receptors (Schmucker and Zipursky 2001; Klein 2004).

Thus, a question arises as to whether Nck1 and other SH2/SH3 domain containing proteins may compete for the same binding sites once EphA3 receptor is activated. To answer this question, we attempted to map the binding locations on EphA3 with Grb2 and RasGAP. Both of these proteins play key roles in the regulation of Ras/MAPK pathway, and it has been known the activity of MAPK pathway is closely correlated with cell behavior like neurite retraction and growth cone collapse (Tong et al, 2003).

Characterization of EphA3 interaction with these proteins would therefore shed light on how SH2 domains of various proteins compete for binding and induce varied phenotypes.

Results

The SH2 domain of both Grb2 and RasGAP bind strongly to EphA3

Grb2 is a small adaptor protein that contains one SH2 domain and two SH3 domains (Fig. 5-1 A). Numerous studies have shown that Grb2 physically interacts with EphA2, EphB1 and EphB2 (Stein et al. 1996; Pratt and Kinch 2002; Moeller et al. 2006). Grb2 is known to bind to Sos, a RasGEF through its SH3 domains (Egan et al. 1993; Buday et al. 1994). When Grb2/Sos complex associates with phosphorylated RTKs, Sos is activated and catalyzes the replacement of GDP with GTP on Ras. Active Ras is then able to up-regulate MAPK activities (Zarich et al. 2000; Avruch et al. 2001).

RasGAP is a 120 KD cytosolic protein that promotes the conversion of GTP to GDP on Ras small GTPases. As a result, Ras is inactivated and the MAPK activity is down-regulated (Elowe et al. 2001; Tong et al. 2003). RasGAP consists of two SH2 domains, one SH3 domain, one pleckstrin homology domain (PH), one protein kinase C2 domain (C2) and one Ras guanine activating protein domain (Ras-GAP) (Fig. 5-1 A). Previous reports have shown that RasGAP associates with EphB2, forming a large protein complex that includes RasGAP, Nck1, and EphB2 (Holland et al. 1997; Tong et al. 2003). These studies also show that the binding occurs mostly through the N-terminal SH2 domain of RasGAP.

The SH2 domain of Grb2 and the N-terminal SH2 domain of RasGAP were each fused to GST peptide in pGEX 4T-2 vectors. Beads conjugated with GST fusion proteins

were used in pulldown experiments. The precipitated proteins were analyzed by Western blot (Fig. 5-1B). The analysis shows that GST-Grb2SH2 is able to precipitate phosphorylated EphA3 receptor, indicating that Grb2 protein interacts with the receptor. We also confirmed the association of RasGAP with EphA3 through the N-terminal SH2 domain (Fig. 5-1 B). The abundance of precipitated EphA3 in both lane 4 and lane 5 indicates that the binding between EphA3 with Grb2 or RasGAP is strong.

Tyrosine 602 on EphA3 is a binding site for Grb2 SH2 domain

After validating the strong binding of EphA3 and Grb2, we conducted another pulldown experiment to identify the binding location of Grb2 on EphA3.

In summary, the tyrosine mutants (Y596F, Y602F, Y779F, Y937F, Y596E, Y602E), EphA3WT and K653R were overexpressed in 293A cells. The cells were treated with ephrin-A5, lysed, and incubated with sepharose beads conjugated to GST-Grb2SH2 proteins. The precipitated proteins were analyzed by Western blot (Fig. 5-2).

The Western blot reveals the binding of Grb2 and EphA3 is dependent on the kinase activity of EphA3 since the kinase dead mutant K653R doesn't show any binding with Grb2 SH2 domain. Among the tyrosine mutants, the Y779F and Y937F mutants both exhibit normal binding with Grb2, while both Y596F and Y602F display no interaction with Grb2, similar to K653R. When the Y596 and Y602 were replaced by glutamate, disparate results were obtained. While Y596E mutant can be precipitated by Grb2 SH2 domain at a normal level, a significantly lower amount of Y602E is precipitated by Grb2 protein (Fig. 5-2). This result was consistent in three separate experiments. Upon

examining the phosphorylation pattern of these mutants (Fig. 3-3A), we conclude that the tyrosine 602 is one of several major binding sites of Grb2 SH2 domain.

RasGAP binds to EphA3 partially through tyrosine 602

When GST-RasGAPNSH2 was incubated with cell lysates containing various EphA3 mutants, similar results were found (Fig. 5-3). Both Y596F and Y602F are not precipitated by RasGAP N-terminal SH2 domain, while other tyrosine to phenylalanine mutants bind to RasGAP N-terminal SH2 domain normally. When the two tyrosine to glutamic acid mutants were tested, we found that Y596E binds normally to RasGAP, while Y602E exhibits only partial binding. This finding suggests that the Y602 in the juxtamembrane domain also mediates binding with the RasGAP N-terminal SH2 domain but other unknown binding sites also exist.

Discussion

EphA3 interacts with multiple SH2 domain containing proteins

A multitude of proteins that have been found to bind to Eph receptors contain SH2 domains (<http://proteoscape.uchicago.edu/sh2/index.html>, Fig. 5-4). Among them are well known adaptor proteins such as Crk, Grb2, Grb10, Nck1, SHC, and SLAP (Pandey et al. 1995; Stein et al. 1996; Holland et al. 1997; Stein et al. 1998; Lawrenson et al. 2002; Pratt and Kinch 2002). These adaptors do not possess catalytic abilities, but contain multiple protein binding domains (Fig. 5-4 A). They serve as scaffold proteins that not only link activated tyrosine receptors to downstream effectors, but also bring downstream effector proteins in close proximity with each others.

Src family kinase (SFK) members are also known to bind to Eph receptors and they include Fyn, Yes, Src, and Abl/Arg (Ellis et al. 1996; Choi et al. 1999; Yu et al. 2001). These proteins possess similar domain structure and catalyze phosphorylation of cytoplasmic substrates (Fig. 5-4 B). For instance, Src kinase phosphorylate R-Ras and reduces cell adhesion by regulating integrin signaling (Zou et al. 2002). Interestingly, it has been shown that EphB2 has the ability to phosphorylate R-Ras as well (Zou et al. 1999).

Another group of SH2 containing proteins are enzymes that are not SFK members. This group of proteins includes p85 PI3 kinase, PLC gamma, RasGAP, SHEP-1, and SHP2 (Holland et al. 1997; Dodelet et al. 1999; Boyd and Lackmann 2001). These proteins (Fig. 5-4 C) are able to play direct roles in regulating signaling pathways. For example, SHEP1 possesses one SH2 domain and one Ras-GEF domain. It binds to R-Ras and Rap1A through the Ras-GEF domain and brings these small GTPases close to EphB2 receptor juxtamembrane domain (Dodelet et al. 1999). Once the R-Ras molecules are located close to the receptor/effector complex near the plasma membrane, they become phosphorylated by the Src kinase or EphB2, as mentioned earlier.

Of special interest to us is the capability of these binding proteins to form complexes among themselves. Grb2 has been found to bind to Crk, SHC, Abl/Arg, PLC gamma, PI3 kinase, SHP2, and RasGAP (Saleem et al. 1995; Wang and Moran 1996; Wong and Johnson 1996; Pei et al. 1997; Riordan et al. 2000; Lamprecht et al. 2002; Plattner et al. 2004). These multilateral interactions may enhance the association with Eph receptors and give rise to a tightly organized signaling complex through which the receptor signals efficiently.

Grb2 may compete with Nck1 for the binding of EphA3

Using the power of the GST pulldown assay, we confirmed that Grb2 is able to bind phosphorylated EphA3. The characterization of EphA3-Grb2 interaction shows that it is different from the binding of Grb2 with another EphA receptor, EphA2. As reported earlier, Grb2 interacts with activated EphA2 indirectly through another SH2 containing protein, SHC (Pratt and Kinch 2002).

Furthermore, the GST pulldown assays demonstrate that tyrosine 602 in the juxtamembrane domain is one binding site for the Grb2 SH2 association. When this tyrosine residue is mutated to a phenylalanine residue, Grb2 fails to bind EphA3. Replacement at this position with glutamic acid rescues binding. Tyrosine 596, in comparison, modulates the kinase activity of the receptor, but does not serve as a direct binding site. In addition, mutation of tyrosine 779 and 937 did not result in loss of binding with EphA3, suggesting that these residues play an unimportant role in mediating the interaction with Grb2 SH2 domain. It is important to point out that other binding sites also exist since Y602E displays some binding with Grb2. A more comprehensive experiment needs to be performed in order to identify the rest of the interacting tyrosine residues.

Since Grb2 and Nck1 share one identical binding site, they are likely to compete for binding when they are both present in EphA3 expressing cells. Nck1, through competition with Grb2, could compromise the Sos/Ras/MAPK pathway and lead to down-regulated MAPK activity. However, because Grb2 is able to bind to multiple

locations and also indirectly through other proteins, the effect of this competition is not known at present but it would be interesting to find out.

RasGAP binds to EphA3 through multiples sites including tyrosine 602

Our experiment data shows that p120RasGAP also binds to tyrosine 602 in the juxtamembrane domain, while tyrosine 596 does not play a part in the interaction with RasGAP. However, tyrosine 602 is only responsible for mediating part of the interaction, as evidenced by the mutation of residue 602 to glutamate, which did not entirely abrogate binding with RasGAP. It can be inferred that other binding sites for RasGAP exist on EphA3. Similar to our finding, Elowe et al have reported that mutation of tyrosine 604 and 610 residues in EphB2 (equivalent to 596 and 602 of EphA3) to glutamates, results in a reduction rather than complete loss of binding with RasGAP (Elowe et al. 2001). However, a different group reported that when both the juxtamembrane domain tyrosines and tyrosine 667 were mutated on EphB2, a complete loss of binding with RasGAP occurred (Tong et al. 2003). Since tyrosine 659 of EphA3 is equivalent to tyrosine 667 of EphB2, it is probable that tyrosine 659 in the kinase domain is the other binding site that mediates the recruitment of RasGAP to the activated EphA3. More research has to be conducted in order to test this hypothesis.

In the current study, we only tested the binding of N-terminal SH2 domain of RasGAP. Even though previous studies show that the C-terminal SH2 domain usually does not interact with activated tyrosine kinase receptors (Holland et al. 1997), there exists a possibility that this domain mediates synergistic binding with N-terminal SH2 domain. Furthermore, indirect binding through Crk and Nck1 has been reported.

Therefore, even though tyrosine 602 is a common site for both Nck1 and RasGAP to bind, the two proteins may be capable of multilateral binding, similar to the association of RasGAP and Nck1 with EphB2 (Holland et al. 1997). Furthermore, it has been shown that p62dok assists the association of RasGAP and Nck1 since both proteins bind to p62dok through their SH2 domains (Tang et al. 1997).

As we discussed earlier, RasGAP directly regulates the activity of Ras and consequently, reduces both integrin signaling and ERK activities, critical to Eph receptor signaling. In NG108 cells, activated EphB2 recruits RasGAP and down-regulates MAPK activity (Elowe et al. 2001; Tong et al. 2003), leading to neurite retraction and growth cone collapse. Grb2 on the other hand, activates Ras through Sos, and up-regulates ERK activities. Since our data suggest that both proteins bind to EphA3 through tyrosine residue 602 at least partially, the recruitment of Grb2 or RasGAP onto activated EphA3 may provide an explanation for different responses induced ephrin treatment.

Other SH2 domain proteins that interact with the juxtamembrane domain

Our studies show that Nck1, Grb2 and RasGAP all bind to EphA3 receptor via interaction with tyrosine 602 in the juxtamembrane domain (Fig. 5-5). In addition, previous studies have shown that the juxtamembrane domain is very important in the interaction with many SH2 domain containing proteins such as Fyn, Yes, Src, Crk, SLAP, SHEP-1, PLC gamma, and Abl/Arg kinase (Ellis et al. 1996; Stein et al. 1998; Zisch et al. 2000; Yu et al. 2001). Therefore, we speculate that the preferential association of different sets of SH2 containing proteins with Eph receptor is likely to

translate to context-dependent responses to ephrin stimulation. More research is needed in order to understand how this signal transduction process is regulated.

A.

B.

Figure 5-1. Interaction of EphA3 with Grb2 SH2 domain and RasGAP N-terminal SH2 domain.

(A) Domain structure of wild type Grb2, RasGAP, and the GST fusion proteins used in the pulldown assays. (B) Western blot analysis of the pulldown experiment demonstrating that both GST-Grb2SH2 and GST-RasGAPNSH2 precipitate wild type EphA3.

Figure 5-2. Identification of Tyr602 as one binding site of Grb2 protein.

The EphA3 tyrosine mutants along with wild type EphA3 were employed in a pulldown experiment using GST-Grb2SH2. The results were analyzed by Western blot. Tyrosine 602 on EphA3 is one of several binding sites used by Grb2 protein.

Figure 5-3. Identification of Tyr602 as one of RasGAP binding sites on EphA3.

The results of pulldown assay with GST-RasGAPNSH2 were analyzed by Western blot.

RasGAP binds to EphA3 through tyrosine 602 and other unknown sites.

A. Adaptor proteins

B. Src kinase family

C. SH2 containing enzymes

Figure 5-4. Domain structure of all SH2 domain containing proteins that bind to Eph receptors.

(A) Adaptor proteins. (B) Src family kinases (SFKs). (C) Enzyme that associate with Eph receptors. Abbreviations: C2, protein kinase C conserved region 2; F-actin binding, F-actin binding domain; PH, Pleckstrin homology domain; Phosphatase, phosphotyrosine phosphatase domain; PLCXc, phospholipase C catalytic domain; PTB, phosphotyrosine binding domain, RA, Ras-associated-like domain; Ras-GAP, Ras-GAP domain; Ras-GEF, Ras-GEF domain; RhoGAP, RhoGAP domain; SH2, Src homology-2 domain; SH3, Src homology-3 domain; Y Kinase, tyrosine kinase domain.

Figure 5-5. A schematic representation of the interactions of EphA3 receptor with Grb2 and RasGAP.

Both Grb2 and RasGAP interact through Y602 as well as other phosphotyrosines at unknown sites. Grb2 and RasGAP regulate MAPK activity oppositely through regulating Ras GTPase activity. Nck1 binds solely through Y602 of EphA3, but its role in Ras/Raf/MAPK pathway is still unclear.

Chapter VI. Materials and Methods

Reagents and antibodies

Ephrin-A5-Fc fusion protein was purchased from R&D systems (Minneapolis, MN). The ligand was clustered through antibody cross-linking using anti-human Fc (Jackson ImmunoResearch, West Grove, PA) at an antibody to ephrin ratio of 1 to 5 in weight (1:15 in molar ratio). The mixture was incubated at 4 °C for 8 hours and used to stimulate cells at 1 - 2 µg/ml concentration. Mouse anti-Nck1 monoclonal antibody was purchased from BD Biosciences (San Jose, CA). The rabbit anti-EphA3 polyclonal antibody and mouse anti-HA monoclonal antibody were purchased from Santa Cruz (Santa Cruz, CA). The phosphotyrosine antibody used in analyzing the phosphorylation of the EphA3 receptors was purchased from Cell Signaling Technology (Danvers, MA). For Western blot analyses, these antibodies were used at 1:1000 and for immunocytochemical staining, at 1:200. Secondary antibodies used in Western blotting and immunostaining were acquired from Sigma-Aldrich (St. Louis, MO) and Jackson ImmunoResearch (West Grove, PA) respectively. When re-blotting was required, the nitrocellulose membrane was washed briefly and incubated in Western-blot re-strip buffer from G-Biosciences (St. Louis, MO).

Yeast two-hybrid screen

The Yeast two-hybrid screen was performed with DupLex-A system from Origene (Rockville, MD) according to the instructions. In brief, the intracellular domain of EphA3 receptor was cloned into pEG202-NLS vector, fused to DNA binding protein LexA to

generate the bait plasmid pEG202-NLS-EphA3intra. This plasmid was then transformed into yeast strain EGY188 along with a reporter plasmid carrying a LacZ gene and an embryonic mouse brain cDNA library cloned in the target plasmid pJG4-5. The transformed yeast cells were plated and screened for LacZ transcription through X-gal reaction. All positive clones were then extracted and sequenced. The identity of the positive clones was determined using BLAST searches against public databases. The specificity of the binding between the identified cDNA products and the bait protein used was further confirmed through yeast mating test. Briefly, the isolated target plasmid carrying cDNA fragment was transformed into EGY188 and this strain was mated with an EGY40 strain containing both the reporter plasmid and pEG202-NLS-EphA3intra. Then the offspring cells were lysed and incubated with X-gal solution for 2 hours. Negative controls for mating test were created by using an empty bait plasmid (pBait) or an irrelevant control plasmid (pRHFM) in place of pEG202-NLS-EphA3intra in the test.

Cell culture and DNA transfection

HEK293A cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 U/ml and 100 µg/ml respectively). When cells grew to about 90% confluence, plasmid DNA was transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. About 10 µg DNA and 30 µl Lipofectamine 2000 were used to transfect one 100 mm dish. Six hours later, the transfection mixture was removed and replaced with regular culture medium (DMEM supplemented with 10% fetal bovine serum). Cells were harvested at 48 hours after transfection for assays. In brief, the cells were washed once

with ice cold PBS and lysed in lysis buffer (50 mM Tris•Cl, pH 8.0, 150 mM NaCl, 1% NP-40, 100 µg/ml PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin, 1 mM sodium orthovanadate) for further analysis. The total protein concentration of these lysates was measured using BioRad Dc protein assay (Hercules, CA).

Analysis of the EphA3 receptor phosphorylation

Cell lysates of transfected HEK293A cells were pre-cleared by incubation with 70 µl 50% Protein A beads (Upstate Technology, Lake Placid, NY) for 1 hour at 4 °C. About 500 µg of total protein was incubated with 2.0 µg rabbit polyclonal anti-EphA3 antibody (c-19, Santa Cruz Biotechnology, Santa Cruz, CA). After two hours of incubation with agitation at 4 °C, 70 µl of 50% Protein A beads were added and the incubation continued for 1 more hour at 4 °C. Afterwards, the supernatant was disposed and the beads were washed extensively with IP wash buffer (PBS, 0.1% Triton X-100, 100 µg/ml PMSF, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, and 1 mM Sodium orthovanadate). At the end of the washes, the beads were mixed with equal volume of 2X Laemmli buffer and boiled for 5 minutes. The phosphorylation levels of the various EphA3 mutant proteins were analyzed with Western blot using the anti-phosphotyrosine antibody (Cell Signaling, Danvers, MA).

Preparation of Glutathione S-Transferase (GST) fusion protein

Different GST fusion constructs in pGEX 4T-2 (Li, Meriane et al. 2002) were transformed into competent DH5α bacteria cells. To produce the GST fusion proteins, a single bacteria colony was used to inoculate 5 ml 2xYTA medium (16 g/l Tryptone, 10

g/l Yeast extract, 5 g/l NaCl and 100 µg/ml Ampicillin) and cultured overnight at 30 °C. On the second day, the culture was used to inoculate 500 ml 2xYTA culture medium, which was incubated at 30 °C until the OD at 600 nm reached 0.5-2.0. Then IPTG was added to a final concentration of 1 mM to induce expression of GST fusion proteins. The culture was incubated for another 6 hours at 30 °C. At the end of incubation, the bacteria were collected by centrifugation at 8,000 rpm using a Sorvall centrifuge (Thermo Scientific, Waltham, MA). Bacteria pellet from 100 ml cultures was re-suspended in 5 ml PBS, supplemented with protease inhibitors (100 µg/ml PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin) and sonicated on ice in short bursts. Triton X-100 was then added to the sonicated product at a final concentration of 1% and the cell lysates were incubated at 4 °C for 30 minutes with agitation. Bacteria cell debris was removed by centrifugation and the supernatant was incubated with 200 µl 50% Glutathione Sepharose beads (GE healthcare Pharmacia, Piscataway, NJ) at 4 °C for 1 hour. The beads were washed with PBS containing 0.05% Triton X-100 for 3 times and stored in PBS with protease inhibitors (100 µg/ml PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin) at 4 °C until use.

GST fusion protein pulldown

Transfected 293A cells were stimulated with cross-linked ephrin-A5-Fc (1 µg/ml) for 30 minutes at 37 °C. The cells were washed once with ice cold PBS and lysed in GST pulldown assay lysis buffer (10 mM Hepes, pH 7.4, 1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin, 1 mM sodium orthovanadate). The lysate was collected with a cell scraper and centrifuged at 13,000 rpm for 10 minutes in an

Eppendorf microcentrifuge. After centrifugation to remove cell debris, the supernatant was pre-cleared with 50 μ l of 50% Glutathione Sepharose beads for 30 minutes at 4 °C. Later, Glutathione Sepharose beads conjugated with the desired GST fusion proteins were added to the supernatant and the mixture was incubated at 4 °C for 2 hours. At the end of incubation, the beads were collected by centrifugation and washed 3 times with PBS supplemented with 0.05% Triton X-100, each for 10 minutes. The beads were then boiled in 2X Laemmli buffer for 5 minutes before analysis by Western blot technique.

Co-immunoprecipitation assay

Transfected 293A cells or dissociated neuron cultures were stimulated with or without cross-linked ephrin-A5-Fc for 30 minutes at 37 °C. The cells were washed gently with ice cold PBS once and lysed in cell lysis buffer. The cell lysate then was cleared by centrifugation and the protein concentration of the supernatant determined. A small fraction of the lysate was used later for analysis of protein expression levels. For immunoprecipitation, 2 mg of each lysate were pre-cleared with protein A beads (Upstate USA, Inc., Charlottesville, VA) for 1 hour at 4 °C. Then the desired antibody or control IgG was added to the supernatant. The mixture was incubated on a rocker at 4 °C for 4 hours before 40 μ l 50% protein A beads were added. After continued incubation of 1 more hour, the beads were collected and washed with PBS with 0.05% Triton X-100 for 3 times, each 10 minutes. The precipitated immunocomplexes were boiled in 2X Laemmli buffer for 5 minutes and the samples were analyzed with SDS-PAGE coupled with Western blot technique using different antibodies.

Immunostaining

Cells were stimulated with cross-linked ephrin-A5 for 30 minutes and fixed with freshly prepared 4% paraformaldehyde solution for 30 minutes at room temperature. The fixed cells were washed two times with PBS, each 10 minutes. The cells were then blocked with 5% BSA in PBS plus 0.05% Triton X-100 overnight at 4 °C. On the second day, blocking buffer was replaced with primary antibodies in 0.5 X blocking buffer (2.5% BSA and 0.025% Triton X-100 in PBS). After 4 hours of incubation at room temperature, the cells were washed again 2 times with PBS and the secondary antibody in 0.5 X blocking buffer was added and incubated for 2 hours. Two more washes follow the antibody incubation and DAPI solution (1 µg/ml) was used to stain the cells for 30 minutes at room temperature to visualize the nuclei. After two additional PBS washes, the culture dishes were examined under a fluorescence microscope.

Cellular process retraction assay

HEK293A cells were plated sparsely on culture dishes to allow examination of individual cells and their processes. These cells were transfected with desired plasmids and 48 hours after transfection, the cells were incubated in DMEM medium with or without cross-linked ephrin-A5 for 1 hour at 37 °C. Pictures of transfected cells (N>30) as indicated by EGFP expression, were taken before and after the treatment (L1, L2, respectively). The length of processes was measured from where they exit the cell body to the end of the process using ImagePro software. Using the formula $E \text{ (extension)} = (L2 - L1) / L1$, we calculated the process length change index E as a percentage of initial total process length. A positive E value indicates process extension and a negative number

indicates process retraction. E value of transfected cells was compared to EGFP expressing cells (100%) and then plotted in the bar graph.

Cell migration assay

To prepare for this assay, transwell inserts (24.5mm diameter, 8 μ m pore diameter) purchased from Corning Labware (Corning, NY) were coated on the underside with fibronectin (20 μ g/ml) to facilitate attachment of migrated cells. HEK293A cells were transfected with desired genes using Lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the cells were trypsinized and washed gently with PBS. A small portion of the cells was plated into 6 well dishes for determination of efficiency for each transfection. An equal portion of the cell suspension was used in the migration assay. They were plated on the prepared transwell inserts in DMEM without serum and the lower compartment in the setup was filled with complete culture medium (DMEM supplemented with 10% FBS) with or without 2 μ g/ml cross-linked ephrin-A5. These transwell dishes were then incubated overnight at 37 °C with 5% CO₂. On the second day, cells on inserts were fixed and all the cells that have not migrated were cleaned off using Q-tips. DAPI staining was carried out to visualize the cell nuclei before cell counting was done on a microscope. Five to ten random fields were chosen and the number of all cells that migrated through the membrane (T2) and the number of the transfected cells that migrated (T1) were both quantified. In addition, the transfection efficiency was individually determined and expressed as a percentage ($R = \text{transfected cells/all cells}$). The migration ability (M) of cells expressing different genes was

calculated using the following formula: $M = T1 / (T2 \times R)$. When the data were used in the graph, M was compared with EGFP transfected controls and converted as a percentage of the M value of EGFP control, which is considered to be 1.

Dissection of rat embryos and neuron culture

Embryonic day 18 Sprague-Dawley rats were sacrificed and the brains were taken out. After midbrain was trimmed, the two hemispheres were separated and turned over. The cortices and hippocampi were dissected out and collected. To make dissociated neuron culture, the collected neural tissues were trypsinized at 37 °C for 10 minutes and dissociated further by pipetteing repeatedly with glass pipettes with small opening. At the end, dissociated neurons were counted and plated in neurobasal medium (1 X B27 supplement, 0.5 mM glutamine, 25 µM glutamate, 100 U/ml penicillin and 100 µg/ml streptomycin) at desirable density on culture dishes that were coated with Poly-D-lysine (100 µg/ml). For staining experiment, a low-density culture was used to allow easy observation of individual neurons. For Western blotting and immunoprecipitation, a large quantity of cells was plated at a very high density to ensure enough proteins would be available for assays. To make explant culture, the hippocampi were cut into small pieces until a uniform size of 1 mm in diameter was achieved. These explants were gently pipetted and plated in neurobasal medium on Nunc Lab-Tek 4-well glass chamber slides that were coated with Poly-D-lysine (500 µg/ml) and Laminin (20 µg/ml).

Adenovirus construction

In a nutshell, the various Nck1 genes including Nck1WT, Nck1R308K and Nck1W38/143/229K were cloned into pAdTrack-CMV vector. These plasmids carrying

Nck1 mutants were individually linerized through PmeI digestion and cleaned through agarose gel extraction. A mixture of the linerized pAdTrack-CMV-Nck1 clones and pAdEasy vector was used to transform BL5781 cells in which recombination of the two plasmids took place. Individual colonies were picked out and screened for the right recombinant products, Recombinant adenoviral vectors, pAdEasy-CMV, pAdEasy-CMV-Nck1WT, pAdEasy-CMV-Nck1R308K and pAdEasy-CMV-Nck1W38/143/229K were each digested with PacI enzyme and the digested plasmids were used to transfect adenovirus packaging cell line HEK293A cells. Seven days later, the transfected cells were collected and processed through “freeze-thaw-vortex” procedure to release all adenoviruses from the cells. In brief, harvested cell pellets were resuspended in sterile PBS and frozen in a dry ice/ethanol bath for 5 minutes. The frozen cells were then thawed at 37 °C and vortexed vigorously until a homogenous suspension was obtained. This “freeze-thaw-vortex” procedure was repeated at least three times before being spun at 5000 rpm for 5 minutes. After being ridden of the cell debris, the supernatants were used to infect 293A cells. The infected cells were harvested and processed to releases the amplified adenoviruses. This amplification process was repeated until a high titer of virus stock was obtained. The amplified virus stocks were stored at –80 °C.

Growth cone collapse

Hippocampal explant culture was infected with adenoviruses on the same day they were plated. After overnight infection, fresh culture medium was added to the culture. Four days later, cross-linked ephrin-A5 was used to treat these explants for half an hour before a freshly mixed fixative (4% paraformaldehyde, 0.25% glutaraldehyde, 0.1 M cacodylate buffer pH7.4

and 0.1 M sucrose) was used to fix the cultures. The explant cultures were washed three times with PBS before being incubated in 0.3% Triton-X-100 in PBS for 20 minutes. Texas Red X-Phalloidin was used to stain the cells for 20 minutes at room temperature. The cultures were washed again with PBS until all the residual stain was cleaned. To prepare the slides for inspection on a microscope, the top chambers were removed from the chamber slides and a small amount of 50% glycerol in PBS was added on top of the slide. Glass coverslips were then mounted on these slides and the edges of the coverslips were sealed with nail polish. These slides were wrapped in foil and kept at 4 °C until use.

Chapter VII. Conclusions

Novel binding proteins are implicated in EphA receptor signaling

The mechanisms of Eph/ephrin signaling remain an important topic of research as increasing amounts of data suggest that the complexity of Eph/ephrin signal transduction surpasses our initial expectations. Though many binding proteins have been identified, our understanding of the topic remains incomplete.

Through a large-scale yeast two-hybrid screen, we identify fifteen novel proteins that interact with the cytoplasmic domain of EphA receptors. These proteins are categorized into four groups: membrane trafficking related genes, cytoskeleton regulation related genes, tumorigenesis related genes, and cytoplasmic enzymes. These proteins likely participate in the regulation of various functions Ephs regulate. Further studies on these novel proteins will increase our understanding of the mechanisms involved in modulating a variety of cellular events such as endocytosis, cytoskeletal rearrangements and cell proliferation.

Nck1, Grb2 and RasGAP all bind to tyrosine 602 through their SH2 domains

The present study identifies adaptor protein Nck1 as a major binding protein for both EphA3 and EphA5. Nck1 has been found to bind to EphB1 and EphB2 through direct or indirect interaction (Stein et al. 1998a); however, its involvement in EphA receptors has never been established.

The current study also shows that Nck1 only interacts with tyrosine residue 602 of EphA3 receptor. Tyr602 is located within the juxtamembrane domain of EphA3 receptor

and is highly conserved among all Eph receptors. The interaction of Nck1 with the juxtamembrane domain of Eph receptors is common to many SH2 domain proteins. Since a diversity of biological events are mediated by SH2 domain proteins, the competition between Nck1 and other SH2 domain proteins for binding with the juxtamembrane domain is a probable explanation for the drastically different phenotypes induced by activated EphA receptors.

Grb2 and RasGAP are two SH2 domain proteins involved in the regulation of MAPK activity. Although this study has yet to examine all sites to which Grb2 and RasGAP bind, we show that these two signaling proteins share at least one binding site.. The preferential association with either Grb2 or RasGAP is likely to result in up- or down-regulation of Ras/ERK/MAPK pathway and present an underlying mechanisms for the switch between attractive and repulsive phenotypes (Tong et al. 2003).

It remains to be determined which roles Nck1 plays in this regulatory process. Though Nck1 is known to bind to Sos and induce the activation of Ras activity, Nck1 has also been identified in a RasGAP/Nck1/EphB2 protein complex. Taken together, these data suggest that Nck1 is capable of exerting opposite effects in a context dependent fashion (Tanaka et al. 1995a).

Nck1 is an integral part of EphA3 mediated signaling

Nck1 co-localizes with EphA3 after ephrin stimulation in both HEK 293A cells and primary neuron cultures, indicating the endogenous association of the two proteins. Nck1 binds to activated EphA3 and triggers the activation of downstream signaling proteins such as PAK, NIK, Nap-1, and WASp/WAVE proteins (Fig. 7-1). The activation of these

signaling pathways in turn leads to changes such as cytoskeletal rearrangement and cell adhesion.

When dominant negative Nck1 genes are introduced into 293A cells, the function of endogenous Nck1 is compromised. Furthermore, it is found that the disruption of either Nck1/EphA3 binding or Nck1/effector binding is able to elicit a partial reversal of Eph mediated phenotypes such as inhibition on cell migration and process retraction after ephrin treatment.

Although the significance of Nck1 in EphA3 mediated growth cone collapse wasn't confirmed in this study, it remains probable that Nck1 functions in other aspects of axonal behavior such as axonal branching, neurite retraction, turning and synaptic formation. More research needs to be conducted to elucidate the signaling mechanism of Nck1 in EphA3 expressing neurons.

Figure 7-1. A summary of EphA3 signal transduction pathway involving Nck1, Grb2 and RasGAP.

Signaling pathways downstream of EphA3 that are regulated by Nck1, Grb2 and RasGAP are delineated in the graph. Through multiple pathways, Nck1 binds to Tyr602 and regulates cytoskeletal activities. Grb2 and RasGAP, on the other hand, compete for binding through both Y602 and unknown binding sites, thereby modulating Ras/MEK/ERK pathway.

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Curriculum vitae

Tian-jing Hu

EDUCATION:

- Rutgers University, New Brunswick, NJ
Ph. D in Neurobiology, October 2007
- Nankai University, School of Life sciences, Tianjin, China
B.S in Biochemistry and Molecular Biology, June 1999

PUBLICATIONS:

- Mistargeting Hippocampal Axons by Expression of a Truncated Eph Receptor
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