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EphA Receptors Regulate Growth Cone Dynamics through the Novel Guanine Nucleotide Exchange Factor Ephexin

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Summary

Eph receptors transduce short-range repulsive signals for axon guidance by modulating actin dynamics within growth cones. We report the cloning and characterization of ephexin, a novel Eph receptor-interacting protein that is a member of the Dbl family of guanine nucleotide exchange factors (GEFs) for Rho GTPases. Ephrin-A stimulation of EphA receptors modulates the activity of ephexin leading to RhoA activation, Cdc42 and Rac1 inhibition, and cell morphology changes. In addition, expression of a mutant form of ephexin in primary neurons interferes with ephrin-A-induced growth cone collapse. The association of ephexin with Eph receptors constitutes a molecular link between Eph receptors and the actin cytoskeleton and provides a novel mechanism for achieving highly localized regulation of growth cone motility.

Introduction

The pathfinding of axons during the development of the nervous system is a highly regulated process governed by a complex set of environmental guidance cues. A number of extracellular factors have been identified, including netrins, slits, semaphorins, and ephrins, that act through specific cell surface receptors to control axon guidance. These guidance factors function as either attractive or repulsive guidance cues depending on their ability to induce growth cone extension or retraction. The directional control of axon pathfinding is thought to result from local modulation of actin dynamics within the growth cone that promotes growth toward or away from guidance stimuli. However, the molecular mechanisms that link positional guidance cues from cell surface receptors to the actin cytoskeleton remain unclear. In particular, it is not known how guidance factors regulate the actin cytoskeleton at specific growth cone positions to provide precise control of axon guidance during nervous system development.

We have addressed this question for a set of guidance molecules that have been implicated in short-range axon repulsion, the Eph receptor tyrosine kinases (RTKs). The Eph receptor family constitutes the largest class of receptor tyrosine kinases with 14 members identified to date. The Eph receptors are subdivided into two groups, EphA and EphB receptors, based on their structural properties and ligand binding preferences—EphA receptors bind the GPI-linked ephrin-A ligands, whereas EphB receptors bind the transmembrane ephrin-B ligands. Eph receptors and ephrins are required for the proper formation of specific axon projections in the central nervous system, including the corticospinal tract and the anterior commissure (reviewed in Frisen et al., 1999). The role of Eph receptors in axon guidance has been most thoroughly characterized in the chick retinotectal projection, where gradients of expression of EphA receptors and ligands are necessary for proper topographic mapping (reviewed in Flanagan and Vanderhaeghen, 1998). Eph receptors and ephrins also restrict cell migration during hindbrain segmentation and vasculogenesis, possibly via modulation of integrin function (reviewed in Wilkinson, 2001).

The control of axon guidance by Eph receptors is believed to be mediated by alterations in actin dynamics that lead to growth cone retraction. However, the intracellular mechanisms involved in coupling Eph receptor activation to changes in the actin cytoskeleton have remained unresolved. The various members of the p21 Rho family of small guanosine triphosphatases (GTPases) elicit distinct effects on actin structures (Schmitz et al., 2000) and are likely to be involved in Eph receptor regulation of the actin cytoskeleton. In neuronal cell lines, the activation of the Rho family GTPase RhoA stimulates actinomyosin contractility and stress fiber formation resulting in growth cone collapse, whereas the induction of Cdc42 and Rac1 results in the extension of filopodia and lamellipodia, respectively (Kozma et al., 1997). Recently, Eph receptor activation has been linked to changes in the activities of certain Rho GTPases (Wahl et al., 2000). The activation of EphA receptors in retinal ganglion cells (RGCs) induced the activation of RhoA and the inhibition of Rac1, consistent with a retraction response. Thus, Eph receptors may control growth cone motility by regulating the balance between the activities of individual Rho GTPases.

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The molecular mechanisms by which EphA receptors and other axon guidance receptors regulate the activity of Rho family GTPases remain to be elucidated. It is known that Rho GTPases function as binary molecular switches, shuttling between an inactive GDP bound conformation and an active GTP bound conformation, in which the Rho GTPases bind and activate downstream effectors. Rho GTPases are regulated by the opposing effects of two classes of enzymes, guanine nucleotide exchange factors (GEFs) of the Dbl family and GTPase-activating proteins (GAPs). Dbl GEFs act to enhance the exchange of bound GDP for GTP and thus activate the Rho GTPases, whereas GAPs inhibit Rho family members by potentiating their intrinsic GTPase activity. One intriguing possibility is that the activity of Dbl GEFs and/or GAPs might be regulated by cell surface receptors so as to link extracellular axon guidance cues to highly localized changes in the actin cytoskeleton within a growth cone.

Here we report the cloning and characterization of ephexin, a novel member of the Dbl family of GEFs that interacts directly with EphA4. In the absence of ephrin-A stimulation, ephexin catalyzes guanine nucleotide exchange for RhoA, Cdc42, and Rac1. The activation of EphA receptors inhibits ephexin activity toward Cdc42 and Rac1, yet potentiates its activity toward RhoA. An inhibitory mutant of ephexin suppresses ephrin-A1-induced growth cone collapse in purified RGCs, suggesting an important role for ephexin in EphA regulation of growth cone motility. We propose that spatially restricted regulation of Rho GTPase signaling pathways through the modulation of ephexin activity may underlie localized growth cone responses to ephrin-A.

Results

Cloning of Ephexin, A Novel Member of the Dbl Family of Guanine Nucleotide Exchange Factors (GEFs)

To identify EphA4-interacting proteins that might function to regulate axon guidance, we performed a yeast two-hybrid screen. The bait construct consisted of the intracellular portion of mouse EphA4 fused to the DNA binding domain of LexA (Finley and Brent, 1996) and was autophosphorylated when expressed in yeast (data not shown). We obtained 25 independent interacting clones upon screening a cDNA library from embryonic day 14 (E14) rat spinal cord and dorsal root ganglia. One of the clones encoded a novel member of the Dbl family of guanine nucleotide exchange factors for Rho family GTPases. We named this gene ephexin, for *Eph*-interacting *ex*change prote*in*.

To determine if the ephexin coding region extends further than the 5′ end of the cDNA that we initially identified, we performed 5′-rapid amplification of cDNA ends (5′-RACE) from rat brain mRNA and obtained an additional 395 nucleotides of sequence that matched a mouse expressed sequence tag (EST). Complete sequencing of the mouse EST revealed 94% identity to rat ephexin within the coding region and indicated that it likely represents the mouse ortholog of ephexin. The mouse ephexin clone extended the known sequence to include a consensus Kozak translation initiation site at nucleotide 156. More recently, a partial cDNA encoding mouse ephexin, called Ngef, was identified and was demonstrated to reside on human chromosome 2q37 and mouse chromosome 1 (Rodrigues et al., 2000).

Conceptual translation of the mouse cDNA revealed a protein of 620 amino acids comprising an N-terminal region with no homology to other proteins, a tandem Dbl homology-pleckstrin homology (DH-PH) motif that is conserved in all Dbl GEFs, and a C-terminal SH3 domain (Figure 1A). Immunoblotting with affinity-purified polyclonal antibodies raised against either the N-terminal region, the SH3 domain, or the C-terminal tail of ephexin detected an endogenous protein present in rat telencephalic neurons (Figure 1B) that displayed the same electrophoretic mobility as recombinant murine ephexin expressed in 293T cells (data not shown). Partial V8 proteolysis of lysates from neurons and ephexin-expressing 293T cells followed by immunoblotting with the C-terminal anti-ephexin antibody confirmed that the recombinant and endogenous proteins are identical (data not shown). Taken together, these data indicate that the murine ephexin clone includes the full-length ephexin coding sequence.

The initiator methionine of ephexin is followed by a hydrophobic stretch of 14 amino acids resembling a signal peptide or a signal-anchor sequence (Martoglio and Dobberstein, 1998). We have been unable to detect endogenous or overexpressed rat ephexin in a secreted form, and the hydrophobic sequence is not predicted to be a signal peptide by an algorithm designed to recognize signal peptides (Emanuelsson et al., 2000). Instead, this hydrophobic region may serve as a signal-anchor sequence to localize ephexin to the inner surface of the plasma membrane (Wahlberg and Spiess, 1997). Immunoblotting of cellular membranes from postnatal day 7 (P7) rat brain revealed that ephexin is present both in detergent-soluble cytoplasmic fractions and detergent-insoluble membrane fractions that contain caveolin (Figure 1B), a marker of membrane-associated caveolae in which Eph receptors have been detected (Rothberg et al., 1992; Wu et al., 1997).

Ephexin-related genes were found to be present in invertebrates and vertebrates, and their sequences are highly conserved, suggesting an essential function for ephexin. The genomes of *C. elegans* and *Drosophila* each contain a single gene with a high degree of homology to ephexin (Figures 1C and 1D). In humans, in addition to an ephexin ortholog, there are at least three other genes that are highly homologous to ephexin—the previously characterized Dbl family member TIM (Chan et al., 1994) and two uncharacterized cDNAs, Neuroblastoma and KIAA0915. Using the ClustalX program, we performed phylogenetic analysis of the DH-PH domains of the ephexin-related proteins and compared them to the DH-PH domains of Dbl and Vav1, two other Dbl family GEFs. This analysis revealed that the DH-PH domains of ephexin-related GEFs are more closely related to each other than to the DH-PH domains of Dbl and Vav1, and identified ephexin-related GEFs as a subfamily within the larger Dbl family of GEFs (Figure 1C). Alignment of ephexin subfamily proteins revealed a high degree of conservation within the DH, PH, and SH3 domains, but little or no homology in the N-terminal

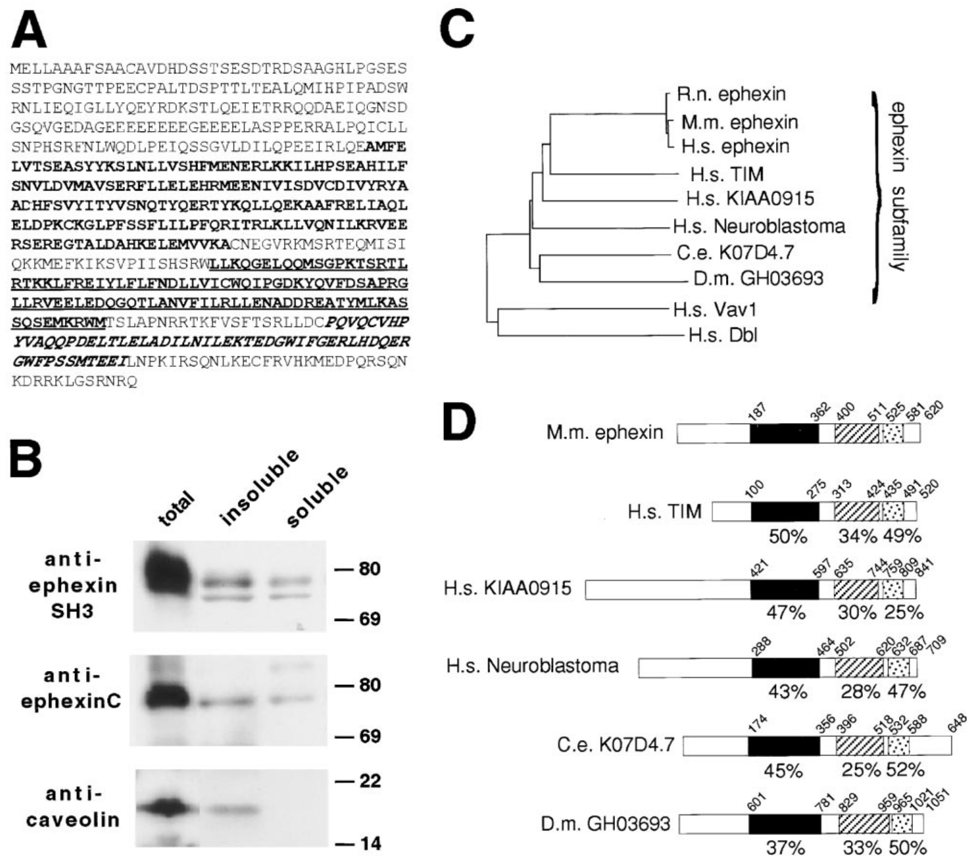

Figure 1. Analysis of Ephexin Amino Acid Sequences

(A) Protein sequence and domain structure of mouse ephexin. DH domain, boldface; PH domain, boldface underscored; SH3 domain, boldface italics. Domains were identified by the program Pfam.

(B) Subcellular fractionation of ephexin. Total cellular membranes from P7 rat brains, the Triton X-100-solubilized membrane fraction, or the insoluble fraction were analyzed by immunoblotting with antibodies to the SH3 domain (top) or C terminus (middle) of ephexin, or to caveolin (bottom).

(C) Phylogenetic analysis of the DH-PH cassettes of ephexin and other Dbl family proteins using the program ClustalX. R.n., *Rattus norvegicus*; M.m, *Mus musculus*; H.s., *Homo sapiens*; C.e., *Caenorhabditis elegans*; D.m., *Drosophila melanogaster*.

(D) Comparisons of individual domains of ephexin subfamily proteins by pairwise BLASTP analysis. DH domain, filled box; PH domain, hatched box; SH3 domain, stippled box. The amino acid positions of the domain boundaries are shown above, and the percent identities to mouse ephexin are shown underneath.

regions, including the 14 amino acid hydrophobic sequence present in ephexin orthologs (Figure 1D).

Ephexin Expression Is Enriched in the Nervous System and Is Developmentally Regulated

We determined the tissue distribution of ephexin to gain insight into possible functional roles for this GEF. Northern blot analysis of ephexin mRNA from P20 rat tissues revealed an approximately 2.8 kb mRNA species with highest levels of expression in the brain and spinal cord and lower levels of expression in testes (Figure 2A). In addition, we detected low levels of a transcript in kidney and liver that migrates more slowly than the neuronal transcripts. These more slowly migrating transcripts may represent alternatively spliced or initiated ephexin mRNA or a closely related family member. Ephexin mRNA was not detected in heart, lung, skeletal muscle, or spleen.

The enrichment of ephexin mRNA in the nervous system at P20 led us to explore its expression in brain at other ages to determine if ephexin expression is developmentally regulated. Ephexin mRNA can first be detected in E15 rat brain and gradually increases throughout embryonic development (Figure 2A). In the early postnatal period, ephexin mRNA levels rise sharply, peak at P10, and then decline. Interestingly, this expression pattern closely matches that previously described for EphA4 (Mori et al., 1995). Ephexin mRNA expression in the E17 rat was found by in situ hybridization to be restricted to the eye and also in deep layers of the developing cortex (Figure 2B). In the eye, ephexin expression was diffuse at E17 and then became highly restricted to the inner nuclear and RGC layers at P6 (Figure 2C). Staining of control and ephexin-transfected P6 RGCs with the affinity-purified polyclonal anti-ephexin N-terminal antibody (anti-ephexinN) revealed that endogenous and overexpressed ephexin protein is localized in RGC axonal growth cones (Figure 2D). Since the topographic mapping of RGC axons to CNS targets in the early postnatal stages of development is regulated by EphA receptors in

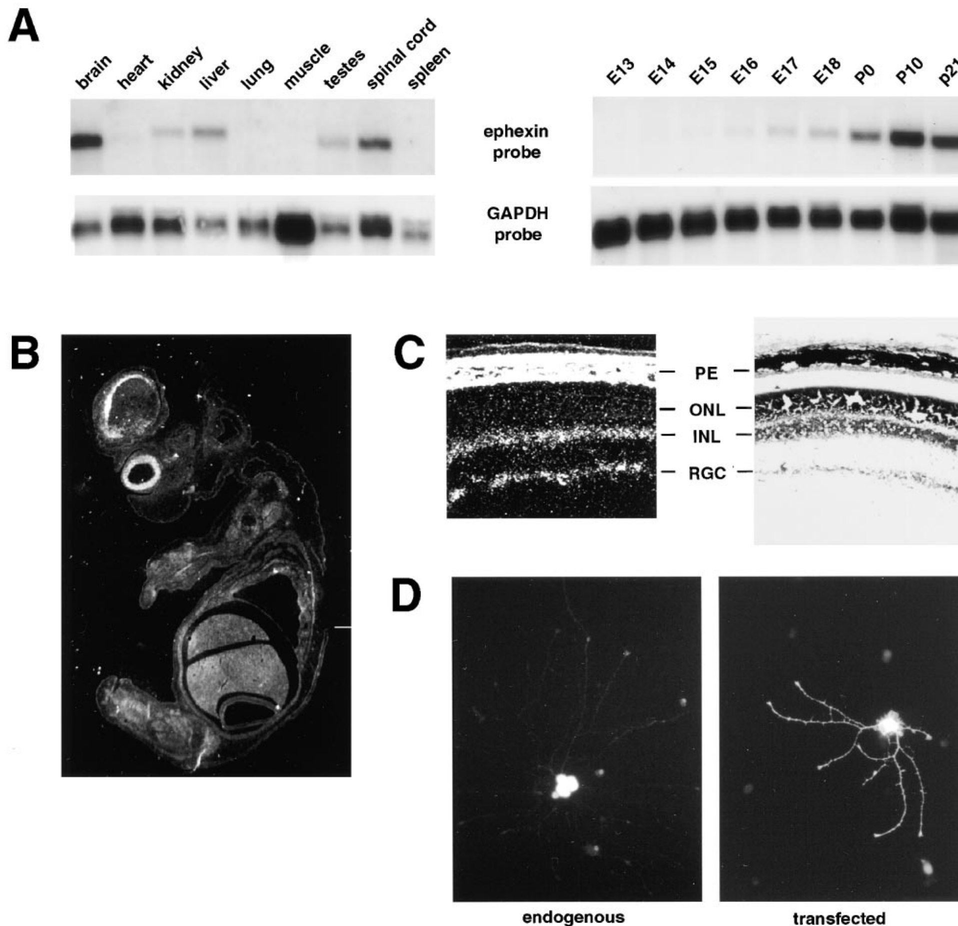

Figure 2. Expression Pattern of Ephexin

(A) Northern blot analysis of ephexin mRNA expression. Blots with mRNA from P20 rat tissues (left) or from total rat brain from different ages (right) were probed with a fragment of rat ephexin corresponding to the PH and SH3 regions.

(B and C) In situ hybridization of ephexin mRNA in rat tissue sections. A sagittal section from an E17 embryo (B) or a coronal section of a P6 eye (C) was probed with the SH3 and 3′ untranslated regions of rat ephexin. An adjacent eye section (C, right) was stained with cresyl violet to identify cell layers. PE, pigmented epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; RGC, retinal ganglion cell layer.

(D) Immunocytochemical staining of ephexin in purified RGCs. Control (left) or ephexin-transfected (right) RGCs were stained with the anti-ephexinN antibody.

the growth cone (Flanagan and Vanderhaeghen, 1998), these findings indicate that ephexin is expressed with an appropriate spatial and temporal profile to mediate important functional effects of Eph receptors.

Interaction and Domain Mapping between Ephexin and EphA4

The observation that ephexin interacts with the cytoplasmic domain of EphA4 in yeast raised the possibility that ephexin and EphA4 interact in mammalian cells. To test this possibility, we cotransfected 293T cells with ephexin and EphA4 expression constructs, and immunoprecipitated detergent cell lysates with the anti-ephexinN antibody. As shown in Figure 3A, the EphA4 receptor was readily detected in anti-ephexinN immunoprecipitates. The coimmunoprecipitation of EphA4 with the anti-ephexinN antibody was dependent on the expression of ephexin (Figure 3A) and was undetectable in immunoprecipitates in which a nonimmune rabbit IgG antibody replaced the anti-ephexinN antibody (data not shown). These findings indicate that ephexin and EphA4 interact when they are coexpressed in mammalian cells.

We also tested whether the interaction between ephexin and EphA4 was dependent on EphA4 tyrosine phosphorylation. We generated an EphA4 mutant in which valine 635 in the ATP binding region was converted to a methionine (V635M), rendering EphA4 kinase inactive (KD) (Shu et al., 1994) (Figure 3A). When expressed in 293T cells, EphA4-KD is not tyrosine phosphorylated (Figure 3A). However, the EphA4-KD mutant was able to interact with ephexin to the same extent as wild-type EphA4 in both 293T cell coprecipitation experiments and in the yeast two-hybrid assay (Figures 3A and 3B). These results indicate that ephexin interacts with EphA4 receptors in both the absence and presence of EphA4 receptor autophosphorylation.

We next identified the domain within EphA4 that mediates the interaction with ephexin. EphA4 constructs were generated in which portions of EphA4 were deleted. The resulting EphA4 mutants were expressed at

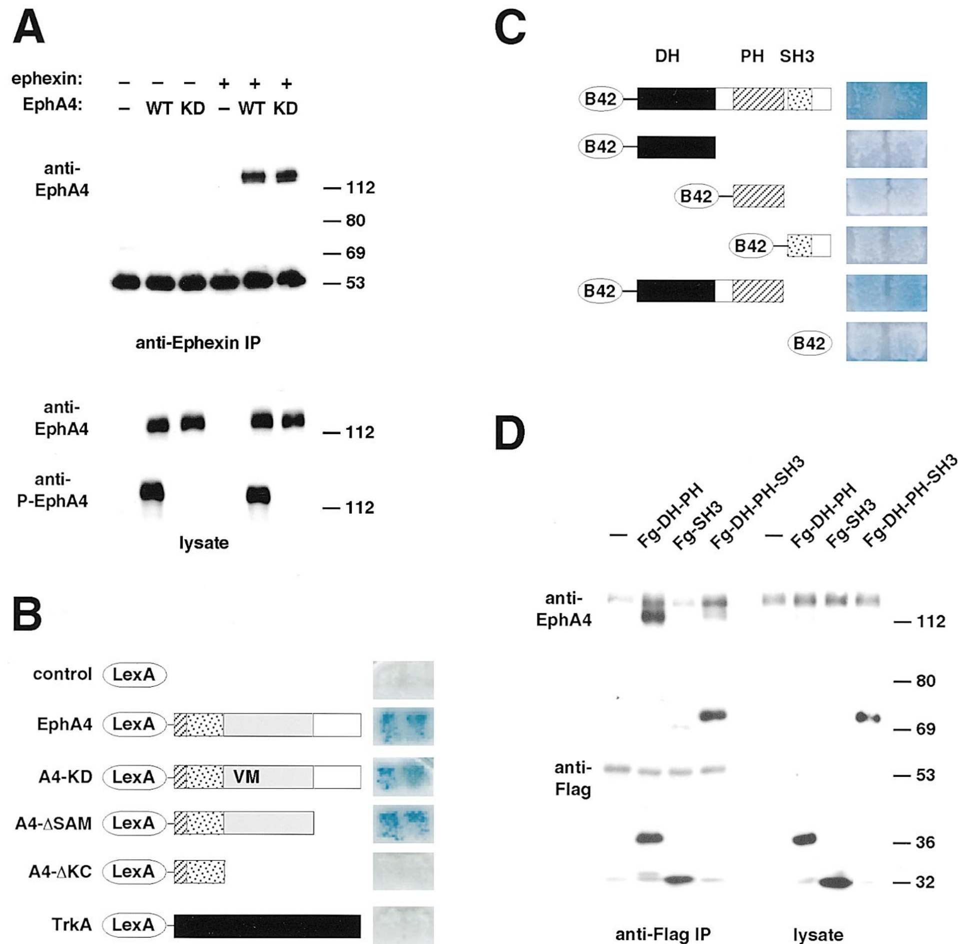

Figure 3. Interaction and Domain Mapping between Ephexin and EphA4

(A) Coimmunoprecipitation of ephexin and EphA4 in 293T cells. 293T cells were transfected with ephexin and/or wild-type (WT) or kinase-dead (KD) EphA4. Two days later, cell lysates were immunoprecipitated with anti-ephexinN and immunoblotted with an anti-EphA4 antibody (top). Whole-cell lysates were also immunoblotted with anti-EphA4 (middle) or a phosphorylation-specific EphA receptor antibody (anti-P-EphA, bottom).

(B) Mapping of the ephexin interaction domain of EphA4. Rat ephexin (amino acids 67–620) was coexpressed in the yeast two-hybrid assay with the LexA DNA binding domain alone or with LexA fusions to the cytoplasmic domain of EphA4, kinase-dead EphA4 (A4-KD), SAM domain-truncated EphA4 (A4-ΔSAM), or C-terminal kinase domain truncated EphA4 (A4-ΔKC), or to the cytoplasmic domain of TrkA. Positive interactions were indicated by the induction of a LacZ reporter. EphA4 juxtamembrane domain, hatched box; amino-terminal kinase lobe, stippled box; carboxy-terminal kinase lobe, shaded box; SAM domain, open box; V635M kinase-inactivating mutation, VM; cytoplasmic TrkA domain, black box.

(C and D) Mapping of the EphA4 interaction domain of ephexin. (C) The EphA4 cytoplasmic domain was coexpressed with the B42 transcription activation domain alone or with B42 fusions to the indicated domains of ephexin. (D) Coimmunoprecipitation of ephexin domains and EphA4 in 293T cells. Flag-tagged domains of ephexin were cotransfected with EphA4 and the cell lysates immunoprecipitated with anti-Flag antibody followed by immunoblotting with anti-EphA4 (top left) or anti-Flag (bottom left). Whole-cell lysates were also immunoblotted with anti-EphA4 (top right) or anti-Flag (bottom right).

comparable levels in the yeast two-hybrid assay (data not shown) and tested for their interaction with ephexin. EphA4 receptors lacking the sterile α motif (SAM) domain in the C-terminal tail thought to mediate homophilic interactions (EphA4-ΔSAM) were capable of interacting with ephexin as effectively as the wild-type receptor, suggesting that the ephexin interaction domain resides within the EphA4 juxtamembrane or kinase domains (Figure 3B). To explore a possible interaction between ephexin and the tyrosine kinase domain of EphA4, we used information from the crystal structures of Src family tyrosine kinases (Sicheri et al., 1997; Xu et al., 1997) to construct an EphA4 mutant that lacks the C-terminal lobe of the EphA4 kinase domain (EphA4-ΔKC). The EphA4-ΔKC mutant failed to interact with ephexin in the yeast two-hybrid assay, indicating that ephexin binds to specific sequences within the C-terminal lobe of the EphA4 kinase domain (Figure 3B). Ephexin interacts efficiently with multiple EphA receptors but poorly with EphB receptors (data not shown) and fails to interact with TrkA (Figure 3B), another RTK expressed in CNS neurons, highlighting the specificity of the EphA4-ephexin association.

We next investigated which regions of ephexin mediate interaction with EphA4. Since the original yeast two-hybrid interacting clone of ephexin lacked amino-termi-

nal sequences, we focused on the DH, PH, and SH3 domains. The individual ephexin DH, PH, and SH3 domains or the DH-PH combined domains were equivalently expressed (data not shown) and tested in the yeast two-hybrid assay to determine which regions of ephexin were sufficient to maintain the interaction with EphA4. The ephexin DH, PH, or SH3 domains alone were unable to bind EphA4 in the two-hybrid assay (Figure 3C). However, the tandem ephexin DH-PH domains were sufficient to mediate an interaction with EphA4, suggesting that portions of both domains are important for the interaction of ephexin with EphA4 (Figure 3C). The domains of ephexin that mediate the EphA4-ephexin interaction were also identified by expressing Flag epitope-tagged regions of ephexin with EphA4 in 293T cells and testing for the presence of EphA4 in anti-FLAG immunoprecipitates. In support of the findings from the yeast two-hybrid assay, the DH-PH module of ephexin, but not the SH3 domain, was found to coimmunoprecipitate with EphA4 when coexpressed in 293T cells (Figure 3D). Taken together, these data identify previously undefined functions for the kinase domain of EphA receptors as well as for the catalytic DH-PH module of a Dbl family GEF in mediating protein-protein interactions.

Ephexin Activates RhoA, Cdc42, and Rac1

To determine the specific Rho GTPases on which ephexin can catalyze guanine nucleotide exchange, we utilized an in vitro assay that measures the ability of ephexin to induce the release of preloaded [^{3}H]GDP from various Rho family GTPases. In this assay, ephexin strongly activated RhoA and also significantly activated Cdc42 (Figure 4A). We also assessed the nucleotide exchange activity of ephexin in neuronal extracts. Since Rho GTPases bind downstream effectors when in the GTP bound state, GST fusions of these effectors can be used to capture active Rho GTPases from cell lysates. Thus, a GST fusion to the Rho binding domain (RBD) of rhotekin, a RhoA effector, can be used to assay levels of active RhoA (Ren et al., 1999), and a GST fusion to the p21 binding domain (PBD) of Pak can be used to assay levels of active Cdc42 and Rac1 (Sander et al., 1998). Using this assay, we observed large increases in RhoA and Cdc42 activities and small but reproducible increases in Rac1 activity in cultured embryonic cortical neurons infected with a herpesvirus expressing ephexin (HSV-ephexin) (Figure 4B).

We also observed the effects of ephexin on the morphology of fibroblasts, in which RhoA, Cdc42, and Rac1 each elicit distinct morphological changes. Specifically, RhoA induces stress fiber formation, Cdc42 filopodia extension, and Rac1 lamellipodia formation and membrane ruffling (Nobes and Hall, 1995; Gauthier-Rouviere et al., 1998). When expressed in REF-52 fibroblasts, ephexin induced morphologic changes consistent with the activation of RhoA, Cdc42, and Rac1 in 28%, 12%, and 15% of transfected cells, respectively (Figures 4C and 5D). Because Cdc42 activation can lead to Rac1 activation in certain cellular contexts (Nobes and Hall, 1995), the low level activation of Rac1 that we observe in multiple assays of ephexin activity may reflect a direct activation by ephexin or a secondary effect of activation of Cdc42. Taken together, both in vitro and cell-based assays indicate that ephexin strongly activates RhoA and Cdc42, and may also promote Rac1 activity in cells.

Ephexin Activates the Rac1 and Cdc42 Effector Kinase, Pak

Since ephexin has strong GEF activity toward different Rho family members, we investigated whether effector enzymes downstream of Rho GTPases might also be induced in response to ephexin activation. Several lines of evidence link activation of the Rac1 and Cdc42 effector Pak to modifications in the organization of the actin cytoskeleton (Bagrodia and Cerione, 1999). To monitor Pak activation, we raised a phosphorylation-specific antibody (anti-P-Pakα) against a peptide encompassing autophosphorylated serine residues 198 and 203 of Pakα (Manser et al., 1997). In immunoblot experiments, anti-P-Pakα recognized Pakα when expressed in 293T cells only in the presence of a constitutively active Rac1 mutant (RacV12), thus demonstrating the specificity of this antibody toward phosphorylated Pak (Figure 5A). Furthermore, the detection of Pakα in the presence of RacV12 is eliminated when Pakα serine residues 198 and 203 are mutated to alanine, demonstrating the site specificity of the antibody (J. Chernoff, personal communication).

To test whether ephexin can activate Pak, we performed immunoblot analysis with the anti-P-Pakα antibody on 293T cells coexpressing ephexin and Pakα. The expression of wild-type ephexin led to a robust increase in Pak phosphorylation comparable to that observed with RacV12 expression (Figure 5A). The activation of Pak was dependent on the GEF activity of ephexin since a point mutation within the DH domain of ephexin (Q254A) that eliminates catalytic activity had no effect on Pak phosphorylation (Figure 5B). The ability of ephexin to activate Pak phosphorylation was also dependent on active Rac1 since coexpression of a dominant-negative Rac1 (RacN17) blocked the ephexin-induced increase in Pak phosphorylation (Figure 5A). We also tested the ability of ephexin to activate endogenous Pak in primary neuronal cultures. When cultured embryonic cortical cells were infected with HSV-ephexin, a significant increase in Pak phosphorylation was observed compared to uninfected neurons (Figure 5C). These results demonstrate that Pak is a downstream target of activated ephexin.

Modulation of Ephexin Activity by EphA Receptors

The association of ephexin with EphA4 led us to investigate whether EphA receptors might modulate ephexin activity toward specific Rho GTPase signaling pathways in a way that is consistent with the growth cone collapsing activity of activated EphA receptors. The high sensitivity of the anti-P-Pak antibody allowed us to measure changes in the Rac1/Cdc42-Pak pathway in response to extracellular stimuli that were below the limits of detection of the PBD pulldown assay (data not shown). Cultured embryonic cortical cells were stimulated with ephrin-A1 and cell lysates were subjected to immunoblot analysis with the anti-P-Pakα antibody to assay the activity of endogenous Rac1 and Cdc42 signaling pathways. Ephrin-A1 stimulation led to a time-depen-

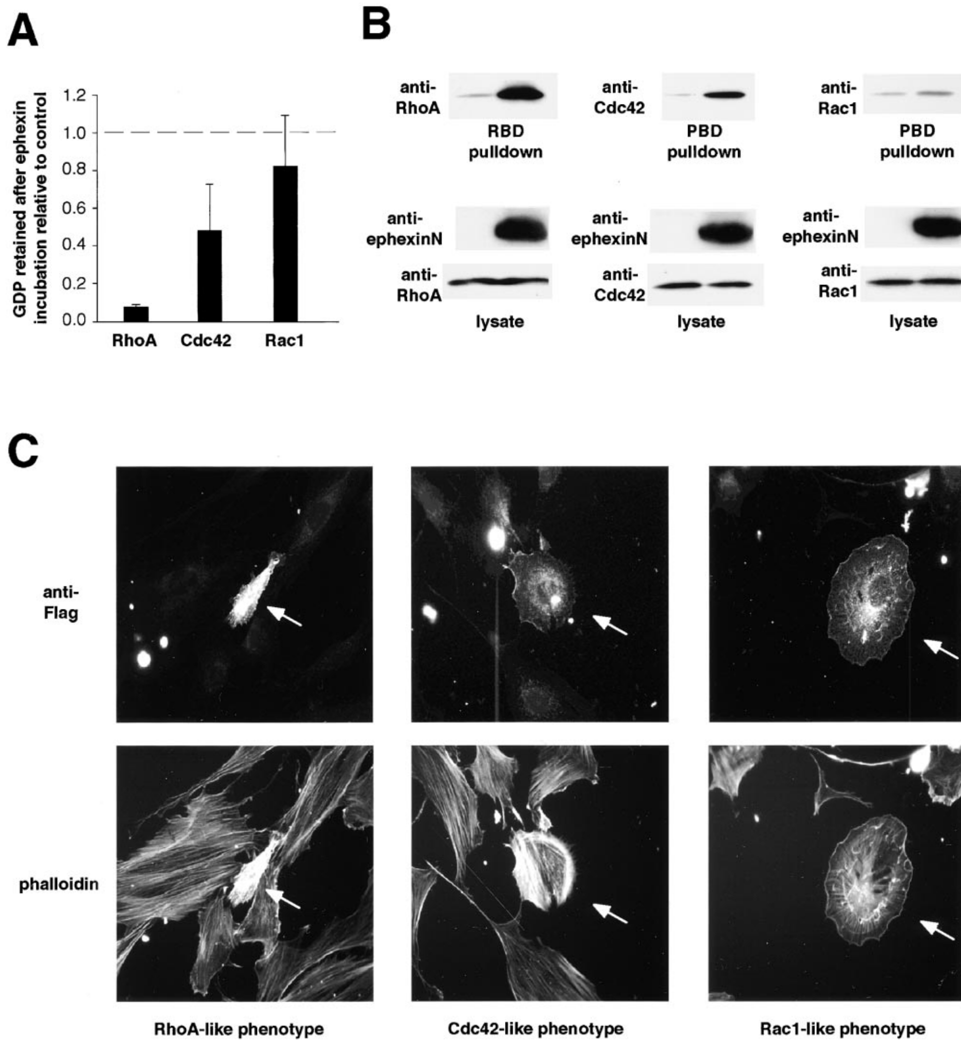

Figure 4. Characterization of Ephexin GEF Activity

The guanine nucleotide exchange activity of ephexin was measured with (A) GDP release assays in vitro, (B) Rho GTPase effector pulldown assays in neurons, and (C) F-actin morphology assays in ephexin-transfected REF-52 fibroblasts.

(A) The exchange activity of rat GST-ephexin (amino acids 67–620) fusion protein was measured as its ability to catalyze the release of [^{3}H]GDP from RhoA, Cdc42, or Rac1. Activity is expressed as the ratio of the amount of GDP retained on each Rho GTPase in the presence of ephexin over the amount remaining in the absence of ephexin. Experiments were repeated at least three times, and each point was performed in duplicate.

(B) The exchange activity of HSV-ephexin expressed in cultured E18 cortical neurons was measured by its ability to increase the levels of activated RhoA, as detected by RBD pulldown (top panels), or to increase the levels of activated Rac1 or Cdc42, as detected by PBD pulldown. Levels of Rac1, Cdc42, RhoA, and ephexin in the cell lysates were determined by immunoblotting (bottom panels).

(C) REF-52 cells were transfected with Flag-tagged ephexin and stained with anti-Flag followed by a FITC-conjugated secondary to detect transfected cells and TRITC-phalloidin to detect F-actin. Transfected cells are indicated by arrows, and display RhoA-like (left), Cdc42-like (middle), or Rac1-like (right) phenotypes.

dent inhibition of Pak phosphorylation that paralleled the time course for Eph receptor activation, suggesting that EphA receptor activation inhibits the Rac1/Cdc42-Pak signaling pathway (Figure 5C). Since ephexin can activate Pak, we asked whether Eph receptors might be inhibiting Pak through the modulation of ephexin activity. Primary cultured cortical neurons were infected with HSV-ephexin and then treated with ephrin-A1 to activate endogenous Eph receptors. The activation of Pak induced by HSV-ephexin as measured with the anti-P-Pakα antibody was significantly suppressed by treatment with ephrin-A1 (Figure 5C). Immunoblot analysis of the same cell lysates with an anti-Pakα antibody indicated that Pakα levels remained constant upon HSV-ephexin infection and ephrin-A1 treatment. These results suggest that EphA receptor activation results in the inhibition of ephexin activity toward the Rac1/Cdc42-Pak signaling pathway.

We also examined the ability of EphA4 receptors to regulate ephexin activity in REF-52 fibroblasts. Ephexin expression induced the formation of stress fibers in approximately 25% of transfected cells, and lamellipodia and filopodia in approximately 10%–20% of transfected cells (Figure 5D). The morphologic effects induced by

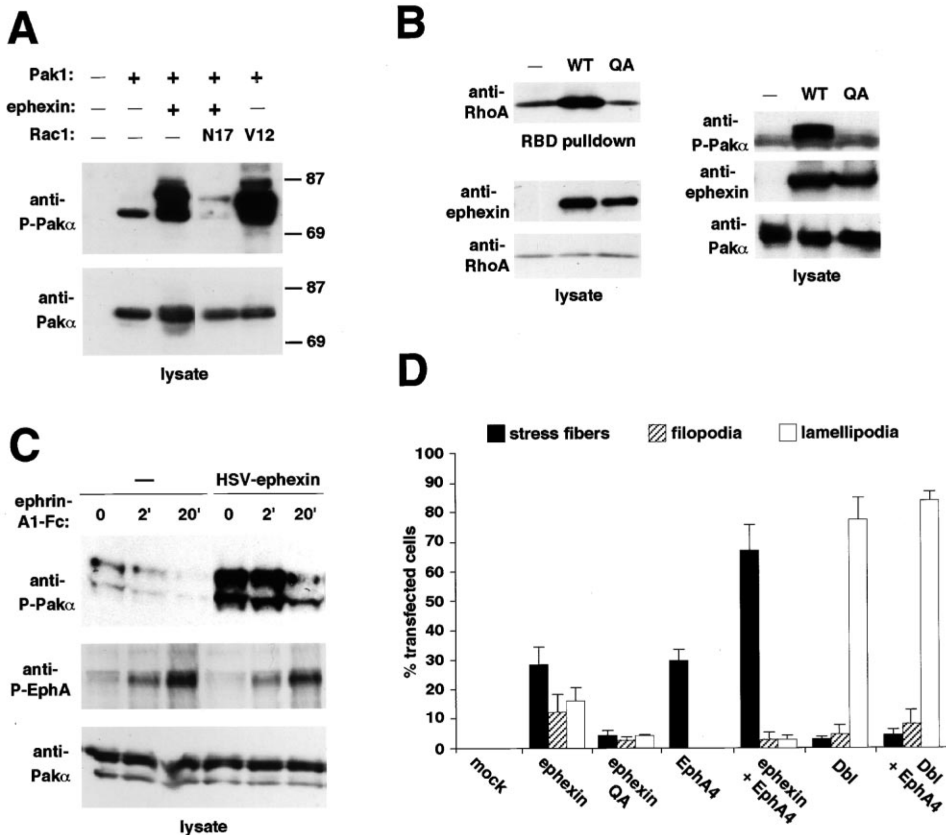

Figure 5. Modulation of Ephexin Activity by EphA Receptors

(A) Characterization of a phosphorylation-specific Pakα antibody, anti-P-Pakα. 293T cells were transfected with Pakα, ephexin, and/or Rac1 expression constructs and cell lysates immunoblotted with anti-P-Pakα (top) or a total Pak antibody, anti-Pakα (bottom).

(B) Characterization of a catalytically inactive mutant of ephexin, ephexinQA. 293T cells were transfected with empty vector, wild-type ephexin, or ephexinQA, and levels of activated RhoA were measured by the RBD pulldown assay (top left). Pakα phosphorylation in similarly transfected cells was assayed by immunoblotting with anti-P-Pakα (top right). Cell lysates were also assayed for the expression levels of wild-type ephexin and ephexinQA (middle panels), RhoA levels (bottom left), or Pakα (bottom right).

(C) Analysis of EphA receptor modulation of ephexin activity as measured with anti-P-Pakα. Cortical neurons from E18 rat were infected with HSV-ephexin or were left untreated (−). 12 hr after infection, the cells were treated with aggregated ephrin-A1-Fc for the indicated time points and cell lysates were immunoblotted with anti-P-Pakα to measure levels of Pak phosphorylation. Lysates were also immunoblotted with anti-P-EphA to measure the state of EphA receptor activation, and with anti-Pakα to measure the levels of Pak expression.

(D) EphA4 receptor modulation of ephexin activity toward Rho GTPases in REF-52 fibroblasts. REF-52 fibroblasts were transfected with wild-type ephexin, ephexinQA, myc-tagged oncogenic Dbl, and/or wild-type EphA4. 24 hr after transfection, the cells were fixed and stained with anti-ephexinN, anti-Myc, or anti-EphA4 antibodies to identify transfected cells, and with TRITC-phalloidin to visualize F-actin. All transfections were repeated at least three times, and an average of 100 cells were examined each time.

ephexin were dependent on ephexin's GEF activity since the catalytically inactive Q254A mutant failed to elicit significant morphologic effects in REF-52 cells (Figure 5D). When expressed in REF-52 cells, EphA4 receptors induced stress fiber formation in approximately 30% of transfected cells (Figure 5D) and were constitutively tyrosine phosphorylated at the important signaling tyrosines Y596 and Y602 (data not shown), suggesting that they were fully activated. The coexpression of EphA4 receptors with ephexin in REF-52 cells resulted in a significant increase in the percentage of cells displaying stress fibers and a marked decrease in the percentage of transfected cells with lamellipodia and filopodia (Figure 5D). In contrast, the coexpression of EphA4 receptors with Dbl, another Rho family GEF, failed to modulate Dbl activity in the REF-52 morphology assay (Figure 5D). These results demonstrate that activated EphA4 receptors enhance the ability of ephexin to activate RhoA and inhibit the ability of ephexin to activate Rac1 and Cdc42, thus resulting in a shift in the balance of Rho GTPase activities.

Ephexin Mediates Ephrin-Induced Growth Cone Collapse

We next established an assay to directly address the role of ephexin in mediating growth cone collapse activity of ephrin-A1 in purified RGCs. In RGCs cultured from both E21 and P6 rat, ephrin-A1 induced dose-dependent growth cone collapse that occurred in 80%–90% of growth cones at maximal doses (Figures 6A and 6B). When wild-type ephexin was overexpressed in RGCs, the growth cone collapse effect of ephrin-A1 was significantly enhanced in ephexin-transfected cells as compared to vector-transfected controls (Figure 6C). To inhibit the

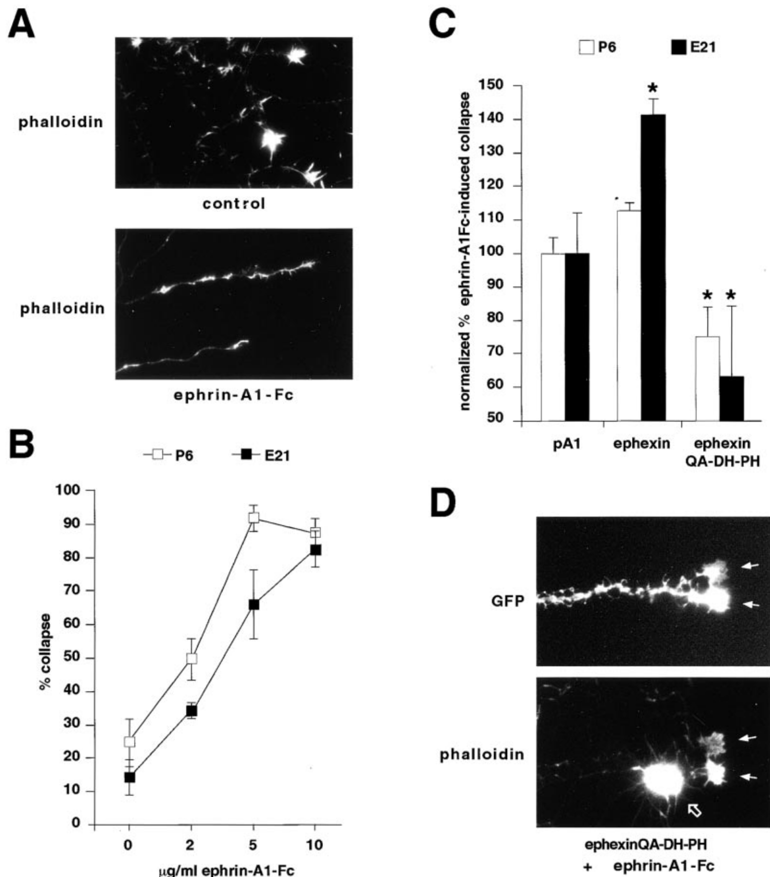

Figure 6. Ephexin Functions in Growth Cone Collapse Induced by Ephrin-A

(A) Ephrin-A1-Fc induces growth cone collapse in cultures of purified rat RGCs. P6 RGCs were treated with either aggregating antibody alone as a control (top) or with 5 μg/ml of aggregated ephrin-A1-Fc (bottom) for 30 min and stained with Alexa 594-conjugated phalloidin.

(B) Dose-response curve of ephrin-A-induced RGC growth cone collapse. Cultured purified P6 or E21 RGCs were stimulated with aggregated ephrin-A1-Fc at the indicated concentrations and the percentage of collapsed growth cones counted. The mean percentage collapse from two separate experiments, each performed in duplicate, is shown.

(C and D) Overexpression of wild-type or mutant ephexin alters growth cone responses to ephrin-A. (C) P6 or E21 RGCs transfected with the empty expression vector pA1, wild-type ephexin, or ephexinQA-DH-PH were treated with 5 μg/ml of aggregated ephrin-A1-Fc. At each age, collapse rates were normalized to those of pA1 transfectants. Asterisks mark collapse rates significantly different ($p < 0.05$) from pA1 transfectants. (D) The axon and growth cones of a P6 RGC transfected with ephexinQA-DH-PH (filled arrows) were detected by visualizing coexpressed GFP (top). Phalloidin staining of the same field shows failure to collapse in response to aggregated ephrin-A1-Fc (bottom). The open arrow indicates an untransfected RGC.

activity of endogenous ephexin, we incorporated the Q254A mutation into the DH-PH module of ephexin (QA-DH-PH) and overexpressed it in RGCs. Since the GEF-inactive QA-DH-PH mutant maintains EphA4 receptor binding activity (data not shown) and lacks the ability to bind to potentially important SH3 binding coregulators, this mutant could uncouple endogenous ephexin from EphA receptor regulation. When expressed in RGCs, QA-DH-PH significantly inhibited ephrin-A1-induced growth cone collapse (Figures 6C and 6D). The expression of wild-type or mutant ephexins did not significantly alter basal rates of collapse in unstimulated RGCs (data not shown), demonstrating that the changes in collapse rates were dependent on EphA receptor activity. Taken together, the gain-of-function and loss-of-function manipulations of ephexin in RGCs suggest that ephexin is an important mediator of ephrin-A1-induced growth cone collapse.

Discussion

EphA receptors and their ligands of the ephrin-A family mediate short-range repulsion of axons during nervous system development. However, the intracellular signaling mechanisms engaged by EphA receptors that lead to localized actin rearrangement and growth cone collapse have previously been poorly understood. Here we describe the cloning of ephexin, a novel member of the Dbl family of GEFs that activates Rho GTPases. Several lines of evidence suggest that ephexin couples EphA receptor activation to modulation of Rho GTPase signaling that leads to growth cone collapse. First, ephexin is expressed specifically in the CNS and is present during embryonic development in regions of the nervous system where EphA receptors function to control axon guidance. Second, ephexin associates directly with EphA receptors. Third, the activation of EphA receptors potentiates the ability of ephexin to activate RhoA yet leads to an inhibition of ephexin activity toward Cdc42 and Rac1. Finally, overexpression of ephexin leads to increased sensitivity of RGCs to ephrin-A1-induced growth cone collapse, whereas a dominant-negative version of ephexin suppresses ephrin-A1-induced collapse. Taken together, these findings suggest that ephexin plays an important role in EphA receptor-initiated axon guidance repulsion.

Rho family GTPases control the formation of distinct actin-based structures in the growth cone, and have been proposed as candidates for regulation by axon guidance receptors. Forward migration of the growth cone may involve a balance between basal RhoA, Cdc42, and Rac1 activities. Activated Cdc42 and Rac1 promote the extension of filopodia and lamellipodia, respectively, and activated RhoA generates contractile forces that may be required for forward translocation of the growth cone body (Schmitz et al., 2000). In contrast, growth cone collapse may result from elevated RhoA activity concurrent with decreases in Cdc42 and Rac1 activity. Increased RhoA-mediated contractility would lead to

the withdrawal of the growth cone body, while reduced Cdc42 and Rac1 activity would result in the retraction of lamellipodia and filopodia from the leading edge of the growth cone. Thus, it is possible that inhibitory axon guidance receptors may induce growth cone collapse by shifting the balance of Rho GTPase activities away from Cdc42 and Rac1 and toward RhoA. Indeed, previous results have demonstrated that ephrin-A5 stimulation of RGCs leads to the inhibition of Rac1 and activation of RhoA (Wahl et al., 2000), but have not elucidated the molecular mechanisms by which EphA receptors signal to Rho GTPases.

Our findings that EphA receptor activation leads to an inhibition of ephexin activity toward Rac1 and Cdc42, but an enhancement of ephexin activity toward RhoA, suggest that the control of Rho GTPases by EphA receptors is mediated in part by ephexin. In the absence of ephrin-A1 stimulation, binding of ephexin to inactive EphA receptors may serve to anchor ephexin at the cell membrane of growth cones in a position to activate RhoA, Cdc42, and Rac1 (Figure 7A). Under these conditions, the net effect from ephexin activity and that of other regulatory GEFs or GAPs would be growth cone extension. When EphA receptors are activated through contact with an ephrin-A-presenting cell surface, ephexin activation of Cdc42 and Rac1 is inhibited, whereas ephexin activation of RhoA is potentiated (Figure 7B). The reduction in Cdc42 and Rac1 activities results in the retraction of lamellipodia and filopodia, while the increase in RhoA activity leads to high levels of contractile forces in the growth cone. Thus, EphA receptor regulation of ephexin may play a role in modulating Rho GTPases to orchestrate the collapse of the growth cone. Control over the balance of Rho GTPase activities may be a general mechanism by which axon guidance factors and their receptors manipulate the actin cytoskeleton to elicit attractive or repulsive growth cone guidance effects.

For growth cones to respond to extracellular guidance cues in a directional manner, guidance receptors must transduce highly localized signals to the actin cytoskeleton specifically in the region of the growth cone where receptor activation occurs. Our finding that ephexin interacts directly with EphA4 receptors independent of receptor kinase activity suggests a mechanism for localized actin regulation by EphA receptors. Specifically, EphA activation would result in the modulation of ephexin activity only at the site of ligand presentation, while ephexin molecules in other regions of the growth cone remain unaffected. Furthermore, because ephexin is directly associated with EphA receptors, its downstream effects on Rho GTPases may remain confined to the vicinity of the activated receptors. Thus, ephexin may be modulated by EphA receptors to elicit local changes in Rho GTPase activity and to induce spatially restricted retraction of the growth cone at points of contact with ephrin-A.

The mechanism through which EphA receptors modulate ephexin activity is currently not clear. One intriguing possibility is that substrate specificity within the catalytic domain of ephexin can be differentially regulated to yield inhibitory effects toward some GTPases and potentiating effects toward others. Alternatively, EphA4 inhibition of ephexin signaling to Rac1 and Cdc42 might

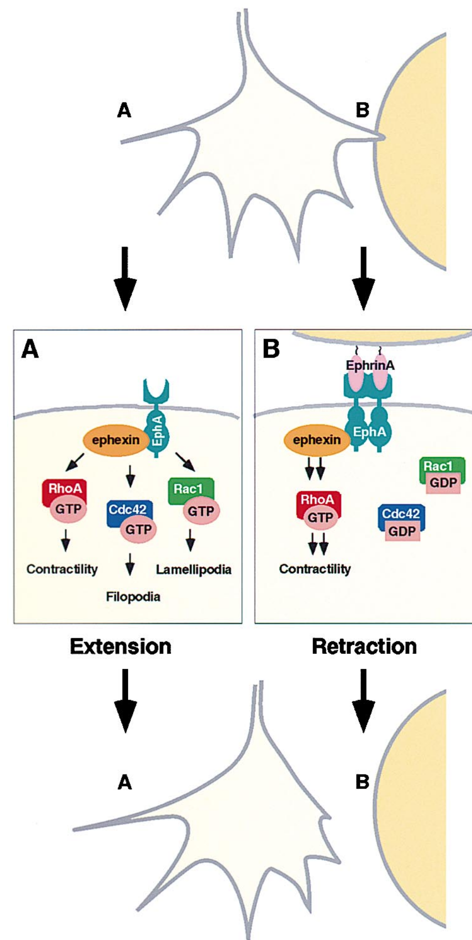

Figure 7. A Model for How EphA and Ephexin May Locally Regulate Actin Dynamics in a Growth Cone during Axon Guidance

(A) In regions of the growth cone not in contact with ephrin-A, ephexin is stably bound to EphA receptors at the membrane, where ephexin contributes to the activation of RhoA, Cdc42, and Rac1. The coordinate activation of these GTPases supports a basal level of actinomyosin contractility and filopodia and lamellipodia, which together generate forward movement.

(B) In regions of the growth cone in contact with ephrin-A, EphA activation leads to a suppression of ephexin activity toward Rac1 and Cdc42 and to an enhancement of ephexin activity toward RhoA. The suppression of Rac1 and Cdc42 may result in the local depolymerization of actin in filopodia and lamellipodia, while unopposed actinomyosin contractility from potentiated RhoA activity may induce localized withdrawal of filopodia and lamellipodia.

be an indirect effect of elevated RhoA activity, or vice versa, as has been previously suggested (Kozma et al., 1997; Leeuwen et al., 1997). In addition, the access of ephexin to different Rho GTPases might be regulated by EphA receptors as a result of changes in Rho GTPase subcellular localization, and this could contribute to the observed changes in Rho GTPase activities.

The EphA receptor-induced change in ephexin activity might result from a posttranslational modification of ephexin. One possibility is that EphA receptor activation leads to changes in the phosphorylation state of ephexin due to the activation of specific kinases or phosphatases. Ephexin might also be modified as a result of

EphA-induced regulation of the PI3-kinase signaling pathway since EphA4 receptors bind to multiple PI3-kinase regulatory subunits (S. M. S., unpublished observations), several Eph receptors are known to activate PI3-kinase (Pandey et al., 1994), and PI3-kinase lipid products have been shown to regulate the activity of Vav and αPIX, a Pak-interacting GEF (Han et al., 1998; Yoshii et al., 1999). Alternatively, since the association of EphA4 receptors occurs in the catalytic DH-PH domains of ephexin, EphA modulation of ephexin might occur through reversible steric or allosteric hindrance of GEF activity. Further studies utilizing specific pharmacologic and dominant-negative reagents as well as more detailed structure-function analyses should help to elucidate the mechanism of regulation of ephexin by EphA receptors.

In summary, the experiments described here identify ephexin as an important molecular link between EphA receptors and the Rho family of GTPases, and suggest a model for how EphA receptors may locally regulate the actin cytoskeleton during axon guidance. Additional studies with specific inhibitors of ephexin and the genetic disruption of ephexin will be required to determine the role of ephexin in axon guidance and cell migration events during development.

Experimental Procedures

Yeast Two-Hybrid Screening

The Interaction Trap yeast two-hybrid system was used as described (Finley and Brent, 1996), with amino acids 570–986 of moues EphA4 as bait. An E14 rat spinal cord/dorsal root ganglia cDNA library consisting of 2×10^6 primary transformants and a TrkA bait plasmid were kindly provided by David Ginty (Johns Hopkins University).

5′-RACE

Additional 5′ sequences of rat ephexin were obtained using a 5′-RACE kit (GibcoBRL). Reverse transcription from P20 rat brain RNA was performed with the 3′ rat ephexin primer TATAGACCGAGAAG TGATCA followed by consecutive PCR reactions with a universal 5′ primer and the nested 3′ rat ephexin primers ATGTTCTCCTCCA TGCGGTGTTC and CATCCAGGACATTGGAGAAGA.

Antibodies

The anti-EphA4 antibody was raised against amino acids 909 to 986 of mouse EphA4 fused to glutathione S-transferase (GST), as previously described (Becker et al., 1995). Rabbit anti-P-EphA was raised against the EphA3 peptide LRTY(p)VDPHRY(p)EDPTQ, in which tyrosine residues 596 and 602 have been phosphorylated. Anti-P-Pakα was raised against the PAKα peptide PEHTKS(p)V YTRS(p)VIEP with phosphates added to serine residues 198 and 203. The following antibodies were raised against peptide sequences of murine ephexin: anti-ephexinN, amino acids 93–108 (KSTLQEIET RRQQDAE); SH3, amino acids 561–576 (FGERLHDQERGWFPSS); and anti-ephexinC, amino acids 598–613 (HKMEDPQRSQNKDRRK). Anti-Pak1N was a kind gift from Bruce Mayer, University of Connecticut Health Center. The following antibodies are commercially available: Rac1 (Transduction Laboratories), Cdc42 (Transduction Laboratories), RhoA (Santa Cruz), TuJ1 (Babco), and caveolin-1 (Santa Cruz).

Membrane Isolation and Fractionation

Cellular membranes were prepared from P7 rats as described (Wyszynski and Sheng, 1999). Membranes were extracted with 1% Triton X-100 for 1 hr at 4°C and then centrifuged at 20,000 g for 10 min. The supernatant, or the pellet after resuspension into the original volume of TBS, was then boiled in sample buffer containing 0.1% SDS and 5% β-mercaptoethanol, and subjected to SDS-PAGE and immunoblotting. An equal beginning volume of membrane was directly boiled in sample buffer and electrophoresed in an adjacent lane.

Expression Plasmids

The following constructs were received as gifts: Pak-α expression plasmid, Bruce Mayer; wild-type, dominant-negative, and constitutive active Rac expression plasmids, Bruce Yanker (Children's Hospital); EphA4 cDNA, John Flanagan (Harvard Medical School); full-length mouse ephexin EST, Katsuyuki Hashimoto (National Institute of Infectious Diseases, Tokyo); and pGEX-PBD and pGEX-RBD, Joan Brugge (Harvard Medical School). The myc-tagged Dbl construct has been previously described (Olson et al., 1996). All other expression plasmids and viral plasmids described were constructed for this study using standard methods. Full sequences of plasmids are available upon request.

Guanine Nucleotide Exchange Assays

In vitro guanine nucleotide release assays were performed as described elsewhere (Debant et al., 1996) using recombinant GST fusions with rat ephexin (amino acids 67–620) or with Rho GTPases produced using standard procedures. To assay Cdc42 or Rac1 activity in cells, 2×10^6 cells were harvested in GEF lysis buffer (20 mM Tris, pH 7.5; 20 mM $MgCl_2$; 0.5% Nonidet P-40; 0.5% deoxycholic acid; 150 mM NaCl; 1 mM dithiothreitol; 1 mM phenylmethyl sulfonyl fluoride; 0.5% aprotinin). Lysates were clarified by centrifugation and incubated with 50 μg GST-PBD prebound to glutathione agarose (Sigma) for 30 min at 4°C. Samples were rinsed with GEF lysis buffer and then immunoblotted with anti-Cdc42 or anti-Rac1. Assays measuring RhoA activity were performed as described (Ren et al., 1999).

Fibroblast Morphology Assays

REF-52 cells were maintained as described (Blangy et al., 2000) and transfected using Lipofectamine (GibcoBRL). 24 hr after transfection, cells were fixed in 3.7% formaldehyde, permeabilized in 0.1% Triton, and stained.

Preparation of Ephrin-A1-Fc

The expression plasmid pJFE14-ephrin-A1-Fc (Davis et al., 1994) was transfected into 293T cells, and ephrin-A1-Fc was purified from conditioned media with protein-A Sepharose as described (Davis et al., 1994). Ephrin-A1-Fc was aggregated at 0.01 mg/ml (cortical neurons) or at 0.1 mg/ml (RGCs) 30 min prior to use with 0.09 mg/ml (cortical neurons) or 0.45 mg/ml (RGCs) goat anti-human Fcγ antibody (Jackson Laboratories).

Growth Cone Collapse

Retinal ganglion cells were purified and cultured as previously described (Meyer-Franke et al., 1995). Expression plasmids were electroporated into RGCs with a modified GenePulser (BioRad) using a variation of the microporation technique (Teruel et al., 1999). After 1–2 days, aggregated ephrins were added to the cultures for 30 min, fixed in 4% paraformaldehyde, stained with Alexa594-conjugated phalloidin (Molecular Probes), and scored for growth cone collapse.

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