BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2009,** *48,* 6369–6378 6369
DOI: 10.1021/bi900831k

Regulation of Process Retraction and Cell Migration by EphA3 Is Mediated by the Adaptor Protein Nck1[†]

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Received May 14, 2009; Revised Manuscript Received June 6, 2009

ABSTRACT: The Eph family of tyrosine kinase receptors and their ligands, the ephrins, participates in the regulation of a wide variety of biological functions under normal and pathological conditions. During embryonic development, interactions between the ligands and receptors define tissue boundaries, guide migrating axons, and regulate angiogenesis, as well as bone morphogenesis. These molecules have also been shown to modify neural activity in the adult nervous system and influence tumor progression. However, the molecular mechanisms underlying these diverse functions are not completely understood. In this study, we conducted a yeast two-hybrid screen to identify molecules that physically interact with Eph receptors using the cytoplasmic domain of EphA3 as "bait". This study identified Nck1 as a strong binding partner of EphA3 as assayed using both GST fusion protein pull down and co-immunoprecipitation techniques. The interaction is mediated through binding of the Nck1 SH2 domain to the phosphotyrosine residue at position 602 (Y602) of the EphA3 receptor. The removal of the SH2 domain or the mutation of the Y602 residue abolishes the interaction. We further demonstrated that EphA3 activation inhibits cell migration and process outgrowth, and these inhibiting effects are partially alleviated by dominant-negative Nck1 mutants that lack functional SH2 or SH3 domains, but not by the wild-type Nck1 gene. These results suggest that Nck1 interacts with EphA3 to regulate cell migration and process retraction.

Eph[1] receptors make up the biggest group of receptor tyrosine kinases, and a total of 16 different receptors have been identified in vertebrates (*1*). These receptors can be grouped into two subclasses based on their sequence homology and their specificity of ligand interactions (*2*). The EphA receptors (EphA1−EphA10) bind to GPI-linked ligands (ephrin-A1−ephrin-A6), and the EphB receptors (EphB1−EphB6) interact with transmembrane ligands (ephrin-B1−ephrin-B3) (*1*, *3*). In general, ligands and receptors interact promiscuously within each subclass (*2*). However, exceptions to this subclass specificity do exist. For example, EphA4 interacts with both the A-ephrins and the B-ephrins, while EphB2 can also bind ephrin-A5 in addition to the B-ephrins (*2*, *4*). It was also reported that ephrin-A1, -A3, and -A4 can bind to EphB1 with low affinity (*5*), although it was not clear whether these interactions would lead to any significant biological functions.

Members of the Eph receptor family and their ligands are strongly expressed in a wide variety of tissues in developing and adult organisms (*3*). Consistent with their wide expression, Eph receptors and their ligands are found to play important roles in many developmental processes, including tissue morphogenesis, vascular network formation, neural crest cell migration, axon fasciculation, axon guidance, and topographic neural map formation (*6*). Ephrin−Eph interaction usually produces repulsive effects that lead to tissue boundary formation and axon retraction (*7*). However, attractive effects were also observed in certain

[†]Research partially supported by grants from the National Science Foundation (0548541), the National Institutes of Health (HD23315), the National Institute of Environmental Health Sciences (P30ES005022), and the New Jersey Commission on Spinal Cord Research.

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[1]Abbreviations: ADF, actin depolymerizing factor; DCC, deleted in colorectal cancer; DMEM, Dulbecco's modified Eagle's medium; Dock, *Drosophila* Nck1 homologue; EGF, epidermal growth factor; EGFP, enhanced green fluorescence protein; Eph, erythropoietin-producing hepatocellular carcinoma kinase; ephrin, Eph family receptor-interacting proteins; FBS, fetal bovine serum; GAPs, GTPase-activating proteins; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; GPI, glycosylphosphatidylinositol; HEK293A, human embryonic kidney 293A; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; JNK, c-Jun amino-terminal kinase; KD, kinase-dead; LIM, lin-11, Isl-1, and Mec-3 domain; LMW-PTP, low-molecular weight phosphotyrosine phosphatase; MLCK, myosin light chain kinase; Nck, noncatalytic region of tyrosine kinase adaptor protein; NIK, nick-interacting kinase; PAK1, p21-activated kinase 1; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SH2, Src homology 2; SH3, Src homology 3; (N)WASP, (neuronal) Wiskott-Aldrich syndrome protein; WT, wild type.

Published on Web 06/08/2009

biological functions (*8*−*12*). Although most of these functions depend on the kinase activity of the receptors, kinase-independent signaling also exists through the receptors (*13*, *14*), or through reverse signaling of the ligands (*15*). In addition to the roles during development, Eph receptors have also been shown to regulate adult functions such as learning and memory (*16*, *17*), and pathological conditions such as tumorigenesis (*18*, *19*).

Several downstream molecules that mediate Eph receptor functions have been identified. They include the Rho family small GTPases, namely, RhoA, Rac1, and Cdc42, as well as the guanine nucleotide exchange factors (GEFs), such as kalirin, Ephexin, Vav, and the FERM domain, including RhoGEFs (FIR) (*20*). The GEFs usually bind to the activated Eph receptors, and the binding leads to RhoA activation. In addition, the activation of Eph receptors has been shown to recruit GTPase-activating proteins (GAPs) for Rac and lead to the inactivation of Rac (*21*−*23*). These modulations of low GTPase activity trigger changes in cytoskeleton dynamics, causing growth cone collapse and dendritic spine remodeling (*20*). There is also evidence that RasGAP and p62 (dok) associate with activated EphB2 to mediate inhibition of Erk-MAP kinase activity induced by ephrin-B1 (*24*). Furthermore, activation of EphA2 recruits protein tyrosine phosphatase Shp2 and triggers dephosphorylation of FAK, which also forms a protein complex with EphA2 (*25*). Several other protein tyrosine phosphatases (PTPs), including the low-molecular weight PTPs (*26*, *27*), the receptor-type phosphatase PTPO (*28*), and Src family kinases (*29*−*32*), have been reported to associate with Eph receptors and modulate their activity as well. Eph receptors were also found to interact with neurotransmitter receptors. For example, EphB2 binds to the NMDA receptor to promote synaptogenesis (*33*, *34*).

In spite of the progress made so far, the understanding of signaling mechanisms underlying different Eph receptor functions remains incomplete. To further identify potential signaling molecules downstream of the Eph receptors, a yeast two-hybrid screen using an EphA receptor intracellular domain as "bait" was conducted. In this study, the SH2 and SH3 domain-containing adaptor protein Nck1 was identified as a strong EphA3 binding protein. We show here that this interaction is mediated through binding of the Nck1 SH2 domain to tyrosine residue 602 of the EphA3 receptor and that blocking Nck1 function also abrogates the inhibition on process outgrowth and cell migration induced by EphA3 activation. These studies indicate that Nck1 is a key downstream signaling molecule that mediates EphA3 functions.

MATERIALS AND METHODS

Reagents and Antibodies. Ephrin-A5−Fc fusion protein was purchased from R&D Systems (Minneapolis, MN). The ligand was clustered by antibody cross-linking using anti-human Fc (Jackson ImmunoResearch, West Grove, PA) at an antibody: ephrin weight ratio of 1:5 (1:15 molar ratio). The mixture was incubated at 4 °C for 8 h and used to stimulate cells at concentrations of 1−2 μg/mL. Mouse anti-Nck1 monoclonal antibody was purchased from BD Biosciences (San Jose, CA). The rabbit anti-EphA3 polyclonal antibody and mouse anti-*myc* 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 a 1:1000 dilution. Secondary antibodies used in Western blotting were acquired from Sigma-Aldrich (St. Louis, MO). When reblotting was required during Western blotting, the nitrocellulose membrane was washed briefly and incubated in Western-blot restrip buffer from G-Biosciences (St. Louis, MO) for 30 min.

Yeast Two-Hybrid Screen. The yeast two-hybrid screen was performed with the DupLex-A system from Origene (Rockville, MD) according to the instructions. In brief, the intracellular domain of the EphA3 receptor was cloned into the 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 pJG4-5 target plasmid. The transformed yeast cells were plated and screened for LacZ transcription through X-gal reaction. Plasmid DNA from the positive clones was then extracted, amplified in *Escherichia coli*, and sequenced. The identity of the positive clones was determined using BLAST searches against public databases. The specificity of the interaction between the identified cDNA products and the bait protein was further confirmed using a yeast mating test. Briefly, the isolated target plasmids carrying the cDNA fragments were transformed into EGY188, and the resulting cells mated with cells of the EGY40 strain containing both the reporter plasmid and pEG202-NLS-EphA3intra. The mated cells were lysed and incubated with X-gal solution for 2 h. Negative controls for the mating test were generated by using an empty bait plasmid (pBait) or an irrelevant control plasmid in place of pEG202-NLS-EphA3intra in the test.

GST Fusion Protein Pull Down. 293A cells transfected with various EphA3 constructs were stimulated with cross-linked ephrin-A5−Fc fusion protein (1 μg/mL) for 30 min at 37 °C. The cells were washed once with ice-cold PBS and lysed in lysis buffer [10 mM Hepes (pH 7.4), 1% Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM sodium orthovanadate]. The lysed cells were collected and centrifuged at 13000 rpm for 10 min in an Eppendorf microcentrifuge. After centrifugation to remove cell debris, 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 h. The beads were then collected by centrifugation and washed three times with PBS supplemented with 0.05% Triton X-100, each for 10 min. The beads were boiled in 2× Laemmli buffer for 5 min before analysis via a Western blot technique.

Co-Immunoprecipitation Assay. Transfected 293A cells were stimulated with cross-linked ephrin-A5−Fc fusion protein for 30 min at 37 °C. The cells were washed gently with ice-cold PBS once and lysed in cell lysis buffer. The cell lysate was then 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, the desired antibody was added to 2 mg of each lysate and incubated at 4 °C for 4 h. The immunoprecipitates were collected with protein A Sepharose beads and washed three times with PBS and 0.05% Triton X-100. The immunocomplexes were boiled in 2× Laemmli buffer for 5 min and analyzed with SDS−PAGE coupled with Western blot analysis using different antibodies.

Cellular Process Retraction Assay. HEK293A cells were plated sparsely on culture dishes to allow examination of individual cells and their processes. The cells were transfected with desired plasmids and, 48 h after transfection, stimulated with or without cross-linked ephrin-A5 for 1 h at 37 °C. Pictures

of transfected cells ($N > 30$) identified via EGFP expression were taken before and after the treatment. The length of the processes was measured from where they exit the cell body to the end of the process using ImagePro. The difference between the length of the processes before and after incubation was then divided by the length before treatment and expressed as the percentage change.

Cell Migration Assay. To prepare for this assay, transwell inserts (20 mm diameter, 8 μm pore diameter) purchased from Corning Labware (Corning, NY) were coated on the underside with fibronectin to facilitate attachment of migrated cells. HEK293A cells were transfected with the desired genes using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the cells were trypsinized and washed with PBS. A portion of cells was plated in six-well dishes for the determination of transfection efficiency. An equal number of transfected cells was plated on the 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 μg/mL cross-linked ephrin-A5. The transwell dishes were then incubated overnight at 37 °C with 5% CO_2. On the second day, cells on the inserts were fixed and all the cells that had not migrated were cleaned off using Q-tips. Cells on the underside of the inserts were stained with DAPI to allow cell counting under a fluorescence microscope. Five to ten random fields were chosen, and the number of all cells that migrated through the membrane (T_2) and the number of transfected cells that migrated (T_1) were both quantified. In addition, the transfection efficiency was individually determined and expressed as a percentage of total cells (R = transfected cells/all cells). The migration ability (M) of cells expressing different genes was calculated using the formula $M = T_1/(T_2R)$. When the data were used in the graph, M was compared with that of EGFP-transfected controls and converted as a percentage of the M value of EGFP control, which is considered to be 100%.

RESULTS

Nck1 Is an EphA Receptor-Interacting Protein. In the yeast two-hybrid screen, the intracellular domain of EphA3 was inserted into the bait plasmid and a whole mouse embryonic cDNA library was screened. In all, ~300 positive clones were isolated and sequenced. Among them, one clone that exhibited the strongest binding encoded a part of the mouse Nck1 protein, including the second and third SH3 domain and the complete SH2 domain (Figure 1). When this clone was further examined in a yeast mating test, the cDNA protein product had the ability to interact with the EphA3 bait protein and turn on LacZ transcription, while neither an empty bait plasmid (pBait) nor a negative control plasmid could do so (Figure 1A), suggesting a specific interaction between Nck1 and EphA3.

Coincidentally, when the intracellular domain of EphA5 was used in a yeast two-hybrid screen, Nck1 was also one of the

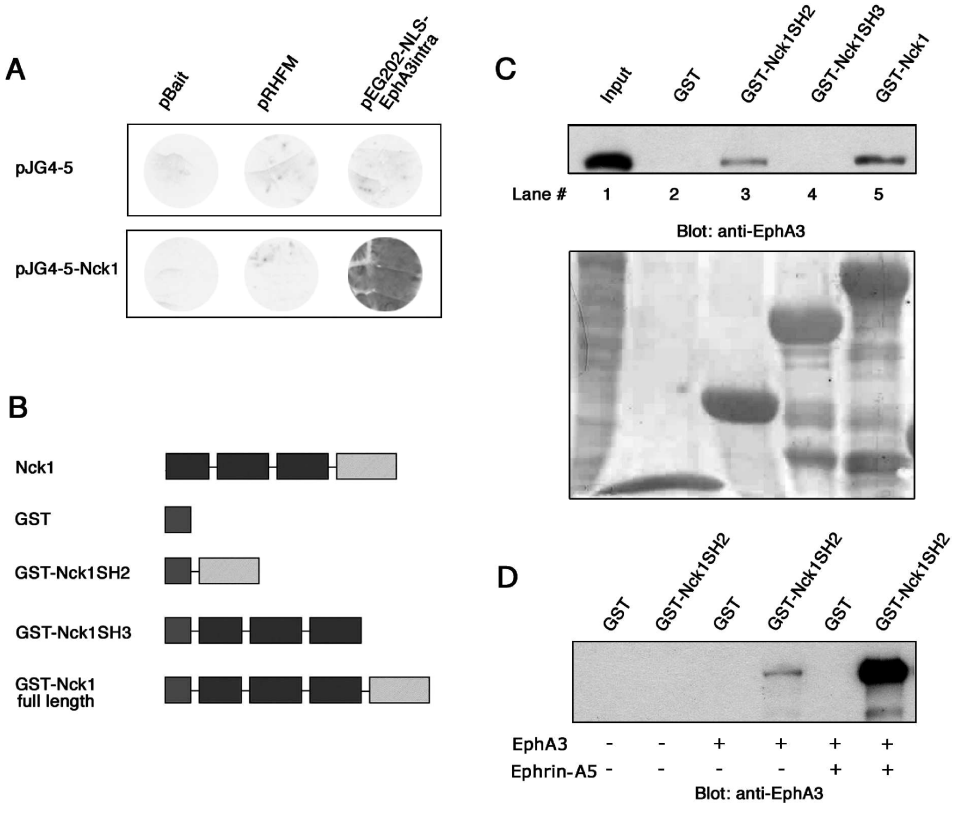

FIGURE 1: Identification of Nck1 as a major binding protein of EphA3. (A) Yeast mating test. Yeast cells carrying pJG4-5-Nck1 cDNA, the LacZ reporter plasmid, and either an irrelevant control plasmid (pRHFM), an empty bait plasmid (pBait), or pEG202-NLS-EphA3intra were lysed and incubated with LacZ substrate X-gal. Only the transformation of both pJG4-5-Nck1 and pEG202-NLS-EphA3intra was capable of turning on LacZ gene expression. (B) Domain structures of the wild-type Nck1 protein and Nck1 fusion proteins used in the GST pull-down assay. (C) 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 subjected to Western blot analysis with anti-EphA3 antibody. "Input" shows the expression of EphA3 in the cell lysate of transfected HEK293A cells. The blot was stained with a Ponceau S solution to show the levels of GST fusion proteins used in the assay (bottom panel). (D) Ephrin-A5 stimulation enhances interaction between EphA3 and Nck1. GST fusion proteins were incubated with cell lysates from stimulated or unstimulated EphA3-transfected 293A cells, and the pull-down complex was analyzed by Western blotting using the anti-EphA3 antibody.

strongest interacting proteins, as determined by the LacZ colorimetric assay (data not shown). These findings collectively show that Nck1 interacts with both EphA3 and EphA5 and probably mediates critical functions of EphA receptors.

Interaction between Nck1 and EphA3 Is Mediated by Binding of the Nck1 SH2 Domain to Tyrosine 602 (Y602) of EphA3. To map the binding site of Nck1, wild-type EphA3 (EphA3-WT) was transfected into HEK293A cells, and 48 h after transfection, the cells were stimulated with cross-linked ephrin-A5 for 30 min. The lysates were then incubated with GST fusion proteins that contained either full-length Nck1 (GST−Nck1), the SH2 domain (GST−Nck1SH2), or the SH3 domain (GST−Nck1SH3) (Figure 1B). Both GST−Nck1 and GST−Nck1SH2 proteins precipitated EphA3 (Figure 1C), while GST control and GST−Nck1SH3 did not. Experiments performed with cell lysates of unstimulated EphA3-expressing 293A cells showed a much reduced level of binding of EphA3 by GST−NckSH2 (Figure 1D). These pull-down studies indicate that the interaction with EphA3 is mediated by the SH2 domain of Nck1.

SH2 protein domains typically bind to phosphorylated tyrosine residues (*35*). To identify the tyrosine residues on EphA3 that the Nck1 SH2 domain binds, several cytoplasmic tyrosine residues of EphA3 known to be phosphorylated upon activation (Y596, Y602, Y736, Y779, and Y937) (G. Shi and R. Zhou, unpublished data) were mutated to phenylalanine using the in vitro mutagenesis method. In addition, a mutant containing a lysine to arginine mutation at amino acid position 653, which was known to inactivate EphA3 kinase activity, was used as a negative control (K653R). These EphA3 mutants, along with wild-type EphA3, were each transiently expressed in HEK293A cells, and the cell lysates were then incubated with GST−Nck1SH2 protein. Among the tyrosine mutants, Y596F and Y602F exhibited no binding to the Nck1 SH2 domain, while the others displayed clear binding (Figure 2A, lanes 1−5). The kinase dead mutant EphA3-K653R also failed to bind the Nck1 SH2 domain compared to wild-type EphA3 (Figure 2A, lanes 6 and 7). Since Y596 and Y602 may regulate EphA3 kinase activity, the loss of binding we observed could be due to either a complete loss of all tyrosine phosphorylation or the absence of the key tyrosine residues. To differentiate between these two possibilities, two additional mutants (Y596E and Y602E) were generated, with Y596 and Y602 being replaced with glutamic acid, respectively. This glutamic acid replacement was shown previously to mimic both the size and charge of a phosphorylated tyrosine and restore kinase activity of similar mutants (*36*). Pull-down studies using these mutants showed that only Y602E failed to bind GST−Nck1SH2 (Figure 2A, lanes 8−10).

To further establish that the loss of Y602 phosphorylation, not the loss of kinase activity, is responsible for the loss of the binding, we examined the ability of EphA3 mutants to autophosphorylate. Wild-type and mutant EphA3 constructs were transiently transfected into 293A cells. Two days after transfection, the cells were treated with ephrin-A5 and lysed, and EphA3 proteins were immunoprecipitated with a rabbit polyclonal anti-EphA3 antibody. The immunoprecipitates were further analyzed for tyrosine phosphorylation using a Western blot technique with a monoclonal anti-phosphotyrosine antibody. This analysis showed that indeed both Y596F and K653R mutants lacked kinase activity (Figure 2B, lanes 1 and 6), correlating with their inability to bind to the Nck1 SH2 domain. Replacement of Y596 with glutamic acid in the Y596E mutant restored both kinase activity and Nck1 SH2 domain binding, suggesting that Y596 is required for EphA3 kinase activity but not SH2 binding. In contrast, both Y602F and Y602E mutants maintained kinase activity (Figure 2B, lanes 2 and 9) but lost the ability to interact with the Nck1 SH2 domain (Figure 2A). Thus, we conclude that Y602 is the critical residue, which serves as the Nck1 docking site when Epha3 is phosphorylated.

Co-Immunoprecipitation of EphA3 and Nck1. To examine whether Nck1 interacts with EphA3 in mammalian cells,

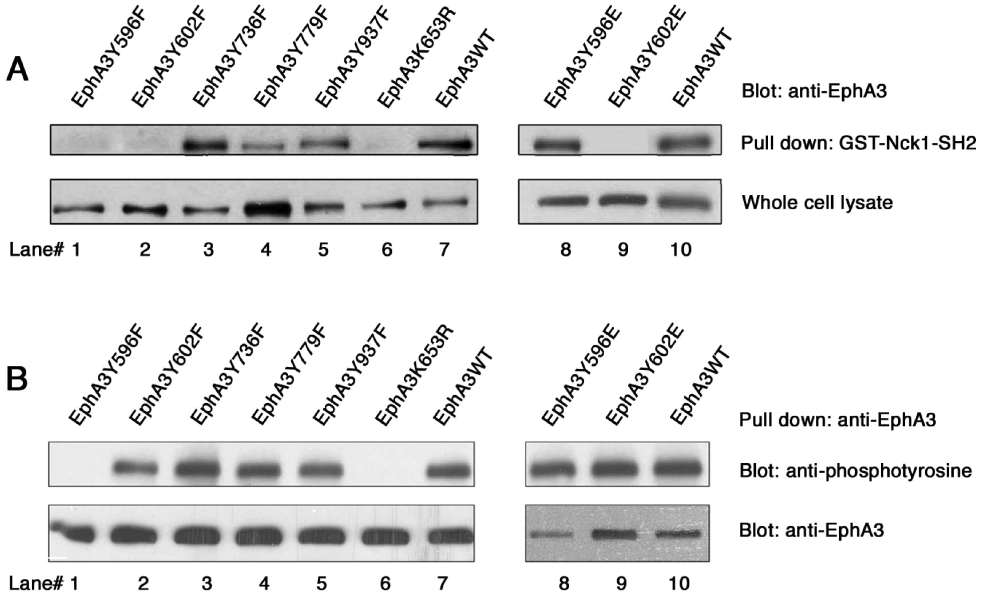

FIGURE 2: Identification of the Nck1 binding tyrosine residue of EphA3. (A) Y602 mutation inactivated Nck1 binding, as shown through a GST fusion protein pull-down assay. The GST−Nck1SH2 fusion protein was incubated with the lysate of HEK293A cells expressing wild-type or mutant EphA3 proteins. The transfected cells were stimulated with cross-linked ephrin-A5−Fc fusion protein for 30 min (1 μg/mL) before cell lysis. The precipitated protein complex was subjected to Western blot analysis with the anti-EphA3 antibody. (B) Autophosphorylation of EphA3 mutants used to map the binding site. Five tyrosine-to-phenylalanine mutants (Y596F, Y602F, Y736F, Y779F, and Y937F), two tyrosine-to-glutamic acid mutants (Y596E and Y602E), a kinase inactive mutant (K653R), and wild-type EphA3 (WT) were expressed individually in 293A cells. EphA3 proteins were immunoprecipitated from ephrin-A5-treated 293A cell lysates and analyzed with a Western blot technique.

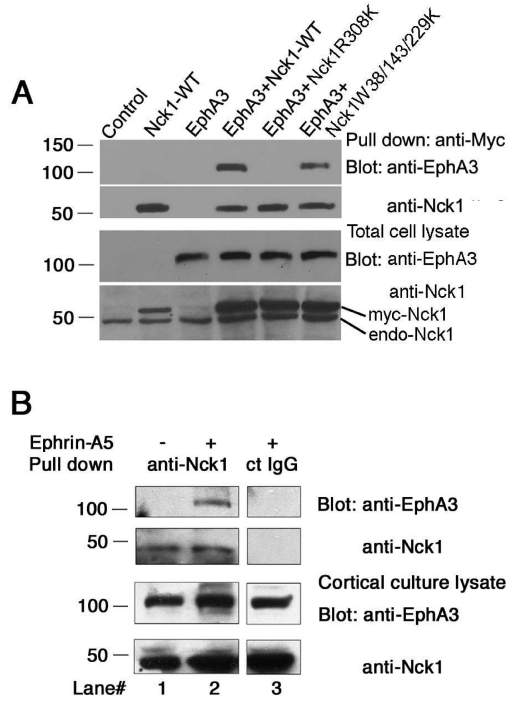

FIGURE 3: EphA3 interacts with Nck1 in both 293A cells and cultured primary neurons. (A) Co-immunoprecipitation of EphA3 and Nck1 in 293A cells. EphA3 and *myc*-tagged Nck1 were cotransfected into 293A cells, and the Nck1 protein complex was pulled down with a monoclonal anti-*myc* antibody. The immunoprecipitates were then analyzed with anti-EphA3 or anti-*myc* Western blots. Expression levels of endogenous (endo-Nck1) and transfected (myc-Nck1) Nck1 in transfected 293A cells are shown in the bottom two panels. (B) Co-immunoprecipitation of Nck1 and EphA3 from primary rat embryonic neuron extracts. E18 cortical neuron cultures were treated with cross-linked ephrin-A5, and the cell lysates were precipitated with either anti-Nck1 antibody or control IgG. Total expression levels of both proteins in the cortical culture lysates are also shown in the bottom two panels.

EphA3 and *myc*-tagged wild-type or mutant Nck1 was transiently transfected into HEK293A cells. The transfected cells were stimulated with 2 μg/mL cross-linked ephrin-A5 for 15 min and lysed with lysis buffer. Nck1 protein was then precipitated from the cell lysates with a rabbit polyclonal anti-*myc* antibody. The immunoprecipitated proteins were analyzed using a Western blot to determine whether EphA3 was coprecipitated. The analysis revealed that a significant amount of EphA3 receptor coprecipitated only with Nck1 containing the wild-type SH2 domain (Figure 3A). Mutations in SH2 domain of Nck1 (Nck1-R308K) abolished the interaction, while mutations in SH3 domains (Nck1-W38/143/229K) had no effect. This observation indicates that Nck1 binds to the activated EphA3 through its SH2 domain in living cells.

To determine whether EphA3 and Nck1 also interact in primary cells, we examined if Nck1 can be immunoprecipitated with EphA3 from the lysates of embryonic cortical neurons, since both proteins are strongly expressed in these cells (*37*, *38*). Indeed, EphA3 could be coprecipitated by an anti-Nck1 antibody (Figure 3B, lane 2) but not by a control mouse IgG (Figure 3B, lane 3). Moreover, it was noted that in the absence of ephrin-A5 stimulation, no coprecipitation occurred (Figure 3B, lane 1). Together, these observations provide further support for EphA3−Nck1 interaction.

Nck1 Mutants Inhibit Ephrin-A5-Induced Cellular Process Retraction. Activation of EphA receptors by ephrin-A5 in

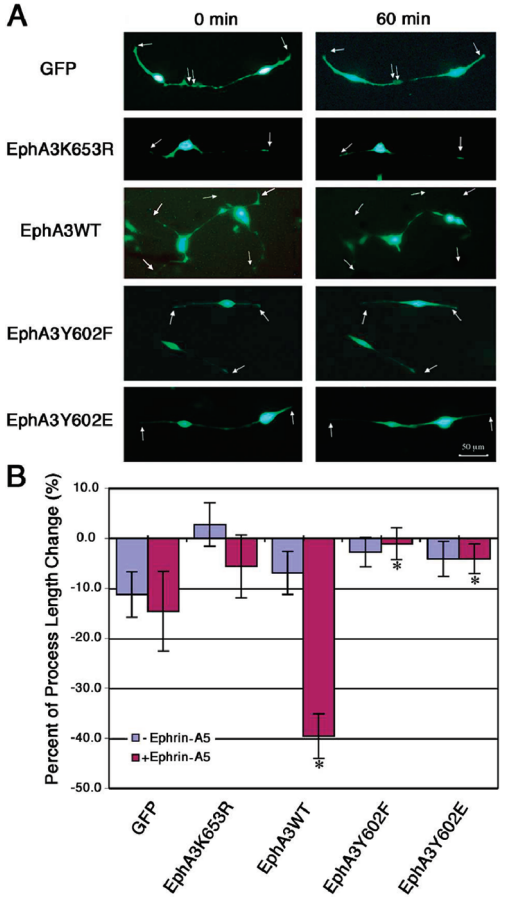

FIGURE 4: Ephrin-A5 induces cellular process retraction in EphA3-transfected cells. (A) Effects of EphA3 mutants on cellular process growth. HEK293A cells were transfected with EGFP or various EphA3 mutants and treated with cross-linked ephrin-A5. Select cells were photographed before and after the treatment and the length of all identifiable processes on all cells was measured. Arrows indicate the end of the processes before treatment. (B) Quantification of the effects of EphA3 mutants. Plotted are changes of process length after treatment, compared to the length before the treatment. A positive number indicates process extension during treatment, while a negative value shows retraction. Asterisks indicate statistical significance, when compared to the control with the same treatment (EphA3K653R; student *t* test, $p < 0.05$).

293 cells has been shown to lead to the retraction of cellular processes when the EphA3 receptor is expressed (*39*) (Figure 4A). This provides a convenient assay for examining whether Nck1 is required for EphA3 function. In this assay, HEK293A cells were plated sparsely on 35 mm tissue culture dishes and transfected with various EphA3 mutants as described in Materials and Methods. Forty-eight hours after transfection, cells were treated with 2 μg/mL cross-linked ephrin-A5 for 1 h. Parallel cultures were treated without ephrin-A5 as controls. Select cells with processes were photographed before and after being treated. The length of the processes before or after treatment was measured and expressed as a quantitative index of the extent of process retraction. Ephrin-A5 treatment of cells transfected with an empty vector expressing EGFP led to an only minimal length change of the cellular processes (Figure 4). Treatment of cells transfected with kinase-dead EphA3 also did not lead to any deleterious effects on the processes (Figure 4). However, when the cells were transfected with EphA3-WT, ephrin-A5 treatment led to a significant retraction of the processes. More importantly, when either EphA3-Y602F or EphA3-Y602E was expressed in

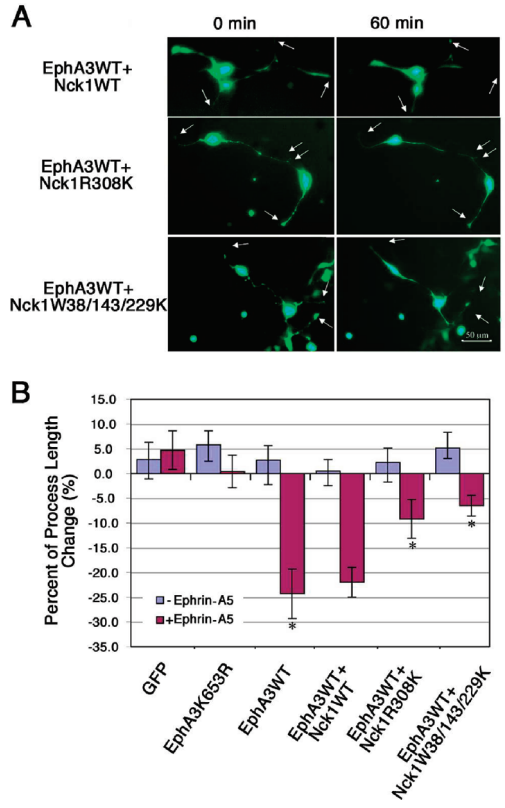

FIGURE 5: Nck1 mutants block EphA3-induced process retraction. (A) Various Nck1 mutants were cotransfected with EphA3-WT to examine their effects on process retraction induced by ephrin-A5 stimulation. Transfected cells were treated and quantified as described in the legend of Figure 4. Arrows show the ends of cellular processes before ligand addition. (B) Quantitative analysis of EphA3-induced process retraction, plotted in a fashion similar to that used for Figure 4B. Asterisks show statistical significance, when results were compared to their proper controls with the same treatment (EphA3-K653R for EphA3-WT, EphA3-WT+Nck1-WT for EphA3-WT+Nck1-R308K and EphA3-WT+Nck-1W38/143/229K; Student's t test, $p < 0.05$).

these cells, process retraction was also minimal, unlike cells expressing wild-type EphA3 (Figure 4). We conclude, therefore, that the kinase activity of EphA3 is required for ephrin-A5-induced retraction and that an intact tyrosine residue at position 602 is critical. These findings are consistent with the notion that Nck1 binding to EphA3 is important for EphA3 function.

To further confirm roles of Nck1 in EphA3-mediated cell retraction, the effects of two dominant-negative mutants, Nck1-R308K and Nck1-W38/143/229K, were analyzed (Figure 5A,B). Both of these mutants have been previously reported to inhibit functions of wild-type Nck1 (*40*). 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 (*41*). As shown in Figure 5A, cotransfection of Nck1-WT with EphA3-WT did not inhibit process retraction of EphA3-expressing cells following ephrin-A5 challenge. The process length was shortened by ~22.0% (±3.0%) as in cells expressing only EphA3-WT. In contrast, when either Nck1-R308K or Nck1-W38/143/229K was coexpressed with EphA3-WT, the process retraction induced by ephrin-A5 was significantly inhibited (Figure 5). In the presence of Nck1-R308K or Nck1-W38/143/229K, the decrease in the process length of cells expressing EphA3-WT was only 9.2 ± 3.9 or 6.5 ± 2.1%, respectively. These results indicate that both the interaction between EphA3 and the Nck1 SH2 domain and the interaction between the Nck1 SH3 domains and other downstream targets are required for EphA3 function.

Nck1 Mutants Downregulate Ephrin-A5-Mediated Inhibition of Cell Migration. To further examine roles of Nck1, we tested the effects of different Nck1 mutants on the regulation of cell migration by EphA3, using the Transwell assay. HEK293A cells were transfected, and 24 h later, the cells were plated on the Transwell inserts in serum-free medium. The lower compartment of the Transwell apparatus was filled with serum containing culture medium supplemented with or without cross-linked ephrin-A5. After being cultured overnight, cells that migrated through the pores onto the underside of the insert were counted. This analysis showed that expression of EphA3-WT caused a sharp decline in the level of cell migration compared to EGFP controls or EphA3KD (Figure 6A). Thus, EphA3 inhibits cell migration, and the kinase activity is indispensable to this regulation. In comparison, neither EphA3-Y602F nor EphA3-Y602E had any effects (Figure 6A), indicating that tyrosine 602 is required for EphA3 function.

To examine whether Nck1 is required for inhibition of cell migration by EphA3, we examined the effects of various Nck1 mutants. Expression of Nck1-WT, Nck1-R308K, or Nck1-W34/143/229K alone showed no significant effects on cell migration (Figure 6B). However, when Nck1-R308K or Nck1-W34/143/229K was coexpressed with EphA3-WT, each mutant significantly alleviated the inhibition of cell migration by ephrin-A5 (Figure 6B). In contrast, Nck1-WT had no effect. Collectively, these observations indicate that interactions through both SH2 and SH3 domains of Nck1 are necessary for regulation of cell migration by EphA3.

Nck1 Acts as a Key Downstream Factor in EphA3-Mediated Signaling. The function of Nck1 in EphA3 signaling was further investigated by using Nck-null mouse embryonic fibroblasts (MEF), which lack both Nck1 and Nck2 expression, in the Transwell cell migration assay. These MEF cells were transfected with wild-type Nck1, EphA3, or both. In the mock transfected cells, no expression of Nck1 or EphA3 proteins was detected (Figure 7A). Nck1 and EphA3 proteins were detected in cells transfected with the respective cDNAs (Figure 7A), showing efficient expression of the transfected genes. In the absence of ephrin-A5 stimulation, expressed EphA3 was partially autophosphorylated (Figure 7A), indicating that overexpression of EphA3 led to partial tyrosine phosphorylation in MEF cells. However, ephrin-A5 stimulation increased the level of EphA3 phosphorylation significantly (Figure 7A) and induced the strongest inhibition of cell migration when both wild-type EphA3 and Nck1 were present in the cell (Figure 7B).

When the Nck-null cells were transfected with EphA3 or Nck1 alone, ephrin-A5 induced only a slight inhibition of cell migration (Figure 7B). However, when both EphA3-WT and Nck1-WT were expressed, the migration of these cells was drastically inhibited by ephrin-A5. In contrast, cotransfection of EphA3 or Nck1 mutants that disrupt the interaction failed to induce strong inhibition by ephrin-A5. When wild-type EphA3 is cotransfected with a Nck1 mutant (Nck1-R308K or Nck1-W38/143/229K) or EphA3-Y602F with wild-type Nck1, ephrin-A5 failed to strongly inhibit migration of the transfected cells (Figure 7B). Taken together, these results further confirm that Nck1 is a critical downstream molecule in mediating the responses to ephrin-A5.

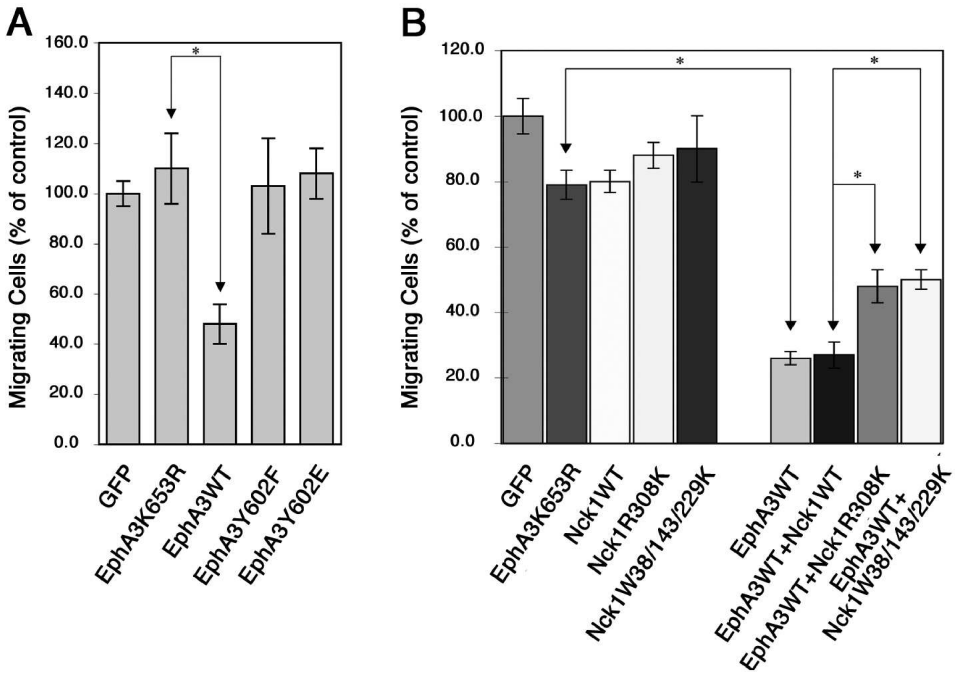

FIGURE 6: Roles of kinase activity, Y602, and Nck in EphA3-induced inhibition of cell migration. HEK293 cells were transfected with various constructs as indicated, and the transfected cells were then assayed for cell migration using Transwell tissue culture cells in the presence of 2 μg/mL cross-linked ephrin-A5−Fc fusion protein in the bottom chamber. (A) EphA3 kinase activity and Y602 are required for inhibition of cell migration by ephrin-A5. (B) Dominant-negative Nck1 mutants reduce the level of EphA3 inhibition on cell migration. Asterisks indicate statistical significance (Student's t test, $p < 0.01$), compared to their proper controls.

DISCUSSION

This study identified Nck1 as a strong binding partner of the EphA receptors and showed that the binding was mediated by interaction of the Nck1 SH2 domain with the phosphotyrosine residue at position 602 of EphA3. Inhibition of Nck1 function using dominant-negative mutants tempered inhibitory effects of EphA3 on cell migration and process extension, and loss of Nck function leads to a significant loss of inhibition of cell migration by ephrin-A5, suggesting that Nck1 is a key downstream mediator of EphA3 functions.

Nck1 Interacts with Activated EphA3. Using EphA3 and EphA5 intracellular domains as bait in a yeast two-hybrid screen, we found that Nck1 exhibited strong interaction with both receptors. An earlier study by Stein et al. showed that Nck1 interacted with the intracellular domain of EphB1 (*42*). It was also reported that EphB2 bound to p62 dok after activation and formed a protein complex that included both RasGAP and Nck1 (*24*). These data together support the notion that Nck1 functions downstream of both EphA and EphB receptor.

Nck1 has no known enzymatic activity and appears to serve as an adaptor protein to link tyrosine phosphorylation signals to downstream effectors. In addition to the SH2 domain, Nck1 also contains three SH3 domains, which mediate binding to multiple downstream molecules that regulate the actin cytoskeleton (*43*). Consistent with the observation in the yeast two-hybrid experiments, we found that Nck1 protein was coprecipitated with EphA3 from both transfected cells and primary neurons. The interaction was mediated by the Nck1 SH2 domain binding to the receptor. These findings indicate that the interaction between Nck1 and EphA3 is similar to its interaction with the PDGF receptor and Crk-associated substrate ($p130^{Cas}$) in PDGF-stimulated cells (*44*−*46*), but different from its binding to DCC and Robo, which takes place through the SH3 domains instead (*47*). Mutagenesis coupled with in vitro pull-down experiments conducted in this study allowed us to localize the key interaction site of EphA3 for Nck1. Indeed, EphA3-Y596F and EphA3-Y602F failed to bind to Nck1. However, the EphA3-Y596F mutant lacks kinase activity for autophosphorylation as well as phosphorylation of exogenous substrate (Figure 2A and data not shown). Replacement of Y596 with glutamic acid restored the kinase activity and interaction with Nck1, showing that Y596 is required for activation of the receptor but not for Nck1 binding. On the other hand, neither EphA3-Y602F nor EphA3-Y602E has any Nck1 binding activity, although both mutants have normal kinase activity as measured by autophosphorylation and phosphorylation of exogenous substrate (Figure 2A and G. Shi and R. Zhou, unpublished observations). Thus, tyrosine residue 602 of EphA3 is indispensable for interaction with Nck1. In addition, the Nck1 SH2 domain has been shown to bind strongly to the sequence of "pY-hydrophilic-hydrophilic-I/P" through an unbiased screen of a degenerate phosphopeptide library (*48*). The EphA3 amino acid sequence after tyrosine 602 (pYEDP) but not 596 (pYVDP) fits this description. Earlier studies showed that Nck1 interacted with EphB1, through the SH2 domain binding to Y594 (pYIDP, the equivalent of Y596 of EphA3) (*49*), indicating that there are variations in molecular interactions among different Eph receptors.

We also showed that activation of EphA3 by ephrin-A5 treatment enhanced the interaction in primary neurons. A small amount of EphA3 was precipitated with GST−Nck1SH2 fusion protein from protein lysates of transfected but unstimulated 293A cells (Figure 1D). This was probably due to the presence of activated EphA3 in transfected cells because of high levels of expression, which has been shown to activate the EphA3 receptor (*39*).

Nck1 Mediates Regulation of Process Retraction and Cell Migration by EphA3 Activation. In both the process retraction assay and the cell migration assay, we showed that

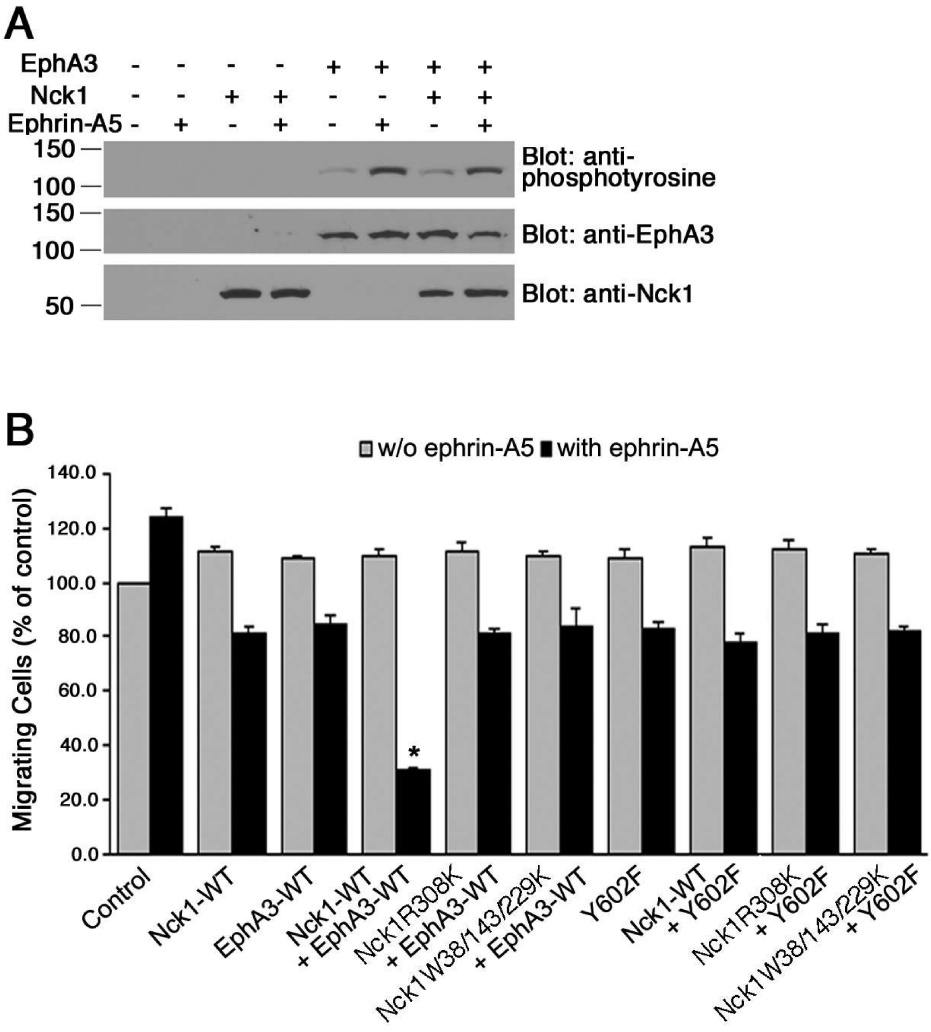

FIGURE 7: Nck1 acts as a downstream mediator in EphA3-induced inhibition of cell migration. (A) Expression of EphA3 and Nck1 in transfected Nck-null MEF cells. The MEF cells were transfected with the specified cDNAs, and the lysates were analyzed for EphA3 and Nck1 expression. In addition, EphA3 phosphorylation was analyzed using an anti-phosphotyrosine antibody. (B) The expression of wild-type Nck1 enables EphA3-mediated inhibition of cell migration. Asterisks indicate statistical significance (Student's t test, $p < 0.05$), when compared to their respective controls.

introduction of either of the dominant-negative Nck1 mutants, Nck1-R308K and Nck1-W38/143/229K, was able to inhibit effects of EphA3 activation, while the Nck1 wild type had no effects. Since these two dominant-negative genes were deficient in either SH2-mediated binding or SH3-mediated binding (*40*, *41*), these observations suggest that both the recruitment of Nck1 onto activated EphA3 through the SH2 domain binding and the interaction with downstream effectors through the SH3 domain are important steps in the transduction of EphA3 signals. A potential concern in using dominant-negative mutants in this study is that the effects observed were due to nonspecific inhibition of binding of other SH2 or SH3 domain-containing proteins by the mutants. However, if the effects of the mutants were nonspecific and Nck1 had no roles in EphA3 signaling, one would expect expression of wild-type Nck1 should also block EphA3 function. Our observation that wild-type Nck1 did not block EphA3 function indicates that the mutants specifically inhibit Nck1 function. Therefore, the fact that both mutants inhibited effects of EphA3 suggests Nck1 is an important signaling intermediate in the pathway of Eph receptor signaling. Analysis using Nck-null MEF cells further confirms this conclusion since ephrin-A5 has minimal effects on the migration of these cells.

In the cell migration assays, we found no increase in the level of inhibition by EphA3 when exogenous Nck1 is also expressed. Since HEK293 cells do express significant levels of endogenous Nck1, it is possible that this level was already sufficient in mediating EphA3 effects, which may explain why a further increase in levels did not lead to any enhancement. Introduction of wild-type Nck1, not the SH2 or SH3 mutants, into the Nck-null MEF cells restores the inhibitory effects of ephrin-A5 on cell migration. This observation indicates that interaction with Nck1 leads to the recruitment of a signal transduction cascade leading to the inhibition cell migration. Notably, in the absence of either Nck1 or EphA3, ephrin-A5 stimulation can still mildly inhibit cell migration. This is probably due to other Eph receptors present in MEF cells. In addition, studies in our group have shown that the complete inhibition effect requires the cooperation of Nck1 and at least one other effector (unpublished data). The disruption of either of these two effectors leads to only a partial deficiency in EphA3 function.

From Nck to Cytoskeletal Regulations. Nck1 has been shown to interact with the p21-activated kinase (PAK1) via its second SH3 domain (*50*, *51*) and translocate PAK1 from the cytosol to the plasma membrane. As a result, PAK1 is brought into the proximity of Rac1 and Cdc42 and becomes activated (*52*).

Active PAK1 phosphorylates substrates, including LIM (Lin-11, Isl-1, and Mec-3 domain) kinase, which in turn inactivates actin depolymerizing factor (ADF) family proteins such as cofilin, and myosin light chain kinase (MLCK), important regulators of actin/myosin organization (*53*, *54*). Inactivation of PAK1 leads to cofilin dephosphorylation and actin depolymerization. Indeed, PAK1 activity is reduced in ephrin-A1 treated cortical neurons (*55*), although it is not known if ADF/cofilin activity is increased. The existence of a Nck1−PAK1 pathway is also supported by genetic data from *Drosophila*: the guidance and targeting of photoreceptor growth cones requires activity of both Nck1 and PAK *Drosophila* homologues (*56*). It would be interesting in future experiments 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 consists of the WASP family proteins, including WASP and N-WASP, via interaction with its SH3 domains (*57*−*59*). WASP mutations were first discovered in an X chromosome-linked immunodeficiency disease (Wiskott-Aldrich syndrome) (*60*), which was characterized by cell abnormalities and actin cytoskeleton defects. The WASP family proteins possess a C-terminal motif that binds to Arp2/3 and activate actin reorganization (*59*). When the Nck1 SH3 domain was overexpressed in cells along with N-WASP and Arp2/3, an unusually high rate of actin nucleation was observed (*57*). Indeed, clustering of the Nck1 SH3 domain alone is sufficient to induce localized actin polymerization (*46*), suggesting that the SH3 domains mediate actin cytoskeleton regulation. N-WASP is highly enriched in the brain and regulates neurite growth (*59*). Thus, Nck1 may function through the WASP pathway to regulate cell morphology and migration following EphA receptor activation.

It has been reported that EphB stimulation led to the formation of a protein complex that included Nck1, NIK, Dok1, RasGAP, and the activation of the Nck1-interacting kinase (NIK), which in turn activated the c-Jun amino-terminal kinase (JNK) (*61*). Interestingly, when the *Drosophila* NIK homologue, Misshapen, was mutated, the photoreceptor axon targeting was disrupted (*62*), in a manner similar to that of the phenotype of Dock (Nck1 homologue in *Drosophila*) knockout (*63*). Nck1 has been shown to interact with more than 30 proteins, most of which have been implicated in actin cytoskeleton reorganization (*64*). Although the Nck1 downstream proteins that are responsible for the effects of ephrin-A5 are not known, our data strongly support a critical role of Nck in regulating cell migration and process outgrowth by Eph receptors, a notion reinforced by recent findings that Nck-null mice have phenotypes similar to that of EphA4-null mice (*65*).

ACKNOWLEDGMENT

We thank Dr. Tony Pawson for the permission to use the Nck-null cells in the study. We also thank A. Son for his constructive comments.

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