EphB1-mediated Cell Migration Requires the Phosphorylation of Paxillin at Tyr-31/Tyr-118*

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Cécile Vindis‡, Thalia Telí, Douglas P. Cerretti§, Christopher E. Turner¶, and Uyen Huynh-Do‡

From the §Division of Nephrology and Department of Clinical Research, University of Bern, CH-3010 Bern, Switzerland, and ¶Department of Cell and Developmental Biology, State University of New York Upstate Medical University, Syracuse, New York 13210

Interactions between Eph receptors and their membrane-bound ligands (ephrins) are of critical importance for key developmental processes such as boundary formation or vascular development. Their downstream signaling pathways are intricate and heterogeneous at several levels, the combined effect being a highly complex and flexible system. Here we demonstrate that activated EphB1 induces tyrosine phosphorylation of the focal adhesion protein paxillin at Tyr-31 and Tyr-118 and is recruited to paxillin-focal adhesion kinase (FAK) complexes. Pretreatment with the specific Src inhibitor PP2, or expression of dominant-negative, kinase-dead c-Src abrogates EphB1-induced tyrosine phosphorylation of paxillin. Cells transfected with the paxillin mutant Y31F/Y118F displayed a reduced migration in response to ephrin B2 stimulation. Furthermore, expression of an LD4 deletion mutant (paxillin ΔLD4) significantly reduces EphB1-paxillin association, paxillin tyrosine phosphorylation, as well as EphB1-dependent cell migration. Finally, mutation of the Nck-binding site of EphB1 (Y594F) interrupts the interaction between Nck, paxillin, and EphB1. These data suggest a model in which ligand-activated EphB1 forms a signaling complex with Nck, paxillin, and focal adhesion kinase and induces tyrosine phosphorylation of paxillin in a c-Src-dependent manner to promote cell migration.

The Eph family of receptor tyrosine kinases and their membrane-bound ligands, the ephrins, are key regulators of several developmental processes, including cell migration, boundary formation, axonal guidance, synaptogenesis, and angiogenesis. Both receptor- and ligand-initiated signals mediate repulsion, adhesion, and de-adhesion mechanisms involved in the motility of adherent cells. After activation by their cognate ligands, Eph receptors are phosphorylated at specific tyrosine residues in the cytoplasmic domain, thus displaying new docking sites for known signaling molecules. Indeed, a variety of Src homology domain (SH2/SH3)-containing proteins have been identified as Eph receptor-binding partners, including the adaptor proteins Nck, Grb10, Grb7, SHEP-1, SLAP, the low molecular weight phosphorylserine phosphatase LMW-PTP, the tyrosine kinases Crk, Fyn, and Src, the phosphatidylinositol-3-kinase, and the Ras GTPase-activating protein (reviewed in Ref. 1). Furthermore, PDZ-binding motifs located at the C terminus of the Eph receptors bind PDZ domain containing proteins such as AF6, Ryk (an RTK-interacting protein), Pick1, syntenin, and the two glutamate receptor-interacting proteins, Grip1 and Grip2 (2).

Importantly, many of the proteins identified in the Eph signaling pathways have been implicated in the regulation of cell morphology, adhesion, and motility. Activation of Eph receptors and ephrins has been shown to affect cell attachment by means of integrin and focal adhesion protein-dependent mechanisms, but contradictory results have been described for the interplay between these systems. In previous works, we showed that EphB1 receptor regulates integrin-dependent cell adhesion through activation of c-Jun kinase by means of Nck-interacting Ste20 kinase (NIK) in endothelial and neuronal cells (3, 4). Subsequently, Miao et al. (5) showed that stimulation of EphA2 receptor with soluble ephrin A1 ligand results in an inhibition of integrin function and cell adhesion. However Carter et al. (6) demonstrated that ephrin A1 induces cell adhesion and actin cytoskeletal changes in fibroblasts in a focal adhesion kinase (FAK)-dependent and p130Cas-dependent manner through activation of EphA2 receptor. Furthermore, activation of ephrinA5 in transfected NIH-3T3 cells enhanced cell adhesion and formation of focal adhesions (7). More recently, we demonstrated that ligand-activated EphB1 forms a signaling complex with c-Src kinase and p52^Six^ adapter protein to promote extracellular signal-regulated kinases (ERK)2/3 activation and chemotaxis (8).

Because cell migration is a highly coordinated process involving continuous re-organization of the cell cytoskeleton, we hypothesized that molecules involved in focal adhesion signaling represent potential targets for activated EphB1. In this study, we provide evidence that ligand-stimulated EphB1 associates with the focal adhesion proteins paxillin and FAK and induces their tyrosine phosphorylation. Furthermore, c-Src-mediated paxillin phosphorylation at Tyr-31 and Tyr-118 is required for EphB1-induced cell migration. In addition, we show that the paxillin LD4 motif and the Nck-binding site of EphB1 (Tyr-594) are necessary for EphB1-paxillin interaction. Expression of an LD4 deletion mutant (paxillin ΔLD4) decreases tyrosine phosphorylation of paxillin at Tyr-31 and Tyr-118, as well as cell migration in response to EphB1 stimulation. Based on these data, we propose a model in which the concerted actions of EphB1, Nck, and paxillin regulate cell migration in an Src-dependent manner.

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To whom correspondence should be addressed: Div. of Nephrology, Dept. of Clinical Research, University of Bern, CH-3010 Bern, Switzerland. Tel.: 41-31-632-3141 or -3144; Fax: 41-31-632-9734; E-mail: uyen.huynh-do@insel.ch.

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EXPERIMENTAL PROCEDURES

Reagents—Cell culture reagents and fibronectin were obtained from Invitrogen AG (Basel, Switzerland); ephrin B2/Fc was obtained from Amgen (Seattle, WA). Control human IgG1 and suramin were purchased from Sigma-Aldrich Chemie (Steinheim, Germany), and PP2 and PP3 were obtained from Calbiochem (La Jolla, CA). The following antibodies were used: rabbit anti-paxillin (Santa Cruz Biotechnology, Santa Cruz, CA), phosphospecific anti-p-Tyr-31-paxillin and anti-p-pTyr-118-paxillin (BIOSOURCE, Camarillo, CA), monoclonal anti-Nck and anti-FAK (Transduction Laboratories, Lexington, KY), goat anti-EphB1 (R & D Systems, Minneapolis, MN), monoclonal anti-hemagglutinin (HA) (12CA5; Roche Applied Science), polyclonal anti-active mitogen-activated protein kinase (MAPK; Promega, Madison, WI), and anti-ERK1/2 and anti- phosphotyrosine 4G10-HRP (Upstate Biotechnology, Lake Placid, NY). ECL chemiluminescence kit and ECL phosphorylation module anti-phosphotyrosine horseradish peroxidase (HRP)-conjugate were obtained from Amersham (Buckinghamshire, UK).

Cell Culture and Transfection—P19 cells were cultured in α-minimum Eagle's medium (MEM) with 10% fetal calf serum; primary human renal microvascular endothelial cells (HRMEC) were isolated and cultured as described (11). Chinese hamster ovary (CHO)-EphB1 cells expressing HA-tagged EphB1 receptor have been described previously (8) and were cultured in Dulbecco's modified Eagle's medium-F12 with 10% fetal calf serum containing Zeocin (Invitrogen AG). For transient transfections, CHO cells were seeded onto 100-mm plates and incubated in culture medium for 24 h before transfection. Cells were transfected using LipofectAMINE Plus with the plasmids encoding vector, wild-type paxillin, paxillin LD4 or LD2 deletion mutants (paxillin LD4 or paxillin LD2) (9), or paxillin Y31F(Y118F mutant) (10). The EphB1 plasmids and dominant-negative Src have been described previously (8). Cotransfection of the plasmid enhanced green fluorescent protein (pEGFP) was used at 1 μg/100-mm plate as a marker of transfection efficiency (about 70%). Cells were used for biochemical or functional assays 48 h later.

Western Blotting and Immunoprecipitation—Cells were serum-starved for 24 h (P19, HRMEC) or 48 h (CHO-EphB1) in Opti-MEM and then stimulated for the indicated times at 37 °C with 0.5–1 μg ml⁻¹ of ephrin B2/Fc as described previously (8). For assessment of ERK1/2 activation and paxillin phosphorylation, cells were lysed in radioimmunoprecipitation assay buffer and 40 μg of proteins were loaded on an SDS-10% PAGE. After transfer to Immobilon/polyvinylidene difluoride membranes (Millipore, Bedford, MA), the membranes were blocked 1% in bovine serum albumin. Phosphorylated ERK1/2 was detected with phosphospecific anti-phospho MAPK, whereas tyrosine-phosphorylated paxillin was detected with antibodies specifically recognizing phosphorylated Tyr-31 and Tyr-118 of paxillin. Membranes were stripped and reprobed with anti-ERK1/2 and anti-paxillin antibodies, respectively. For co-immunoprecipitations, cells were lysed in buffer containing 1% Triton X-100, 20 m M HEPES, pH 7.5, 150 m M NaCl, 10 m M NaF, 5 m M EDTA, 60 m M N-octyl-glucoside, 0.1% SDS supplemented with 1 m M orthovanadate and protease inhibitors. Immunoprecipitations were performed with the indicated antibodies for 2 h at 4 °C as described previously (11).

Cell Migration—For migration assays, a modified Boyden chamber system was used as described previously (8). Briefly, serum-starved cells were resuspended in Opti-MEM and seeded on fibronectin-coated Transwell inserts with 8-μm pore size membranes (Costar, Corning, NY). 1–2 μg ml⁻¹ clustered ephrin B2/Fc was added to the lower chamber, and then cells were allowed to migrate for 6 h at 37 °C. Non-migrating cells were removed with a cotton swab, and migrated cells were fixed with glutaraldehyde, stained with 0.5% crystal violet, and then eluted with acetic acid. Cell migration was quantified by a spectrophotometric reading at A500.

RESULTS

Ligand-activated EphB1 Induces Phosphorylation of Paxillin at Tyr-31 and Tyr-118—Cell movement requires a cytoskeletal re-organization involving phosphorylation of cytoskeleton-associated tyrosine kinases and the formation of focal adhesions. Because of the key role of EphB1 receptor in cell-cell and cell-matrix interactions, we anticipated that EphB1 activation would not only induce integrin “inside-out” activation (3), but that it would also affect focal adhesion complexes. In the present study, using two well established cellular systems expressing EphB1 (8, 12), we found that paxillin, a focal adhesion-associated docking protein, was tyrosine phosphorylated in response to EphB1 stimulation. Time-course analysis showed that this was an early event, beginning as soon as 5 min after exposure to ephrin B2/Fc and lasting for at least 60 min (Fig. 1A). Using phosphospecific antibodies, we furthermore demonstrated that EphB1 induces paxillin phosphorylation at Tyr-31 and Tyr-118 (Fig. 1B), two tyrosine residues which have been shown to play an important role in cell migration in various cancer cell lines (10, 13).

EphB1 Associates with Paxillin and Promotes Tyrosine Phosphorylation of FAK—Because paxillin can be phosphorylated either by c-Src directly or through the intermediary of FAK (reviewed in Ref. 14), we next assessed the phosphorylation status of FAK. Upon EphB1 activation, FAK also exhibited an increased tyrosine phosphorylation, although to a lesser degree than paxillin, beginning 15 min after exposure and lasting for 30–60 min (Fig. 2A). Moreover, ephrin/B2-stimulation of EphB1 led to the recruitment of paxillin to EphB1-signaling

FIG. 1. Activated EphB1 induces phosphorylation of paxillin at Tyr-31 and Tyr-118. Serum-starved P19 (left panels) or CHO-EphB1 (right panels) were stimulated with 0.5–1 μg ml⁻¹ ephrin B2/Fc for the indicated times (see “Experimental Procedures”). A, paxillin-immunoprecipitates were immunoblotted with anti-phosphotyrosine 4G10-HP or anti-paxillin. B, 40 μg of lysates were immunoblotted with anti-pTyr-31 or anti-p-pTyr-118 paxillin as indicated; stripped blots were reprobed with anti-paxillin. Blots are representative of at least three independent experiments.
complexes, concomitantly to the tyrosine phosphorylation of paxillin. We also found the protein FAK in paxillin immunocomplexes after EphB1 stimulation (Fig. 2).

EphB1-induced Paxillin Phosphorylation at Tyr-31/Tyr-118 Requires c-Src Activity and Is Necessary for Cell Migration—We showed previously (8) that ligand-activated EphB1 recruits c-Src and induces its active conformation by phosphorylation of Tyr-418. We also linked c-Src activation to two important biological endpoints: EphB1-dependent ERK activation and chemotaxis. Because Src family kinases are also implicated in regulating paxillin phosphorylation, we next investigated the potential role of the EphB1/c-Src signaling complex in promoting tyrosine phosphorylation of paxillin. Indeed, we observed that paxillin tyrosine phosphorylation clearly decreased when cells were pretreated with the Src tyrosine kinase inhibitor PP2 (Fig. 3A), but not with control PP3 (data not shown).
We confirmed these inhibition studies by performing dominant-negative experiments: transfection of CHO-EphB1 with dominant-negative, kinase-dead c-Src clearly reduced paxillin phosphorylation (Fig. 3B). Taken together, these data demonstrate that c-Src kinase activity is essential for the EphB1-induced tyrosine phosphorylation of paxillin.

Finally, to address the role of paxillin phosphorylation in EphB1-mediated cell migration, we overexpressed a Y31F/Y118F paxillin mutant in CHO-EphB1. We showed a 40% reduction in cell migratory behavior as compared with vector-transfected cells (Fig. 3C). Moreover, paxillin immunoprecipitates were immunoblotted with anti-HA; stripped blots were reprobed with anti-paxillin. Together these findings show that in CHO-EphB1 and P19 cells, paxillin tyrosine phosphorylation is required for proper cell migration.

**EphB1-paxillin Interaction Is Mediated by Nck and Requires the LD4 Motif of Paxillin**—Paxillin contains a number of motifs that mediate protein-protein interactions, including multiple SH2- and SH3-binding domains that mediate association with the Crk, Src, and Csk families, four LIM domains that target paxillin to focal adhesions, and five leucine-rich sequences with the consensus \( \text{LD}X\text{LLXXL} \) (termed LD motifs). These paxillin LD motifs mediate interactions with several structural and regulatory proteins that are important for coordinating changes in the actin cytoskeleton associated with cell motility and cell adhesion (reviewed in Refs. 14 and 15). Transfection of CHO-EphB1 with an LD4 deletion mutant (paxillin \( \Delta \text{LD4} \)) clearly reduced the level of paxillin phosphorylation at Tyr-31 and Tyr-118 (Fig. 4A). Moreover, paxillin \( \Delta \text{LD4} \) showed a reduced cell migration in response to ephrin B2 stimulation (Fig. 4B). Interestingly, overexpressing a paxillin LD2 deletion mutant (paxillin \( \Delta \text{LD2} \)) lacking an important FAK-binding site (9) did not inhibit EphB1-induced cell migration, nor did it disrupt the EphB1-paxillin association. This finding is in keeping with previous studies (9) and suggests that in the present cellular systems, FAK is not a key player in EphB1-directed cell migration, although it is phos-
phorylated by and associates with EphB1.

Analysis of the EphB1 sequence did not reveal any known paxillin-binding subdomain (16). However, we previously showed that EphB1 binds to the adaptor protein Nck through its Tyr-594 residue (11). Also, the LD4 motif of paxillin. On the other hand, c-Src is activated after binding to Tyr-600 of EphB1. Autophosphorylated c-Src (Tyr^418-cSrc) then phosphorlates Tyr-31 and Tyr-118 of paxillin, promoting EphB1-dependent cell migration.

**DISCUSSION**

In this study, we provide evidence that tyrosine phosphorylation of two major phosphorylation sites of the adaptor protein paxillin plays a central role in EphB1-mediated cell migration. We show that activated EphB1 induces the phosphorylation of Tyr-31 and Tyr-118 residues of paxillin in a c-Src-dependent manner.

Paxillin becomes phosphorylated on tyrosine in response to various receptor tyrosine kinases, including platelet-derived growth factor, epidermal growth factor, and nerve growth factor (14). Recently, Carter et al. (6) identified EphA2, FAK, and p130Cas as the most heavily tyrosine-phosphorylated proteins in NIH-3T3 cells plated on ephrin A1-coated surface. The authors also showed that paxillin too was inducibly phosphorylated in response to ephrin A1 stimulation. However, because paxillin was not the major ephrin A-dependent phosphorytosyl protein, they did not further characterize the phosphorylated tyrosine residues. Our findings suggest that, in contrast to EphA2 signaling, ligand-activated EphB1 preferentially targets paxillin at Tyr-31 and Tyr-118. Importantly, expression of a paxillin mutant lacking these two important phosphorylation sites resulted in a reduced migration in response to ephrin B2 stimulation. To date, the role of Tyr-31/Tyr-118 on cell motility has been quite controversial: Yano et al. (18) showed that in MM1 hepatoma cells, a paxillin mutant with substitutions of the four tyrosine phosphorylation sites of paxillin had no effect on cell invasive behavior. In contrast, Petit et al. (10) demonstrated that in NBT-II bladder carcinoma cells, expression of Tyr-31/Tyr-118 paxillin impaired cell motility on collagen by ~50%. More recently, Tsubouchi et al. (13) showed that paxillin Tyr-31/Tyr-118 were essential for efficient membrane spreading and ruffling during adhesion and migration of NmuMG cells. Differences in the cellular context or the experimental design may account for these disparate results.

In an effort to dissect the molecular mechanisms underlying the interaction between EphB1 and paxillin, we performed another series of dominant-negative experiments. We demonstrated that kinase active c-Src is necessary for the tyrosine phosphorylation of paxillin in response to EphB1 stimulation. We confirmed these results by performing pharmacological inhibition experiments with the Src-specific inhibitor PP2. These findings highlight a new aspect of the role of c-Src in EphB1 signaling. Indeed, in a recent publication (8), we showed that EphB1 associates with the adaptor protein p52Shc and activates the MAPK/ERK pathway in a c-Src-dependent manner. Furthermore, expression of dominant-negative, kinase-dead c-Src inhibited EphB1-dependent cell adhesion and migration.
The data presented here suggest that in addition to MAPK activation, c-Src also regulates EphB1-dependent chemotaxis through tyrosine phosphorylation of paxillin. Finally, we showed that, not only in CHO-EphB1, but also in P19 and primary human renal microvascular endothelial cells, ligand-activated EphB1 forms a complex with Nck and paxillin. This interaction was interrupted by the expression of either a paxillin ΔLD4 deletion mutant or of Y594F-EphB1 lacking the Nck-binding site. Taken together, these experiments strongly suggest that EphB1 indirectly associates with paxillin through the intermediary of the adaptor protein Nck and the LD4 motif of paxillin.

In conclusion, we have made the novel observation that EphB1 induces tyrosine phosphorylation of paxillin at Tyr-31 and Tyr-118 in a c-Src-dependent manner. Furthermore, ligand-activated EphB1 builds a complex with Nck, paxillin, and FAK in an interaction requiring Tyr-594 of EphB1 and the LD4 motif of paxillin. The concerted actions of EphB1, Nck, and paxillin, in turn, appear to play a significant role in EphB1-mediated cell migration (Fig. 5).

Because paxillin has been shown to bind in vitro and in vivo to the cytoplasmic part of integrins (14), our results suggest that paxillin itself may be an integrating element linking EphB1 to inside-out integrin activation. Furthermore, paxillin undergoes extensive tyrosine phosphorylation in the developing chick embryo (15), which raises the interesting possibility that it could play an important role in coordinating cell migration and tissue remodelling in the embryo in response to Eph-ephrin interactions. Elucidating the molecular mechanisms linking EphB1-mediated paxillin phosphorylation to integrin inside-out signaling will be the subject of future studies.

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