Inhibition of Integrin-mediated Cell Adhesion but Not Directional Cell Migration Requires Catalytic Activity of EphB3 Receptor Tyrosine Kinase

ROLE OF RHO FAMILY SMALL GTPases*

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Genetic studies have shown that Eph receptor tyrosine kinases have both kinase-dependent and kinase-independent functions through incompletely understood mechanisms. We report here that ephrin-B1 stimulation of endogenous EphB kinases in LS174T colorectal epithelial cells inhibited integrin-mediated adhesion and HGF/SF-induced directional cell migration. Using 293 cells stably transfected with wild type (WT)- or kinase-deficient (KD)-EphB3, we found that inhibition of integrin-mediated cell adhesion and induction of cell rounding was kinase-dependent. Unexpectedly, in two independent assays, both KD- and WT-EphB3 significantly inhibited directional cell migration. Upon ephrin-B1 stimulation, the activities of Rac1 and Cdc42 were reduced in both WT- and KD-EphB3-expressing cells that were induced to migrate. Pharmacological evidence demonstrates that a relative increase in RhoA signaling as a result of decreased Rac1/Cdc42 activities contributes to the inhibitory effects. Furthermore, EphB3-mediated inhibitory effect on cell adhesion but not migration was abolished by the integrin activating antibodies, suggesting that the inhibition of cell migration is not because of down-regulation of integrin function. These results uncover a differential requirement for EphB3 catalytic activity in the regulation of cell adhesion and migration, and suggest that while catalytic activity of EphB3 is required for inhibition of integrin-mediated cell adhesion, a distinct signaling pathway to Rho GTPases shared by WT- and KD-EphB3 receptor mediates inhibition of directional cell migration.

Cell migration is critical for many physiological and developmental processes including wound healing, immune response, and embryonic morphogenesis. Abnormal cell migration contributes to disease processes such as tumor invasion and metastasis. Microscopically, cell migration in vitro can be divided into four distinct steps: 1) cell polarization and membrane protrusion mediated by cytoskeleton reorganization; 2) integrin-mediated adhesion at the tip of the membrane protrusion; 3) contraction forces pulling cell content forward; and 4) de-adhesion at the trailing end involving integrin inactivation and membrane ripping (1, 2). The molecular mechanisms underlying the directional cell motility in vitro have come under extensive investigation, which has led to the identification of numerous cytoskeletal, signaling, and adaptor proteins involved in various steps of cell migration. Similar mechanisms are likely to be involved in vivo, where the migratory movements of cells and axonal growth cones are subject to more complex regulation by both attractive and repulsive cues (3). The combined effects of these cues determine the timing and direction of cell locomotion. By regulating membrane protrusions and actomyosin-mediated contraction, the Rho family of small GTPases are a key converging point for the mechanisms regulating cell migration (4, 5).

Eph receptor tyrosine kinases and their membrane-anchored ligands, called ephrins, have recently emerged as important regulators of cell motility (6–10). The 16 known vertebrate Eph receptors are divided into EphA and EphB subfamilies based on sequence similarities of the extracellular domains and the specificity of ligand binding (eph-nomenclature.med.harvard.edu/table_1.html) (11). In general, the EphA subfamily binds to glycosylphosphatidylinositol-anchored ligands (ephrin-A), whereas EphB receptors bind ligands containing transmembrane domains (ephrin-B). An exception is EphA4, which binds to both ephrin-A and ephrin-B ligands (12). More recently, ephrin-A5 has been shown to activate EphB2 in addition to EphA receptors (13). Because ligands for Eph kinases are also membrane-anchored, Eph/ephrin interactions take place upon contacts between Eph- and ephrin-presenting cells. Previous studies have shown that in addition to signaling by Eph kinases (forward signaling), ephrins on opposing cells can also transmit signals to the cell interior when they are engaged with Eph kinases (14–16). The signaling by ephrins has been referred to as reverse signaling. One well-characterized function of Eph receptors is to mediate cell contact-induced repulsive guidance of axons and neural crest cells during development, although cell adhesion and attraction mediated by Eph receptors and ephrins have also been described (9, 17).

In addition to the extensively studied nervous system, Eph receptors and ephrins are widely expressed in endothelial and epithelial cells. We found that activation of endogenous EphA2
in PC-3 prostate epithelial cells inhibits integrin-mediated cell adhesion and suppresses haptotactic migration of PC-3 cells toward fibronectin and laminin (18). Recently, Batlle et al. (19) demonstrated that EphB kinases and ephrin-B ligands are expressed in opposing gradient in the crypts of the intestine. Genetic studies using EphB2 and EphB3 knock-out mice revealed that the compartmentalized expression contributes to crypt morphogenesis and the positioning of epithelial cells along the crypt. In vitro, stimulation of endogenous EphB kinases in the LS174T colorectal epithelial cell line induces retraction of lamellipodia and filopodia followed by cell de-adhesion and rounding. Biochemical analysis revealed rapid tyrosine dephosphorylation of focal adhesion kinase and paxillin as well as Rac1 inactivation following EphB kinase activation (19). The cellular and biochemical changes were similar to those described in PC-3 cells following EphA2 activation (18).

Multiple previous studies in Caenorhabditis elegans and mouse model systems have revealed that Eph receptors possess both kinase-dependent and kinase-independent functions (20–25). Thus, kinase-independent activities of EphB2 mediate commissural and retinal axon-pathfinding (26, 27) as well as synaptic plasticity (23). Furthermore, EphA4 mediates the formation of major axon tracts in both kinase-dependent and kinase-independent manners (24). The mutations in C. elegans Eph receptor, VAB-1, cause embryonic arrest and swollen bursa of male tail at least in part through kinase-independent mechanisms (20, 21, 22). Whereas some of the kinase-independent functions can be attributed to the reverse signaling through ephrins, it has been suggested that kinase-deficient Eph receptors may also regulate cellular processes by receptor ligation-induced signaling. To test this possibility, here we transfected wild type (WT) and kinase-deficient (KD) EphB3 into 293 cells that express little endogenous EphB kinases. Ligation of WT-EphB3, but not KD-EphB3, inhibited integrin-mediated cell adhesion. In contrast, both KD- and WT-EphB3 can suppress directional cell motility, suggesting that the repulsive guidance of cell motility by EphB3 is kinase-independent. Moreover, Rac1 and Cdc42 activities were reduced upon ephrin-B1 stimulation of KD-EphB3 during wound-induced migration, suggesting that inhibition of Rac1 and Cdc42 mediate kinase-independent regulation of cell motility. Two of the known vertebrate Eph kinases, EphB6 and EphA10, contain kinase domains that are predicted to be catalytically inactive (11, 29). In addition, some of the naturally occurring splice variants of Eph kinases lack the kinase domain. Indeed analyses of human or addition, some of the naturally occurring splice variants of Eph domains that are predicted to be catalytically inactive (11, 29). In vertebrate Eph kinases, EphB6 and EphA10, contain kinase.

The mammalian expression vectors for WT- and KD-EphB3 with a single mutation in the kinase domain (K665R) have been described previously (32). They were transfected into 293 cells using SuperFect transfection reagent (Qiagen). Clonal G418-resistant cells were isolated and expanded. Expression of WT- or KD-EphB3 was analyzed by immunoblotting with anti-EphB3 antibody. GFP-Rac1 plasmid was kindly provided by Dr. Mark Philips, and transiently transfected into WT-, KD-EphB3-, or vector-expressing cells by using Lipofectamine Plus reagent (Invitrogen).

Cell Adhesion Assay—Standard cell adhesion assays were carried out as described previously (18). Briefly, 96-well plates were coated with laminin or fibronectin (FN) at a series of concentrations starting at 10 g/ml at 4 °C overnight. Nonspecific binding sites were blocked with 1% BSA/phosphate-buffered saline (PBS) containing 0.1% BSA. 105 cells were plated in adhesion medium containing 10 g/ml ephrin-B1-Fc or Fc as control and allowed to adhere at 37 °C for 30 min. In some experiments, ephrin-B1-Fc or Fc was further clustered with goat anti-human Fc at a 1:5 ratio on ice for 30 min before adding cell adhesion medium. Adherent cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. A500 was measured from the extracted dye using an enzyme-linked immunosorbent assay reader.

Morphologic and chemotactic cell migration assays using modified Boyden chambers were performed essentially as described (18, 33). To test the migration of LS174T cells, the underside of filter inserts (6.5 mm diameter, 8 mm porous membrane, Costar) was coated with 1 g/ml of laminin in 10% PBS, washed with PBS, and incubated overnight at 4 °C. 25 µl of cell adhesion medium containing 10 g/ml of ephrin-B1-Fc or Fc was added onto the top of the insert and allowed to migrate through the filter at 37 °C overnight. 1 g/ml of Ephrin-B1-Fc or Fc pre-clustered with goat anti-human Fc was added to the lower chamber containing 20 ng/ml of HGF. To test migration of transfected 293 cells, the underside of filter inserts was coated with 200 ng of FN in 10 g/ml of phosphate-buffered saline. Nonspecific binding sites were blocked with 1% BSA/phosphate-buffered saline. 105 cells in serum-free medium containing 10% FBS were plated in the top of the insert and allowed to migrate through the filter at 37 °C overnight. Ephrin-B1-Fc or Fc at 1 g/ml was added to the lower chamber. Cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Cells that went through the filter and stayed on the underside of inserts were counted.

Scratch Wound Assay—Cells were cultured in 12-well plates until confluence. The monolayer of cells was scratched using a fine pipette tip. Pictures were taken before and 20 h after the addition of 1 g/ml ephrin-B1-Fc or Fc using an inverted Leica DM-IRE2 microscope equipped with a Ruper CoolSnap cf digital camera. For biochemical analysis, 1.2 × 105 cells were plated on FN-coated 10 cm dishes and cultured overnight to reach fresh confluence. Cells were induced to migrate by repeated scratch wounding using a razor blade as described previously (34). Four hours after scratch wounding, cells were stimulated with ephrin-B1-Fc or Fc for 15 min and subjected to Rho GTPase pull-down assays. To visualize the cellular localization of Rac1 during cell migration, cells transiently transfected with a GFP-Rac1 expression plasmid were wounded and stimulated on coverslips.

Time-lapse Imaging—Cells were plated on 6-well dishes. After overnight culture in a cell culture incubator at 37 °C with 5% CO2, they were placed in temperature/CO2 control chambers attached to a Leica DM-IRE2 inverted microscope. After 2 h acclimation, time-lapse images were collected for 1 h with 20-s intervals using a Ruper CoolSnap cf camera operated by the Multidimensional Acquisition Package of MetaMorph 6.1r4 software. Ephrin-B1-Fc or Fc control were added to 1 g/ml, and recording was continued for another hour.

Immunoprecipitation and Immunoblotting—Cells were lysed in lysis buffer containing 25 mM Tris (pH 7.4), 50 mM NaCl, 25 mM NaF, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 2 µg/ml leupeptin, and 2 µg/ml aprotinin for 30 min at 4 °C. Lysates were clarified at 13,000 × g for 5 min. Immunoprecipitation was carried out using antibodies at 1–2 µg/ml of total protein at 4 °C for 1 h. Immunoprecipitates were collected using鸟 bind beads (Amerham Biosciences) for 1 h at 4 °C. The beads were then washed with immunoprecipitation washing buffer containing 20 mM Tris-HCl (pH 7.4), 10% glycerol, 50 mM NaCl, 0.2% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM Na3VO4. The samples were boiled in reducing SDS sample buffer and separated on 4–20% Tris glycine gradient gel. Proteins were transferred to Immobilon-P filter membranes (Millipore) and probed with the indicated antibodies.

*The abbreviations used are: WT, wild type; HGF/SF, hepatocyte growth factor/scatter factor; FN, fibronectin; ROCK, Rho-associated kinase; KD, kinase-deficient; GST, glutathione S-transferase.
Kinase-independent Regulation of Cell Migration by EphB3

**RESULTS**

**Activation of EphB Kinases on LS174T Colorectal Epithelial Cells Inhibits Integrin-mediated Cell Adhesion and Suppresses HGF/SF-induced Cell Migration**—Recent in vivo studies using EphB3 knock-out mice have established the critical role of EphB kinase in restricting Paneth cells to the bottom of the intestinal crypt, suggesting it may negatively regulate cell motility (19). In vitro, ephrin-B1-Fc stimulation of endogenous EphB kinases in LS174T cells induced cell rounding. However, it remains to be determined how EphB kinase activation in these cells may impact directional cell migration and regulate integrin-mediated cell adhesion. Whereas LS174T cells exhibit little basal cell migration toward laminin, they could be induced to migrate by presenting HGF/SF in the lower chamber in the modified Boyden chamber cell migration system. We found that ephrin-B1-Fc potently suppressed HGF/SF-induced migration of LS174T cells (Fig. 1A). Moreover, in a conventional cell adhesion assay, integrin-mediated cell adhesion to laminin was attenuated by ephrin-B1-Fc but not Fc control (Fig. 1B). Consistent with induction of rounding of the already spread cells reported by Battle et al. (19), LS174T cells also failed to initiate spreading on laminin in the presence of ephrin-B1-Fc even when laminin was coated at high enough concentrations to support cell adhesion (not shown).

**The Kinase Activity of EphB3 Is Required for Inhibition of Integrin-mediated Cell Adhesion**—To investigate the role of EphB3 catalytic activity in the regulation of integrin-mediated cell adhesion and migration, we turned to 293 cells stably transfected with WT- and KD-EphB3 receptor as a model system. Unlike LS174T cells that express several members of EphB kinase including high levels of EphB2, EphB3, and low levels of EphB1, 293 cells have undetectable or very low level expression of these kinases (not shown), allowing direct comparison between exogenous WT- and KD-EphB3. Immunoblot of total cell lysates showed that both WT-EphB3 and KD-EphB3 were stably expressed in 293 cells (Fig. 2A), and clones expressing similar levels of EphB3 were selected for further analyses. Ephrin-B1 stimulation resulted in increased activation of WT-EphB3, as shown by the elevated tyrosine phosphorylation of the precipitated receptor (Fig. 2B). A basal level of EphB3 tyrosine phosphorylation was detected as have been observed with cells transfected with other EphB receptors (37, 38). The juxtamembrane regions of most Eph kinases contain two highly conserved tyrosine residues that become phosphorylated upon ligand stimulation, and play a key role in Eph kinase signaling (38). Using antibodies against phosphorylated peptide in this region, we found that these sites were preferentially phosphorylated upon ligand stimulation compared with global changes in tyrosine phosphorylation (e.g. compare lanes 3 and 4 in Fig. 2B with lanes 6 and 7 in Fig. 2C).

As expected, little tyrosine phosphorylation was observed for KD-EphB3 with either anti-phosphotyrosine or anti-phosphospecific antibodies (Fig. 2, B and C).

Immunofluorescence analysis revealed equal cell surface presentation of WT- and KD-EphB3 (Fig. 2D). To further confirm that the WT- and KD-EphB3 were functional, we performed cell adhesion to immobilized ligand. Both WT- and KD-EphB3-transfected cells but not vector control, could mediate adhesion to immobilized ephrin-B1-Fc (Fig. 2E), again suggesting they were properly presented on the cell surface and were capable of binding to ligand with similar affinity. All three cell types adhered to poly-L-lysine and FN equally well (Fig. 2E). The results also demonstrate that the EphB3-ephrin-B1 interaction is sufficient to cause mechanical tethering of cells. This observation prompted us to use soluble rather than immobilized ephrin-B1 to study how EphB3 activation modulates integrin-mediated cell adhesion to extracellular matrix proteins.

A conventional cell adhesion assay was carried out to investigate the role of EphB3 kinase activity in regulating integrin-dependent cell adhesion. Similar to endogenous EphB kinases...
in LS174T cells (Fig. 1), the exogenous WT-EphB3 also inhibited cell adhesion to fibronectin upon stimulation with ephrin-B1-Fc but not Fc control (Fig. 3A). The effect required EphB3 catalytic activity, as adhesion of cells transfected with KD-EphB3 was not affected. No significant change was observed in the adhesion of vector-transfected control cells. Clustering of ephrin-B1-Fc with anti-Fc antibodies is known to enhance the biological effects of EphB receptors, presumably by increasing aggregation of the receptors by the multimeric ligands (39, 40).

Clustering of ephrin-B1-Fc with anti-Fc antibodies caused an enhanced inhibition of cell adhesion to FN in WT-EphB3-transfected cells. Clustered ephrin-B1-Fc again did not affect inte-
grin-mediated adhesion in KD-EphB3-expressing cells (Fig. 3A). We conclude that inhibition of integrin-mediated cell adhesion requires the catalytic activity of EphB3.

The effect of WT-EphB3 receptor on cell adhesion was not because of changes in integrin expression, because there was no significant difference in integrin expression profiles in WT-EphB3-transfected cells compared with vector control cells (not shown). We next investigated whether inhibition of integrin-mediated cell adhesion by ephrin-B1-Fc is because of a conformational change of integrins. It is well known that integrins exist in a dynamic conformational equilibrium between high and low affinity states for ligand binding (41, 42); interference with this equilibrium may change cell attachment to integrin ligands. To test this possibility, the β1-integrin-activating antibody 8A2 (43), which can lock the integrin in an active state, was used together with ephrin-B1 to treat cells. As expected, the 8A2 antibody itself promoted cell adhesion (Fig. 3B). Addition of 8A2 antibody completely reversed the inhibitory effects of ephrin-B1-Fc on the adhesion of WT-EphB3 expressing cells to FN (Fig. 3B), whereas a control antibody did not have any effect. These results indicate that EphB3 activation converted integrins to an inactive conformation, thereby decreasing cell binding to FN. Taken together, these data suggest that EphB3 negatively regulates integrin-mediated cell adhesion, and that EphB3 kinase activity is required for this effect.

The EphB3-Ephrin-B1 Interaction Inhibits Cell Migration in a Kinase-independent Manner—We next investigated the effects of EphB3 activation by ligand stimulation on cell motility. A modified Boyden chamber assay was performed to evaluate haptotactic cell migration toward immobilized FN. Fig. 4A shows that migration of WT-EphB3-expressing cells was significantly inhibited by ephrin-B1-Fc compared with Fc. Surprisingly, migration of cells expressing KD-EphB3 was similarly reduced in six independent clones from two different transfections (Fig. 4A and data not shown). In contrast, no change in migration was observed in vector-transfected cells (Fig. 4A). Therefore, the catalytic activity of EphB3 is necessary for suppression of integrin-mediated cell adhesion, but dispensable for inhibition of cell motility.

This kinase-independent function of EphB3 was also examined in a scratch wound-induced cell migration assay. Cells expressing WT-EphB3, KD-EphB3, or vector control were grown to confluence and the monolayer of cells was then scratched with a pipette tip. Without any treatment, all cell lines were able to close the wound within about 24 h. However, in the presence of ephrin-B1-Fc but not Fc, the wound closing process was drastically retarded in WT-EphB3-expressing cells. Ephrin-B1-Fc also inhibited the wound closure of KD-EphB3-expressing cells. In contrast, ephrin-B1-Fc stimulation did not inhibit the wound closing process in vector control cells (Fig. 4B). Together these data demonstrate that the EphB3-ephrin-B1 interaction negatively regulates directional cell migration in a kinase-independent manner.

Induction of Cell Rounding Requires Catalytic Activity of EphB3, whereas Inhibition of Membrane Protrusions and Loss of Cell Polarity Are Kinase-independent—Next we recorded the
Fig. 4. Ephrin-B1 inhibits cell migration in EphB3 kinase activity-independent manner. A, Boyden chamber cell migration assay. The lower side of Transwell inserts was coated with 100 ng of FN. About 1 × 10^5 cells transfected with wild type or kinase-deficient EphB3 were plated in the top chamber and allowed to migrate toward the lower chamber filled with medium containing 1 μg/ml ephrin-B1-Fc or Fc overnight. Cells were fixed and stained with crystal violet. Numbers of cells migrating to the undersides of the inserts from six randomly selected high-power fields were counted under an inverted microscope. Values represent mean ± S.D. B, ephrin-B1 impedes directional cell migration in wound scratching assay in both WT- and KD-EphB3-expressing cells. Cells were cultured in 12-well clustered plates until confluence. The monolayer was scratched with a fine pipette tip to create a wound. Pictures from the same fields were taken before and 20 h after adding ephrin-B1-Fc or Fc using Leica microscopy system.

Ephrin-B1-induced morphological changes of the cells using time-lapse imaging. Cells expressing WT-EphB3, KD-EphB3, or vector control were plated on FN-coated dishes and cultured overnight to allow complete spreading. A time-lapse movie revealed that in the absence of ephrin-B1, all three cell types exhibited dynamic cell movement, but maintained spread morphology (Fig. 5A). Upon stimulation with ephrin-B1, WT-EphB3-expressing cells started to round up as early as 5 min. By 20 min, nearly half of the cells became rounded and some were detached from the substratum. This was preceded by the retraction of membrane protrusions in the forms of both lamellipodia and filopodia. Forty-five min after stimulation cells started to re-adhere and re-spread (Fig. 5A, middle row). Cell rounding and detachment was consistent with the inhibition of integrin function upon ligand stimulation of WT-EphB3 receptors (Figs. 1 and 3). Unlike WT-EphB3 cells, ephrin-B1-stimulated KD-EphB3 cells remained spread and attached to the substratum throughout treatment. However, careful inspections revealed that many KD-EphB3 cells exhibited a reduced dynamic extension of lamellipodia and filopodia, culminating in retraction of prominent membrane protrusions and the loss of polarized morphology. This was accompanied by aggregation of cells into clusters (Fig. 5A, bottom row). Fig. 5B shows a quantitative analysis of the morphological changes, both for cell rounding observed in WT-EphB3 cells and loss of membrane extensions in both WT- and KD-EphB3 cells. Vector-expressing cells did not show any significant morphology change in response to ephrin-B1. We conclude that induction of cell rounding requires the catalytic activity of the EphB3 receptor, whereas inhibition of membrane protrusion and loss of cell polarity are independent of the kinase activity.

Rho GTPases Mediate EphB3 Kinase-dependent and Kinase-independent Regulation of Cell Migration—The actin cytoskeleton provides the driving force for cell migration. The Rho family GTPases regulate actin dynamics and play key roles in coordinating the cellular response required for cell migration, including extension of lamellipodia and filopodia (1, 44). The kinase-independent morphological changes including retraction of membrane processes observed above (Fig. 5) led us to test whether Rho GTPases are involved in EphB3 kinase-dependent and -independent regulation of cell migration. To measure Rho GTPase activity in actively migrating cells, we adopted a previously described assay in which quiescent monolayers of cells on culture plates are induced to migrate by repeated scratch wounding with a multichannel pipette (34). Four hours following such scratch wounding, most WT, KD, and vector control cells on the edges of the wounds were polarized and actively migrating (not shown). Ephrin-B1-Fc or Fc was added and the cells were lysed 15 min later. Active Cdc42 and Rac1 were pulled down by using GST-PAK-CD and active RhoA was pulled down by using GST-Rhotekin RBD (35). Strikingly, a dramatic reduction in Cdc42 and Rac1 activities was observed in the KD-EphB3-expressing cells (Fig. 6A). A significant reduction in Rac1 activity, and a trend of reduction in Cdc42 activity was also observed in WT-EphB3 cells, but not vector control cells. A quantitative analysis was given in Fig. 6B. No significant changes in RhoA were detected in any of the transfected cells (Fig. 6). In contrast to the cells induced to undergo directional motility by repeated scratch wounding, little change in Rac1 and Cdc42 activities upon ephrin-B1 stimulation was seen in cells not induced to migrate (not shown).

Localization of Rac1 and Cdc42 to the leading edge of migrating cells is known to play a critical role in mediating cell polarization and directional migration (45). To assess the impact of ligand stimulation of EphB3 receptor on Rac1 localization in actively migrating cells, GFP-tagged Rac1 was transfected into vector, WT- and KD-EphB3 cells, plated on FN and scratch wounded to induce cell migration. Four hours later, when cells at the leading edge were actively migrating into the wound, cells were stimulated with ephrin-B1-Fc for 15 min and fixed to visualize the location of GFP-Rac1. Fig. 7 shows that in Fc control-stimulated cells, GFP-Rac1 was accumulated at the leading edge of migrating cells in all three cell types. In contrast, ephrin-B1-Fc stimulation led to a reduced localization of
GFP-Rac1 at the leading edge in both WT- and KD-EphB3 expressing cells, whereas no such effects were observed in vector cells. Thus ligation of WT- and KD-EphB3 cells resulted in reduced activity and redistribution of Rac1. The decrease in Rac1 and Cdc42 activity and constant RhoA activity is expected to lead to an altered balance between these GTPases in favor of RhoA, and a relative increase in RhoA activity has been previously linked to inhibition of cell motility (46–49). To test whether a shift in the balance of activated RhoA versus Rac1 and Cdc42 mediates the inhibitory effects of EphB3 on cell migration, cells were treated with Y27632, an inhibitor of the Rho effector ROCK. Fig. 8 shows that Y27632 attenuated the negative effects of ephrin-B1 on the migration of both WT-EphB3 and KD-EphB3 expressing cells (from 79 to 27% and from 66 to 29% inhibition of migration, respectively) (Fig. 8). In contrast, the ROCK inhibitor did not affect migration of vector control cells. This result suggests that the predominance of Rho signaling resulting from inhibition of Rac1 and Cdc42 was a major contributing factor to the inhibition of cell migration by EphB3.

In WT-EphB3 cells, ephrin-B1 stimulation led to a transient inhibition of integrin-mediated cell adhesion and decreased Rac1 activity, both of which could contribute to the suppression of cell motility. Whereas results in the last two sections (Figs. 6–8) established a major role of Rac1 inactivation in inhibiting cell migration, it remains possible that inhibition of integrin function could also contribute to the effects, at least in WT-EphB3 cells. To test this possibility, we performed Boyden chamber cell migration assays in the presence of β1-integrin activating antibody (8A2) in the lower chamber. Fig. 9 shows...
that locking integrin in an active state with 8A2 antibody enhanced the basal migration. However, it did not affect either WT- or KD-EphB3-mediated inhibition of cell migration (Fig. 9), suggesting that transient inhibition of integrin-mediated cell adhesion did not play a major role in the prolonged suppression of cell migration. Instead, ligation of WT- or KD-EphB3 with its cognitive ephrin-B1 ligand inhibits cell motility by differentially regulating Rho GTPases.

**DISCUSSION**

Here we show that ligand stimulation of EphB kinases on LS174T colorectal epithelial cells inhibits integrin-mediated cell adhesion and HGF/SF-stimulated directional cell migration. By using a kinase-deficient EphB3 mutant receptor, we surprisingly found that the inhibition of adhesion but not of migration requires EphB3 kinase activity. Cellular and biochemical analyses demonstrate that inhibition of Rac1 and Cdc42 plays a major role in kinase-independent regulation of cell motility by EphB3 receptor.

Genetic studies in vivo have shown that Eph receptors possess both kinase-dependent and kinase-independent functions (20–27). In addition, some Eph receptors, including EphA6, EphA7, and EphB1, have kinase-deficient variant forms that originate by alternative splicing (50). Two other Eph receptors, EphB6 (29) and the recently discovered EphA10 (11), have kinase domains that apparently lack catalytic activity and, therefore, these receptors may only have kinase-independent functions. However, neither the biological function that can be regulated by Eph kinases in a kinase-independent manner nor the underlying molecular mechanisms are completely understood.

There are at least three possible explanations for the kinase-independent function of Eph receptors. Because Eph-ephrin
interactions initiate bidirectional signaling, one explanation for the kinase-independent functions of Eph receptors is that the receptor extracellular domains stimulate reverse signaling through the ephrins. Examples in support of this notion include the fact that the EphB2 ectodomain alone in the absence of the entire cytoplasmic domain is able to guide commissural axon projection and retinal ganglion cell axon pathfinding, most likely through ephrin-B reverse signaling (26, 27). Alternatively, the interaction between Eph receptors and ephrins can mediate mechanical tethering or adhesion between cells. A good example is the cell-cell adhesion mediated by the interaction between ephrin-A5 and kinase-truncated EphA7 receptor at the midline of dorsal neural fold (51). Finally, kinase-deficient receptors may still be able to activate downstream pathways upon ligand stimulation. Signaling by EphB6 that is naturally kinase-deficient belongs to this category (29). Ligation of endogenous EphB6 by ephrin-B1 or -B2 can trigger signaling leading to the activation of p98 mitogen-activated protein kinase in primary T cells (52) and dephosphorylation of Cbl in Jurkat cells (53). The point mutations in Vab-1 (C. elegans Eph receptor) that inactivate the catalytic activity have also been suggested to have some signaling capacity (20–22). Our results show that EphB3 receptor forward signaling can take place in both a kinase-dependent and kinase-independent manner. Whereas catalytic activity of EphB3 is required for inhibition of integrin-mediated cell adhesion, a signaling pathway shared by WT- and KD-EphB3 receptor mediates inhibition of the activity and membrane localization of Rac1, which contribute to suppression of cell migration.

The Rho family GTPases regulate actin dynamics and play key roles in coordinating the cellular responses required for cell migration (44). Rac1 and Cdc42 stimulate the formation of lamellipodia and filopodia at the leading edge of migrating cells, respectively, whereas RhoA stimulates the formation of focal adhesion and stress fibers. It is believed that the balance between Rac1/Cdc42 and RhoA is a key determinant in the regulation of cell motility, i.e. the dominance of Rac1/Cdc42 signaling promotes cell migration, whereas enhanced RhoA activity inhibits it (46–49). Here we show that binding of ephrin-B1 to EphB3 either in the wild type or kinase-deficient form results in marked cell morphological changes characterized by reduced dynamic extension of filopodia and lamellipodia, culminating in retraction of prominent cell protrusions and loss of cell polarization. Biochemical analyses revealed a decrease in Rac1 and Cdc42 activities in cells induced to migrate. Although we did not observe an absolute increase in RhoA activity by ephrin-B1 stimulation on migrating cells, ephrin-B1-induced inhibition of cell migration could be significantly attenuated by an inhibitor of ROCK. Together these results suggest that an increased ratio between RhoA and Cdc42/Rac1 activities, resulting from the decreased Rac1 and Cdc42 activity, indirectly enhances the effects of RhoA signaling leading to inhibition of cell migration.

Similar to what we have previously observed with EphA2 in PC-3 cells (18), stimulation of WT-EphB3 receptor induced rapid cell rounding and detachment from the substratum. Such cell rounding and de-adhesion upon activation of wild type Eph kinases have also been reported in a number of other experimental systems, including transfected EphB2 in 293 cells (37, 54), endogenous EphA2 in MDA-MB-231 breast cancer cells (55), transfected EphA3 in 293 cells (56) as well as endogenous EphB kinases in LS174T colorectal epithelial cells (19). Cell detachment from the substratum can result from reduced integrin-mediated cell adhesion. Indeed previous studies have documented a down-regulation of integrin affinity by Eph receptors (18, 37). Similarly, activation of
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EphB3 on Jurkat T cells reduced integrin-mediated cell adhesion to fibronectin (57). Here we found that ephrin-B1 stimulation of endogenous EphB kinases in LS174T cells and exogenous WT-EphB3 in 293 cells inhibits integrin-dependent adhesion. Moreover, an integrin-activating monoclonal antibody that locks β1-integrins in an active conformation could overcome the effects of ephrin-B1. Inactivation of the catalytic activity of EphB3 abolished the inhibitory effects, suggesting that the down-regulation of integrin affinity requires the kinase activity of EphB3. Rho GTPases has recently been identified as key mediators for integrin functions such as cell adhesion (28, 58), which raises the possibility that the inhibitory effects mediated by EphB3 activation on cell migration may be secondary to that on cell adhesion. However, the integrin activating antibody that can completely reverse the inhibitory effect on cell adhesion does not have any effects on inhibition of cell migration mediated by EphB3. These results indicate that the inhibition of cell migration is not because of down-regulation of integrin affinity or inhibition of cell adhesion.

EphB3 and ephrin-B1 have recently been shown to be expressed in complementary compartments in the neonatal intestinal epithelium where they establish a boundary between proliferative and differentiated cells (19). Furthermore, EphB3 knock-out mice display a highly penetrant phenotype characterized by Paneth cell mislocalization throughout the intestinal crypts instead of being restricted at the bottom of the crypt in wild type animals (19). Our results suggest that mutations of other Eph receptors, the recent physiological conditions. Given the existence of native kinase-deficient EphB6 and EphA10 as well as the prevalence of kinase-deficient splicing variants of other Eph receptors, the results presented here are likely to have important physiological implications.

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