THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 2, Issue of January 14, pp. 923–932, 2005
Printed in U.S.A.

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*

Received for publication, October 5, 2004, and in revised form, November 4, 2004
Published, JBC Papers in Press, November 9, 2004, DOI 10.1074/jbc.M411383200

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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

* This work was supported by National Institutes of Health Grants CA96533 and CA92259, United States Department of Army Grant DAMD17-99-1-9019, and an award from CaP CURE (to B. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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)[1] 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 mouse kinomes revealed that about 10% of mammalian "kinases" are catalytically inactive because of the lack of one of three conserved catalytic residues (11, 30). The novel signaling mechanisms by the kinase-dead EphB3 receptor characterized here may have important physiological implications.

EXPERIMENTAL PROCEDURES

Reagents—Ephrin-B1-Fc was produced as described (18). The Fc fragment of human IgG and goat anti-human Fc were purchased from Jackson ImmunoResearch. Mouse monoclonal anti-phosphotyrosine PY99, rabbit polyclonal anti-RhoA, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody against EphB3 was raised against the last 100 amino acids fused to GST. Rabbit polyclonal anti-phospho-EphA/B was obtained from Cell Signaling. Mouse monoclonal anti-Rac1 and anti-Cdc42 were purchased from BD Biosciences. Y27632 was purchased from Calbiochem.

Cell Culture and Transfection—LS174T cells were maintained in RPMI 1640 medium and HEK293 cells were maintained in Dulbecco's modified Eagle's medium. Both media were supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin and L-glutamine. 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 fibronection (FN) at a series of concentrations starting at 10 μg/ml at 4 °C overnight. Nonspecific binding sites were blocked with 1% BSA/phosphate-buffered saline at room temperature for 1–2 h. Cells were detached with 2 mM EDTA and washed with adhesion medium (serum-free medium containing 0.1% BSA). 1×10^5 cells were plated in each well in adhesion medium containing 1 μg/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. A_{550} was measured from the extracted dye using an enzyme-linked immunosorbent assay reader.

Cell Migration Assay—Haptotactic 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 μm porous membrane, Costar) was coated with 1 μg of laminin in 10 μl of phosphate-buffered saline and allowed to air dry. Both sides of the filter were coated with 10 μg/ml collagen I at 4 °C overnight. 2×10^5 cells in serum-free medium containing 0.1% BSA were plated in the top of the insert and allowed to migrate through the filter at 37 °C overnight. 1 μg/ml Ephrin-B1-Fc or Fc pre-clustered with goat anti-human Fc was added to the lower chamber containing 20 ng/ml HGF. To test migration of transfected 293 cells, the underside of filter inserts was coated with 200 ng of FN in 10 μl of phosphate-buffered saline. Nonspecific binding sites were blocked with 1% BSA/phosphate-buffered saline. 1×10^5 cells in serum-free Dulbecco's modified Eagle's medium containing 0.1% BSA 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 μg/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 undersides 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 μg/ml ephrin-B1-Fc or Fc using an inverted Leica DM-IRE2 microscope equipped with a Ruper CoolSnap-cf digital camera. For biochemical analysis, 1.2×10^7 cells were plated on FN-coated 10-cm dishes and cultured overnight to reach fresh confluency. Cells were induced to migrate by repeated scratch wounding using a multichannel pipette 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% CO_2, they were placed in temperature/CO_2 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 μg/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 Na_3VO_4, 2 μg/ml leupeptin, and 2 μg/ml aprotinin for 30 min at 4 °C. Lysates were clarified at 13,000 $\times$ *g* for 5 min. Immunoprecipitation was carried out using antibodies at 1–2 μg/mg of total protein at 4 °C for 1 h. Immune complexes were collected using γ-Bind beads (Amersham 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 Na_3VO_4. 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.

[1] 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.

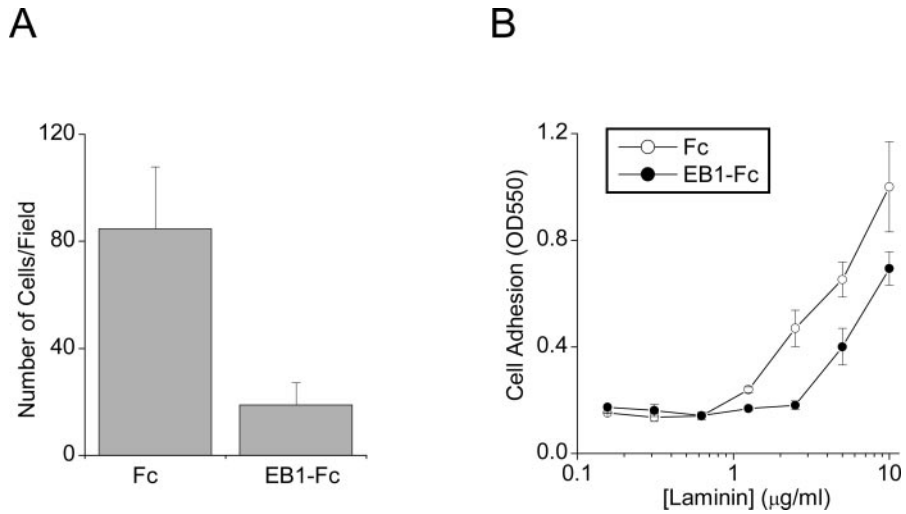

FIG. 1. **Ephrin-B1 stimulation inhibits HGF/SF-induced directional migration and integrin-mediated adhesion of LS174T cells.** *A*, lower side of Transwell inserts was coated with 1 μg of laminin. Both sides of the inserts were coated with 10 μg/ml collagen I. About 2×10^5 LS174T cells were plated in the top chamber and allowed to migrate toward the lower chamber filled with medium containing 20 ng/ml HGF/SF and pre-clustered 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 the mean ± S.D. *B*, 96-well plates were coated with laminin in series dilutions. LS174T cells were plated in the presence of 1 μg/ml Fc or ephrin-B1-Fc pre-clustered by anti-Fc antibody. After 30 min incubation at 37 °C, adherent cells were stained with crystal violet. Values represent mean A_{550} of extracted dye from triplicate wells ± S.D.

Rho GTPases Activity Assay—RhoA and Rac/Cdc42 activities were determined as described previously (35, 36). Monolayers of cells with multiple wounds were stimulated with 1 μg/ml ephrin-B1-Fc or Fc for 15 min and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl, 0.5% sodium deoxycholate, phosphatase inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin on ice for 5 min. Cell lysates were immediately incubated with GST-Rhotekin RBD or GST-PAK-CD coupled on glutathione-Sepharose 4B beads for 45 min at 4 °C. The beads were then washed and re-suspended in SDS sample buffer. The GTP-bound RhoA, Rac1, or Cdc42 were analyzed by immunoblotting using anti-RhoA, anti-Rac1, or anti-Cdc42 antibodies, respectively. Band densities were quantified using ScionImage software.

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 EphB3 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. 1*A*). Moreover, in a conventional cell adhesion assay, integrin-mediated cell adhesion to laminin was attenuated by ephrin-B1-Fc but not Fc control (Fig. 1*B*). Consistent with induction of rounding of the already spread cells reported by Batlle *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. 2*A*), 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. 2*B*). 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. 2*B* with *lanes 6* and *7* in Fig. 2*C*). 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. 2*D*). 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. 2*E*), 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. 2*E*). 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

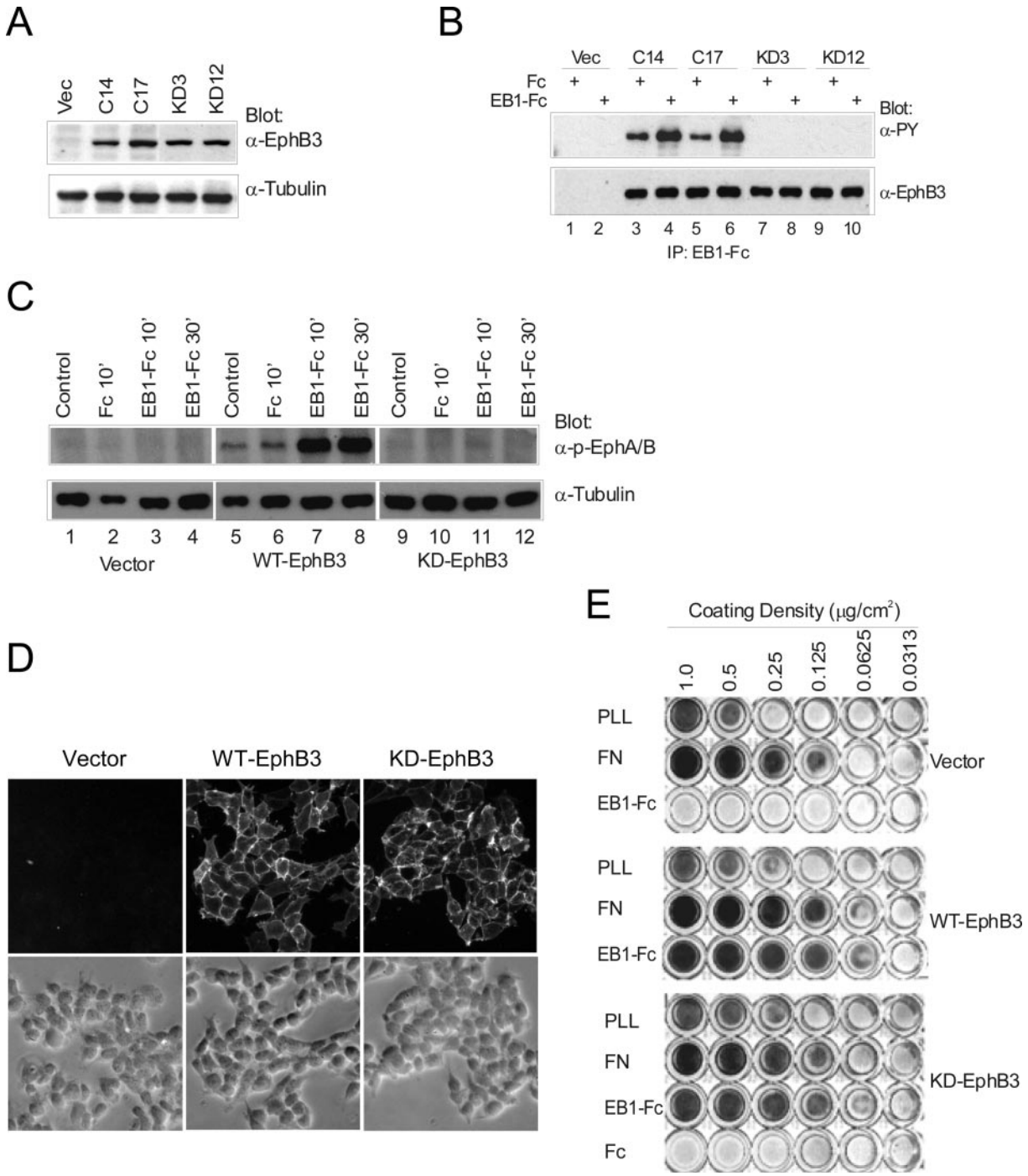

FIG. 2. **Expression of functional EphB3 receptors in 293 cells.** *A*, expression of EphB3 receptors. The expression vector containing cDNA coding WT- or KD (K665R)-EphB3 was stably transfected into 293 cells. Lysates from clonal cell populations (*C14*, *C17*, WT-EphB3; *KD3*, *KD12*, KD-EphB3) were analyzed by immunoblotting with anti-EphB3. *B*, induction of tyrosine phosphorylation of EphB3. Clonal cells were stimulated with 1 μg/ml ephrin-B1-Fc or Fc control for 15 min. EphB3 was immunoprecipitated from total cell lysate and blotted for phosphotyrosine. *C*, phosphorylation of tyrosine residues in the juxtamembrane region of EphB3 by ephrin-B1-Fc stimulation. One clone of each type of cells was stimulated with 1 μg/ml ephrin-B1-Fc or Fc for the indicated times and lysed. The total cell lysates were analyzed by immunoblotting with phosphospecific antibody against peptides derived from the juxtamembrane region of Eph kinase. *D*, immunofluorescence detection of cell surface expression of WT- and KD-EphB3. The indicated cell types were plated onto FN-coated coverslips and cultured overnight to allow spreading. Cells were fixed and stained with 2 μg/ml ephrin-B1-Fc for 1 h followed by detection with goat anti-human Fc conjugated with fluorescein isothiocyanate. *E*, immobilized ephrin-B1 mediates adhesion of WT- and KD-EphB3 cells but not vector control cells. Serial dilutions of the indicated proteins were immobilized on 96-well plates coated with nitrocellulose. EphB3-transfected 293 cells were detached with 2 mM EDTA, and plated onto coated plates for 30 min. Adherent cells were fixed and stained.

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. 3*A*). 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-

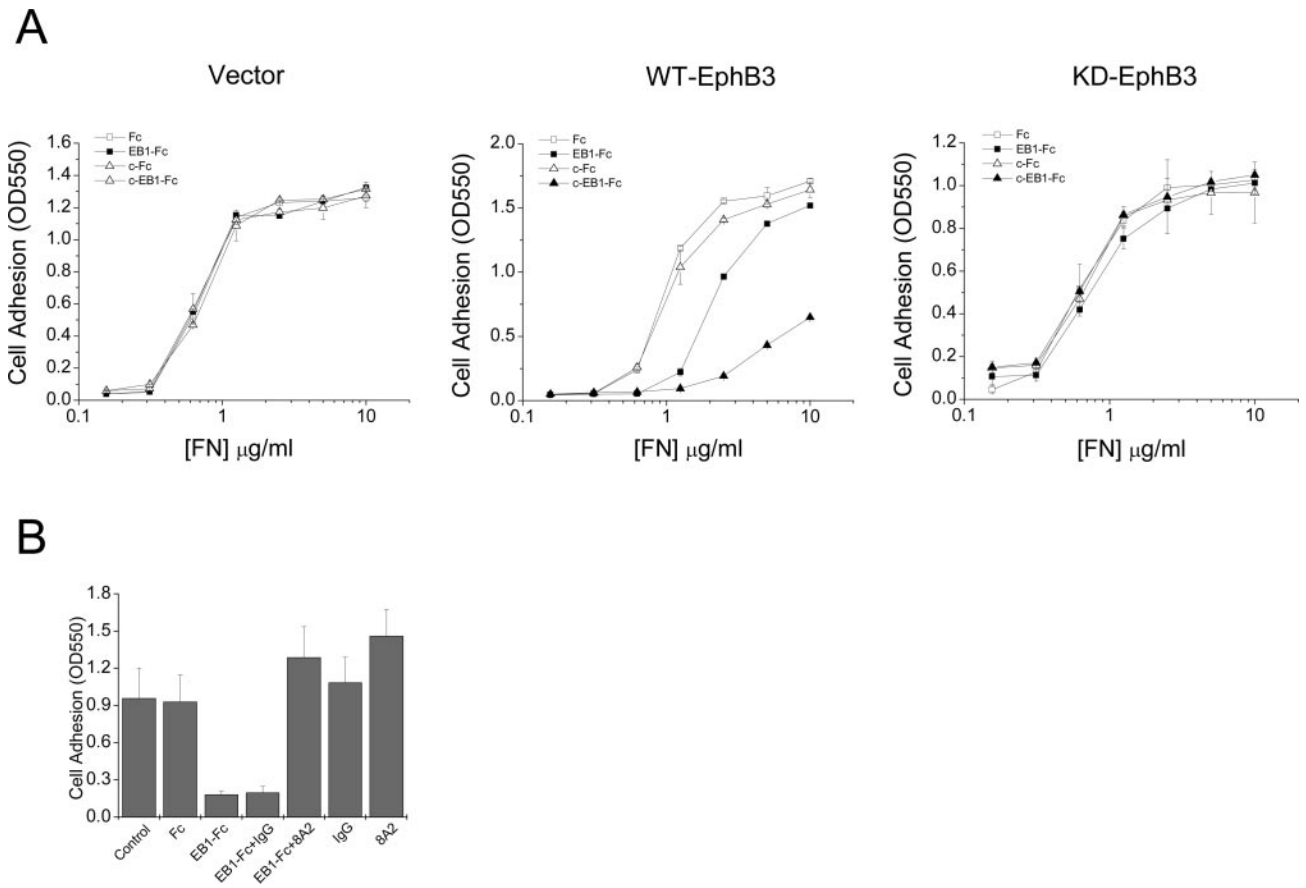

FIG. 3. *A*, ephrin-B1 stimulation of wild type but not kinase-deficient EphB3 inhibits cell adhesion to FN. Ninety-six well plates were coated with FN at serial concentrations. Different cell lines were plated in the presence of either soluble dimeric ephrin-B1-Fc or pre-clustered ephrin-B1-Fc by anti-Fc antibody as described under "Experimental Procedures." Fc was used as control. After 30 min incubation at 37 °C, adherent cells were stained with crystal violet. Values represent mean A_{550} of extracted dye from triplicate wells ± S.D. *B*, the inhibitory effect of ephrin-B1 on cell adhesion to FN can be reversed by a β_1-integrin activating antibody. Cells were plated on the FN-coated 96-well plate. After 20 min, various antibodies were added to a final concentration of 1 μg/ml. The incubation was continued for 15 more min. Cells were then fixed and stained. Values represent mean ± S.D. A_{550} of extracted dye from triplicate wells.

grin-mediated adhesion in KD-EphB3-expressing cells (Fig. 3*A*). 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 β_1-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. 3*B*). Addition of 8A2 antibody completely reversed the inhibitory effects of ephrin-B1-Fc on the adhesion of WT-EphB3 expressing cells to FN (Fig. 3*B*), 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. 4*A* 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. 4*A* and data not shown). In contrast, no change in migration was observed in vector-transfected cells (Fig. 4*A*). 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. 4*B*). 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

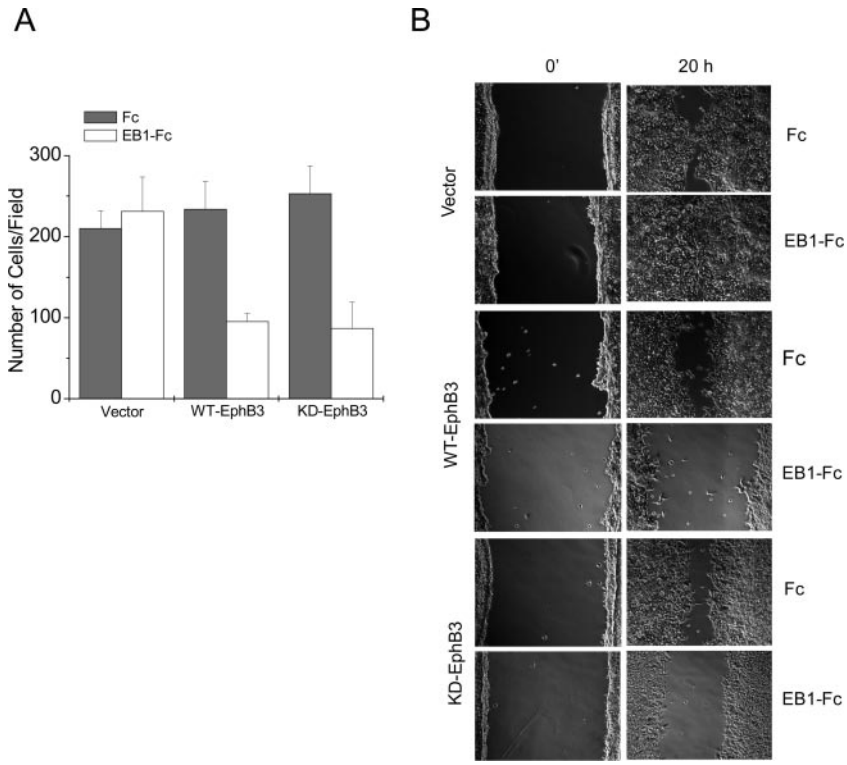

FIG. 4. **Ephrin-B1 inhibits cell migration in EphB3 kinase activity-independent manner.** *A*, Boyden chamber cell migration assay. The lower side of Transwell inserts was coated with 100 ng of FN. About 1×10^5 cells transfected with wild type or kinase-deficient EphB3 were plated in the top chamber 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-expresing 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. 5*A*). 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. 5*A*, *middle row*). Cell rounding and detachment was consistent with the inhibition of integrin function upon ligand stimulation of WT-EphB 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. 5*A*, *bottom row*). Fig. 5*B* 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. 6*A*). 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. 6*B*. 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

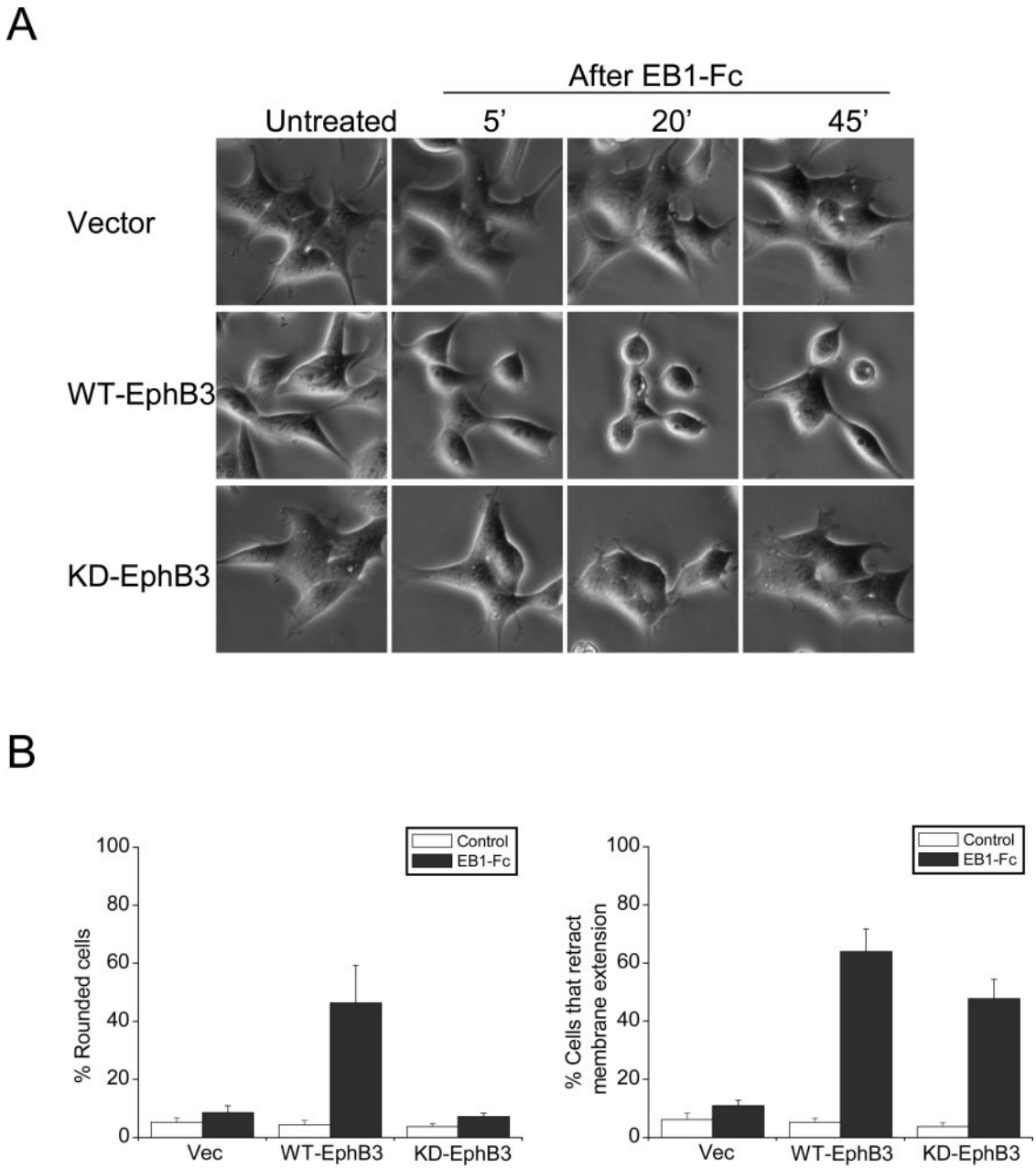

FIG. 5. **The catalytic activity of EphB3 is required for induction of cell detachment from substratum, but not for retraction of membrane protrusions.** Cells were cultured in dishes coated with 5 μg/ml FN overnight to allow spreading. Basal cell morphology was recorded using the time-lapse image system for 1 h with 20-s intervals without ligand first. Ephrin-B1-Fc (1 μg/ml) was then added to the medium and the recording was continued for about 1 h. The representative time-lapse stills were shown for the untreated cells or cells at 3 different time points after addition of ephrin-B1-Fc (*A*). The numbers of rounded cells and cells that retracted membrane extensions and total number of cells per field were counted. Values represent the mean percentage from three different positions, ± S.D. (*B*).

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 β_1-integrin activating antibody (8A2) in the lower chamber. Fig. 9 shows

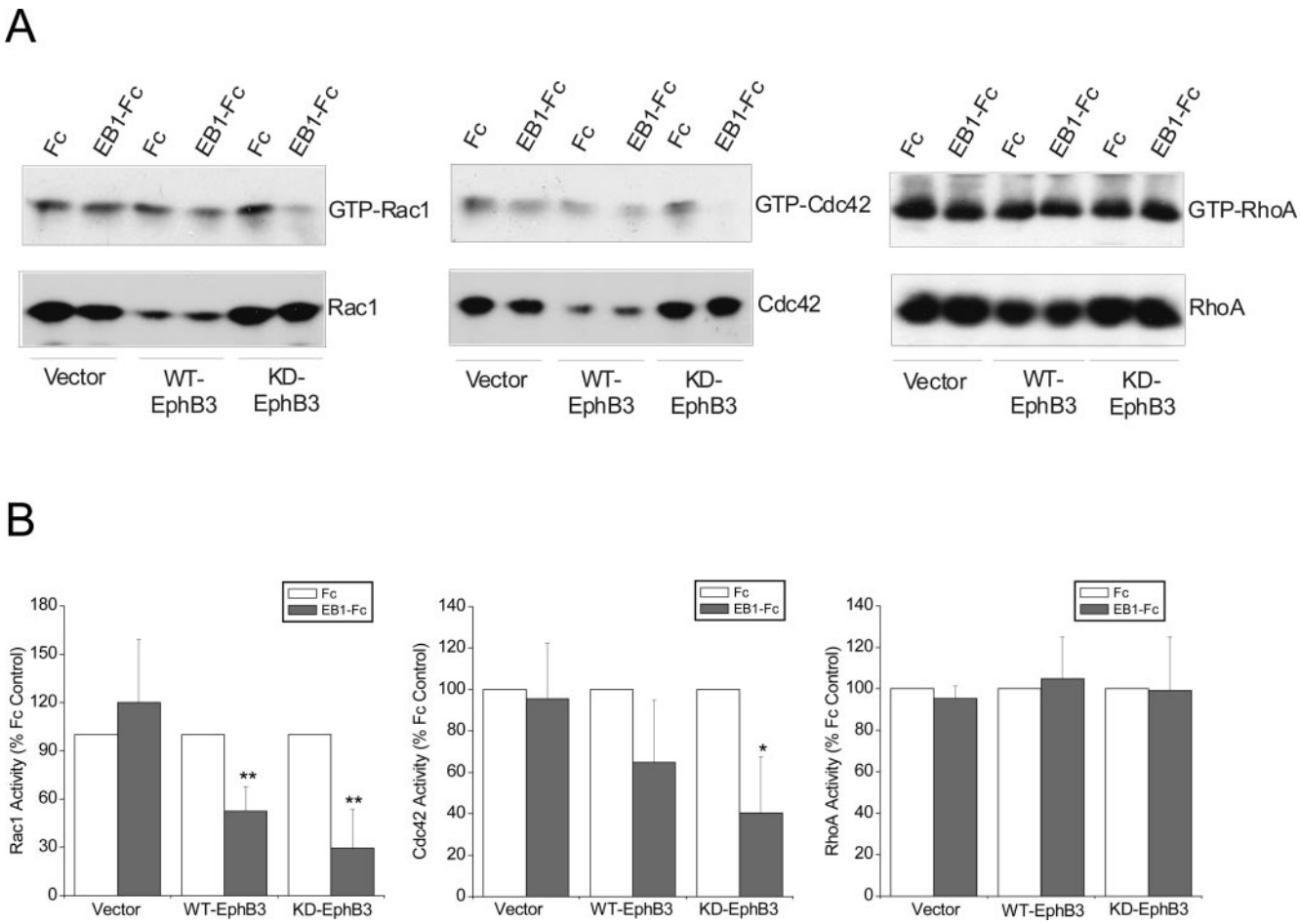

FIG. 6. **The activities of Rho GTPases are regulated by EphB3.** Ephrin-B1 stimulation inhibits Rac1 and Cdc42 activities of migrating cells expressing WT- or KD-EphB3 while having no significant effect on RhoA activity (*A*). Monolayer of cells was scratched several times using multichannel pipette. Four hours after wounding, cells were stimulated with 1 μg/ml ephrin-B1-Fc or Fc for 15 min and lysed. Active Rac1/Cdc42 was precipitated by using GST-PAK-CD and RhoA was precipitated by using GST-Rhotekin. The precipitates were analyzed by immunoblotting with anti-Rac1, anti-Cdc42, or anti-RhoA antibodies. For quantitative analysis (*B*), the ratio of GTP-bound over total Rho GTPases from ephrin-B1-Fc-treated cells were normalized to that from Fc control-treated cells. The data represents the mean value from three independent experiments; ± S.D., *, $p < 0.05$; **, $p < 0.01$.

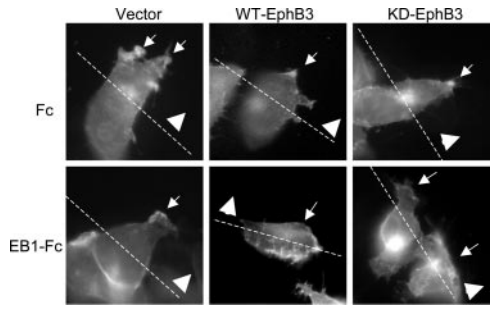

FIG. 7. **Ephrin-B1 treatment diminished GFP-Rac1 localization to the leading edge of migrating cells.** Cells expressing WT-, KD-EphB3, or vector were transfected with GFP-Rac. One day post-transfection, cells were plated onto FN-coated coverslips and cultured overnight to reach confluence. Cells were then wounded and allowed to migrate for 4 h. After stimulation with 1 μg/ml ephrin-B1-Fc or Fc for 15 min to 3 h, cells were fixed and washed. The GFP-Rac-positive wound-edge cells were photographed. Images shown are representatives obtained at 15 min after treatment. *Arrowhead*, direction of cell migration; *arrow*, GFP-Rac1 at leading edge.

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

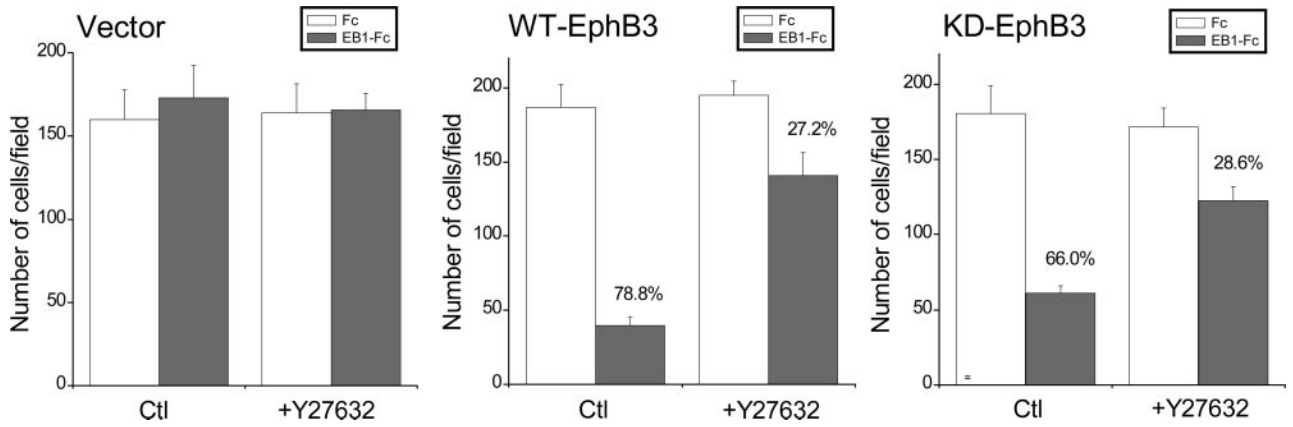

FIG. 8. **Inhibition of ROCK attenuates EphB3-mediated regulation of cell migration.** Boyden chamber migration assay was performed as described in the legend to Fig. 4 except that cells were preincubated with 10 μM Y27632 on ice for 30 min before plating in the top chamber of Transwell. Numbers of cells 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. *Numbers above* the *shaded bar* (EB1-Fc) indicate the percent inhibition compared with *open bars* (Fc). *Ctl*, control.

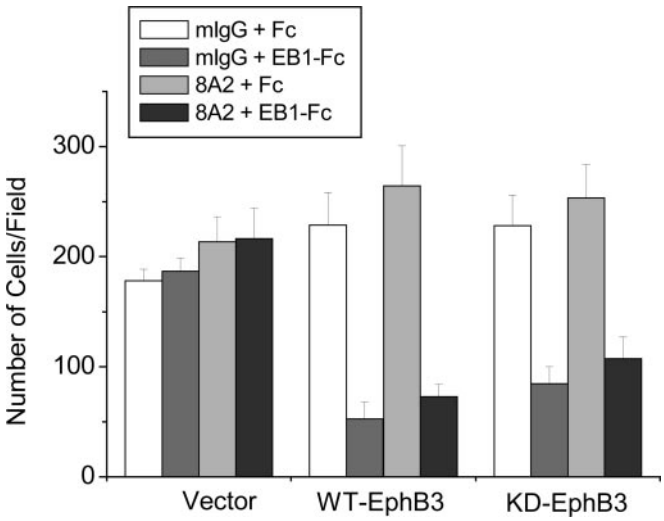

FIG. 9. **The β_1-integrin activating antibodies does not abolish the inhibitory effects of ephrin-B1 on cell migration.** Boyden chamber migration assay was performed as described in the legend to Fig. 4. Integrin activating antibody 8A2 or mouse IgG was presented at a final concentration of 1 μg/ml at the lower chamber in addition to the ephrin-B1-Fc or Fc. 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.

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 p38 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

EphA3 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 β_1-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 neonate 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). These observations suggest that EphB3 signaling sets restrictions to epithelial cell migration. This is analogous to repulsive guidance of growth cones and neural crest cells in developing nervous systems (6, 9, 10). Our results provide direct experimental evidence for the negative regulation of cell migration by the EphB3-ephrin-B1 interaction, which may represent one possible mechanism behind intestinal epithelial cell sorting *in vivo*.

In summary, ligation of EphB3 receptor inhibits cell adhesion in a kinase-dependent manner, whereas inhibition of migration is independent of EphB3 catalytic activity. EphB3 is widely expressed in developing and adult tissues (31). Kinase-dependent and -independent regulatory effects on cell motility may allow this receptor to exert diverse functions under different 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.

REFERENCES

1. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) *Science* **302,** 1704–1709
2. Lauffenburger, D. A., and Horwitz, A. F. (1996) *Cell* **84,** 359–369
3. Tessier-Lavigne, M., and Goodman, C. S. (1996) *Science* **274,** 1123–1133
4. Hall, A. (1998) *Science* **279,** 509–514
5. Nobes, C. D., and Hall, A. (1999) *J. Cell Biol.* **144,** 1235–1244
6. Gale, N. W., and Yancopoulos, G. D. (1997) *Cell Tissue Res.* **290,** 227–241
7. Nakamoto, M., Cheng, H. J., Friedman, G. C., McLaughlin, T., Hansen, M. J., Yoon, C. H., O'Leary, D. D., and Flanagan, J. G. (1996) *Cell* **86,** 755–766
8. Wang, H. U., and Anderson, D. J. (1997) *Neuron* **18,** 383–396
9. Flanagan, J. G., and Vanderhaeghen, P. (1998) *Annu. Rev. Neurosci.* **21,** 309–345
10. Wilkinson, D. G. (2001) *Nat. Rev. Neurosci.* **2,** 155–164
11. Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) *Science* **298,** 1912–1934
12. Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson, D. G., Pawson, T., Davis, S., and Yancopoulos, G. D. (1996) *Neuron* **17,** 9–19
13. Himanen, J. P., Chumley, M. J., Lackmann, M., Li, C., Barton, W. A., Jeffrey, P. D., Vearing, C., Geleick, D., Feldheim, D. A., Boyd, A. W., Henkemeyer, M., and Nikolov, D. B. (2004) *Nat. Neurosci.* **7,** 501–509
14. Bruckner, K., Pasquale, E. B., and Klein, R. (1997) *Science* **275,** 1640–1643
15. Davy, A., Gale, N. W., Murray, E. W., Klinghoffer, R. A., Soriano, P., Feuerstein, C., and Robbins, S. M. (1999) *Genes Dev.* **13,** 3125–3135
16. Holland, S. J., Gale, N. W., Mbamalu, G., Yancopoulos, G. D., Henkemeyer, M., and Pawson, T. (1996) *Nature* **383,** 722–725
17. Wilkinson, D. G. (2000) *Int. Rev. Cytol.* **196,** 177–244
18. Miao, H., Burnett, E., Kinch, M., Simon, E., and Wang, B. (2000) *Nat. Cell Biol.* **2,** 62–69
19. Batlle, E., Henderson, J. T., Beghtel, H., van den Born, M. M., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., van de Wetering, M., Pawson, T., and Clevers, H. (2002) *Cell* **111,** 251–263
20. Chin-Sang, I. D., George, S. E., Ding, M., Moseley, S. L., Lynch, A. S., and Chisholm, A. D. (1999) *Cell* **99,** 781–790
21. George, S. E., Simokat, K., Hardin, J., and Chisholm, A. D. (1998) *Cell* **92,** 633–643
22. Wang, X., Roy, P. J., Holland, S. J., Zhang, L. W., Culotti, J. G., and Pawson, T. (1999) *Mol. Cell* **4,** 903–913
23. Grunwald, I. C., Korte, M., Wolfer, D., Wilkinson, G. A., Unsicker, K., Lipp, H. P., Bonhoeffer, T., and Klein, R. (2001) *Neuron* **32,** 1027–1040
24. Kullander, K., Mather, N. K., Diella, F., Dottori, M., Boyd, A. W., and Klein, R. (2001) *Neuron* **29,** 73–84
25. Dalva, M. B., Takasu, M. A., Lin, M. Z., Shamah, S. M., Hu, L., Gale, N. W., and Greenberg, M. E. (2000) *Cell* **103,** 945–956
26. Henkemeyer, M., Orioli, D., Henderson, J. T., Saxton, T. M., Roder, J., Pawson, T., and Klein, R. (1996) *Cell* **86,** 35–46
27. Birgbauer, E., Cowan, C. A., Sretavan, D. W., and Henkemeyer, M. (2000) *Development* **127,** 1231–1241
28. del Pozo, M. A., Alderson, N. B., Kiosses, W. B., Chiang, H. H., Anderson, R. G., and Schwartz, M. A. (2004) *Science* **303,** 839–842
29. Gurniak, C. B., and Berg, L. J. (1996) *Oncogene* **13,** 777–786
30. Caenepeel, S., Charydczak, G., Sudarsanam, S., Hunter, T., and Manning, G. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 11707–11712
31. Hock, B., Bohme, B., Karn, T., Yamamoto, T., Kaibuchi, K., Holtrich, U., Holland, S., Pawson, T., Rubsamen-Waigmann, H., and Strebhardt, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9779–9784
32. Bohme, B., VandenBos, T., Cerretti, D. P., Park, L. S., Holtrich, U., Rubsamen-Waigmann, H., and Strebhardt, K. (1996) *J. Biol. Chem.* **271,** 24747–24752
33. Miao, H., Nickel, C. H., Cantley, L. G., Bruggeman, L. A., Bennardo, L. N., and Wang, B. (2003) *J. Cell Biol.* **162,** 1281–1292
34. Etienne-Manneville, S., and Hall, A. (2001) *Cell* **106,** 489–498
35. Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999) *EMBO J.* **18,** 578–585
36. Sander, E. E., van Delft, S., ten Klooster, J. P., Reid, T., van der Kammen, R. A., Michiels, F., and Collard, J. G. (1998) *J. Cell Biol.* **143,** 1385–1398
37. Zou, J. X., Wang, B., Kalo, M. S., Zisch, A. H., Pasquale, E. B., and Ruoslahti, E. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13813–13818
38. Holland, S. J., Gale, N. W., Gish, G. D., Roth, R. A., Songyang, Z., Cantley, L. C., Henkemeyer, M., Yancopoulos, G. D., and Pawson, T. (1997) *EMBO J.* **16,** 3877–3888
39. Davis, S., Gale, N. W., Aldrich, T. H., Maisonpierre, P. C., Lhotak, V., Pawson, T., Goldfarb, M., and Yancopoulos, G. D. (1994) *Science* **266,** 816–819
40. Stein, E., Lane, A. A., Cerretti, D. P., Schoecklmann, H. O., Schroff, A. D., Van Etten, R. L., and Daniel, T. O. (1998) *Genes Dev.* **12,** 667–678
41. Hughes, P. E., and Pfaff, M. (1998) *Trends Cell Biol.* **8,** 359–364
42. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 549–599
43. Faull, R. J., Kovach, N. L., Harlan, J. M., and Ginsberg, M. H. (1993) *J. Cell Biol.* **121,** 155–162
44. Etienne-Manneville, S., and Hall, A. (2002) *Nature* **420,** 629–635
45. Ridley, A. J. (2001) *J. Cell Sci.* **114,** 2713–2722
46. Caron, E. (2003) *Nat. Cell Biol.* **5,** 185–187
47. Arthur, W. T., and Burridge, K. (2001) *Mol. Biol. Cell* **12,** 2711–2720
48. Rottner, K., Hall, A., and Small, J. V. (1999) *Curr. Biol.* **9,** 640–648
49. Nimnual, A. S., Taylor, L. J., and Bar-Sagi, D. (2003) *Nat. Cell Biol.* **5,** 236–241
50. Zisch, A. H., and Pasquale, E. B. (1997) *Cell Tissue Res.* **290,** 217–226
51. Holmberg, J., Clarke, D. L., and Frisen, J. (2000) *Nature* **408,** 203–206
52. Luo, H., Yu, G., Wu, Y., and Wu, J. (2002) *J. Clin. Investig.* **110,** 1141–1150
53. Luo, H., Wan, X., Wu, Y., and Wu, J. (2001) *J. Immunol.* **167,** 1362–1370
54. Zisch, A. H., Pazzagli, C., Freeman, A. L., Schneller, M., Hadman, M., Smith, J. W., Ruoslahti, E., and Pasquale, E. B. (2000) *Oncogene* **19,** 177–187
55. Zantek, N. D., Azimi, M., Fedor-Chaiken, M., Wang, B., Brackenbury, R., and Kinch, M. S. (1999) *Cell Growth Differ.* **10,** 629–638
56. Lawrenson, I. D., Wimmer-Kleikamp, S. H., Lock, P., Schoenwaelder, S. M., Down, M., Boyd, A. W., Alewood, P. F., and Lackmann, M. (2002) *J. Cell Sci.* **115,** 1059–1072
57. Smith, L. M., Walsh, P. T., Rudiger, T., Cotter, T. G., Mc Carthy, T. V., Marx, A., and O'Connor, R. (2004) *Exp. Cell Res.* **292,** 295–303
58. del Pozo, M. A., Kiosses, W. B., Alderson, N. B., Meller, N., Hahn, K. M., and Schwartz, M. A. (2002) *Nat. Cell Biol.* **4,** 232–239