Ephrin-B1 transduces signals to activate integrin-mediated migration, attachment and angiogenesis

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Summary

Ephrin-B/EphB family proteins are implicated in bidirectional signaling and were initially defined through the function of their ectodomain sequences in activating EphB receptor tyrosine kinases. Ephrin-B1-3 are transmembrane proteins sharing highly conserved C-terminal cytoplasmic sequences. Here we use a soluble EphB1 ectodomain fusion protein (EphB1/Fc) to demonstrate that ephrin-B1 transduces signals that regulate cell attachment and migration. EphB1/Fc induced endothelial ephrin-B1 tyrosine phosphorylation, migration and integrin-mediated ($\alpha_v\beta_3$ and $\alpha_5\beta_1$) attachment and promoted neovascularization, in vivo, in a mouse corneal micropocket assay. Activation of ephrin-B1 by EphB1/Fc induced phosphorylation of p46 JNK but not ERK-1/2 or p38 MAPkinases. By contrast, mutant ephrin-B1s bearing either a cytoplasmic deletion (ephrin-B1ΔCy) or a deletion of four C-terminal amino acids (ephrin-B1ΔPDZbd) fail to activate p46 JNK. Transient expression of intact ephin-B1 conferred EphB1/Fc migration responses on CHO cells, whereas the ephrin-B1ΔCy and ephrin-B1ΔPDZbd mutants were inactive. Thus ephrin-B1 transduces 'outside-in' signals through C-terminal protein interactions that affect integrin-mediated attachment and migration.

Key words: Ephrin-B1, EphB1, Endothelial, Angiogenesis, Signal transduction

Introduction

Receptors and counter-receptors of the Eph/ephrin family are critical determinants of embryonic patterning (Holder and Klein, 1999), neuronal targeting (Flanagan and Vanderhaeghen, 1998) and vascular assembly (Cheng et al., 2002; Gale and Yancopoulos, 1999). They are implicated in cell-cell recognition processes upon juxtacrine contact and mediate non-proliferative signals that direct cell migration and attachment (Zisch and Pasquale, 1997). Comprising 14 distinct family members, the Eph family of receptor tyrosine kinases, and their nine distinct membrane-bound counter-receptors, the ephrins, are expressed in spatial patterns of reciprocal compartmentalization during development (Gale et al., 1996; Menzel et al., 2001).

Transmembrane Eph receptor kinases are activated upon binding to oligomerized ephrins (Davis et al., 1994), and specific oligomerized forms signal distinct cellular responses (Stein, 1998). EphB subclass receptors (EphB1-6) display overlapping affinity for transmembrane ephrin-B subclass counter-receptors (ephrin-B1-3), whereas EphA subclass receptors (EphA1-8) bind predominantly to the glycerol-phosphatidylinositol-linked ephrin-A counter-receptors (ephrin-A1-6) (Gale et al., 1996; Menzel et al., 2001). Both EphB receptors and ephrin-B counter-receptors have C-terminal sequences capable of interacting with PDZ-domain-containing proteins, including PICK1, syntenin, GRIP (Bruckner et al., 1999; Torres, 1998) and PDZ-RGS (Lu, 2001).

Evidence that ephrin-B subclass counter-receptors signal cell-autonomous responses was first provided by the targeting behavior of axons in EphB2-mutant mice (Henkemeyer et al., 1996). Axonal projections that express ephrin-B2 were misdirected in mice homozygous null for EphB2. However, these axonal projections targeted correctly through migratory fields in animals that expressed EphB2 ectodomains as a tyrosine-kinase-deficient β-galactosidase fusion. Ephrin-B2 cytoplasmic domain sequences are c-src tyrosine kinase substrates that are tyrosine phosphorylated in early embryos and show regulated tyrosine phosphorylation sensitive to EphB2/Fc (Holland et al., 1996) and PDGF (Bruckner et al., 1997) in transfected cell lines. This regulated tyrosine phosphorylation recruits adaptor protein Grb4 and transduces signals to promote changes in the actin cytoskeleton (Cowan and Henkemeyer, 2001).

Ephrin-B2 expression is required for development of the embryonic vascular system where its early expression is restricted to endothelial cells of arterial, not venous, vascular structures (Wang et al., 1998). Homozygous mice null for ephrin-B2 show failure of embryonic vascular development at a stage when extraembryonic yolk sac arterial plexus vessels fail to interconnect with venous plexus vessels that express the EphB4 receptor. A similar failure of vascular embryonic

development is displayed in EphB4-null mice (Gerety et al., 1999) and in mice doubly homozygous for deletions in EphB2 and EphB3 (Adams et al., 1999). More recently, the functional role of the ephrin cytoplasmic domain was demonstrated in ephrin-B2$^{\Delta C/\Delta C}$ mice expressing a mutant ephrin-B2 with a cytoplasmic deletion (Adams et al., 2001). Ephrin-B2$^{\Delta C/\Delta C}$ mice exhibit vascular remodeling defects that are reminiscent of a subset of phenotypes in ephrin-B2-null mice, indicating that signaling through ephrin-B2 is required for vascular development.

Cultured primary microvascular endothelial cells are useful systems for defining EphB1 signaling pathways that impact upon responses relevant to vascular development, including cell attachment, migration and capillary-like assembly responses (Daniel et al., 1996; Huynh-Do et al., 1999; Stein, 1998). Here we have asked whether ephrin-B1 transduces 'outside-in' signals to alter cell attachment or migration in microvascular endothelial cells that express endogenous ephrin-B1 (Stein, 1998). Our findings demonstrate that ephrin-B1 is coupled to integrin function and assign a direct role to the C-terminal ephrin-B1 sequences. The relevance of the in vitro culture results is supported by the in vivo effects of a soluble EphB1/Fc fusion protein that promotes neovascularization.

Materials and Methods

Development of a stable cell line secreting EphB1/Fc

Chinese hamster ovary (CHO) cells (DXB-11) were maintained in DMEM/F12 media supplemented with hypoxanthine and thymidine (Kaufman, 1990). A cDNA fragment encoding a fusion of the extracellular domain of EphB1 to the Fc portion of human IgG_1 (Beckmann et al., 1994) was subcloned into the CHO cell expression vector 2A5ib. Approximately 10 μg of EphB1-Fc-2A5ib expression plasmid was used to transfect DXB-11 cells using Lipofectamine (Gibco-BRL). The selection of DHFR-positive transfectants and the methods used to amplify expression have been described previously (Huynh-Do et al., 1999). Pools of transfectants were amplified in 50-500 nM methotrexate. Roller bottle cultures were grown in DMEM-F12 supplemented with 7.5% fetal bovine serum and 150 nM methotrexate until 80-90% confluent. The media was replaced with DMEM-F12-based serum free media containing peptone, transferrin, lipids and IGF1, and the cells were incubated at 34°C for 10 days or until cell viability was below 50%. The supernatants were harvested, and EphB1/Fc was purified by protein-A-sepharose chromatography.

Antibodies and reagents

Polyclonal rabbit ephrin-B1 antibodies, P1, recognize the C-terminal 15 amino acids of both ephrin-B1 and ephrin-B2, whereas ephrin-B1 antibodies, P2, recognize a non-conserved juxtamembrane spacer domain peptide unique to ephrin-B1 (aa221-238) (Immunex, Seattle, WA). Horseradish-peroxidase-conjugated antibody 4G10-HRP, anti-ERK1/2 and anti-JNK polyclonal antibodies were from Upstate Biotechnology (Lake Placid, NY) and streptavidin-HRP from Jackson (Westgrove, PA). Human IgG_1 and plasma fibrinogen were from Sigma (St Louis, MO), human fibronectin was from Life Technologies. The GRGDTP and GRGESP peptides were from Calbiochem (La Jolla, CA). Anti-integrin blocking mAbs from Chemicon (Temecula, CA) were LM609 ($\alpha_v\beta_3$), P1F6 ($\alpha_v\beta_5$) and JBS5 ($\alpha_5\beta_1$). Polyclonal antibodies against phosphorylated forms of JNK, ERK1/2 and p38MAPK were from Promega (Madison, WI). Other matrix proteins, peptides and antibodies were from previously cited sources (Huynh-Do et al., 1999; Stein, 1998).

Plasmids, cell culture, transfection and FACS analysis

cDNAs encoding ephrinB-1ΔCy (carrying a deletion of the C-terminal 50 amino acids) and ephrinB-1ΔPDZbd (carrying a deletion of four C-terminal amino acids) were amplified by PCR, sequenced and subcloned into expression vector pSRα. Human renal microvascular endothelial cells (HRMEC) were cultured and passaged as described previously (Martin et al., 1997). CHO cells were passaged in DMEM-F12 medium (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). CHO were transfected using the Lipofectamine Plus method (Life Technologies) with vector alone (sRα), plasmids expressing full-length human ephrin-B1, c-myc-tagged ephrin-B1ΔCy (lacking the C-terminal 50 amino acids) or ephrin-B1ΔPDZbd (lacking the four C-terminal amino acids required for interacting with the PDZ domain protein). Migration assays and FACS analysis were performed 48 hours after transfection. Cell surface integrin expression was analyzed 30 minutes after stimulation with IgG (Fc control) or EphB1/Fc (2 μg/ml) using anti $\alpha_v\beta_3$ (LM609, 10 μg/ml). Analysis of ephrin B1 and mutant forms expressed on CHO cell surfaces was conducted using EphB1/Fc (2 μg/ml) followed by FITC-conjugated goat anti-human IgG-Fc (Jackson Labs, 1:200 dilution) on a FACSCaliber (Becton Dickinson, San Jose, CA) instrument using an argon ion laser at 488 nm with detection by a 530±30 nm band pass filter.

Surface biotinylation and ephrin immunoprecipitation

Cell surface proteins were covalently conjugated with biotin by incubation of pre-washed cells for 30 minutes at 4°C with 0.5 mg/ml sulfo-NHS-LC-Biotin in phosphate buffered saline (Pierce, Rockford, IL). Cells were washed, quenched in 0.15 M glycine then lysed, and integrins or ephrin-B1 were immunoprecipitated as described previously (Daniel et al., 1996). Biotinylated proteins were detected following immunoblot transfer using streptavidin-HRP with enhanced chemiluminescence (ECL Western Blotting Detection, Amersham). Data are representative of three independent experiments.

Ephrin-B1 tyrosine phosphorylation

Sixty mm (p60) tissue culture dishes were coated with fibronectin (0.5 $\mu g/cm^2$) overnight at 4°C in bicarbonate buffer (Stein, 1998). Serum-starved HRMEC or CHO cells were replated (15-20×10^5 cells/60 mm dish) for 60 minutes at 37°C. Cells were stimulated with agonists for 15 minutes at 37°C then lysed in 1 ml RIPA buffer. Recovery and tyrosine phosphorylation of endogenous or transfected Ephrin-B1 were assessed by immunoprecipitation with anti-ephrin-B1, followed by anti-ephrin-B1 or anti-phosphotyrosine (4G10-HRP) immunoblots, respectively.

Western blot analysis of MAP kinase phosphorylation

CHO cells stably expressing ephrin-B1 or transiently transfected with no vector (MOCK), full-length human ephrin-B1, ephrin-B1ΔCy or ephrin-B1ΔPDZbd were serum-starved for 24 hours in Opti-MEM, treated with 0.5 mM suramin for 3 hours and then stimulated for the indicated times at 37°C with EphB1/Fc (2 μg/ml) or with varying concentrations of EphB1/Fc for 20 minutes. For assessment of JNK, ERK1/2 and p38 MAPK activation, cells were lysed in RIPA buffer, and 30 μg of proteins were loaded on a 10% SDS-PAGE. After transfer to Immobilon/PVDF membranes (Millipore, Bedford, MA), phosphorylated JNK, ERK1/2 or p38 was detected with antibodies against phosphorylated forms of JNK, ERK1/2 or p38 MAPK. Membranes were then stripped and reprobed with anti-JNK or anti-ERK1/2 to ensure equal loading of proteins.

Cell attachment

Forty-eight-well plates (Falcon) were coated with fibrinogen (1

μg/cm^2) overnight at 4°C in bicarbonate buffer. Two hours prior to the assay, wells were washed twice then blocked at 37°C with 1% BSA. Cells were starved for 48 hours in Opti-MEM, recovered by gentle trypsinization, washed twice in serum-free medium containing 1% BSA, then plated at a density of 0.5-0.8×10^5 cells per well. Controls [no addition, NA or IgG (class-matched human Fc control)] or agonists (EphB1/Fc) were added at the indicated concentrations at the time of plating. After incubation at 37°C for 1 hour, unattached cells were dislodged by brisk vertical contact of the plate with a horizontal surface until cells plated on albumin-coated plates in the absence of matrix (no fibrinogen) were fully detached (four to five slaps). Wells were washed with PBS, and adherent cells were fixed with 2% glutaraldehyde, stained with 0.5% crystal violet (in 0.2 M boric acid) and quantified by OD reading at 570 nm. In some experiments, cells were preincubated with the indicated peptides (100 μM) or integrin-specific antibodies (5 μg/ml) for 15 minutes at room temperature before plating. The data represent three independent experiments and are expressed as means of values from four wells ± s.e.m.

Wound closure assay

Replicate circular 'wounds', or defects (600-900 μm diameter), were generated in confluent HRMEC or CHO cell monolayers using a silicon-tipped drill press, as described previously (Daniel et al., 1999). Serum-free medium was supplemented with the indicated agonists at the time of wounding. Residual fractional 'wound' areas were measured at the indicated times using a Bioquant (Nashville, TN) software package calibrated to a Nikon Diaphot microscope. Mean fractional residual areas of three wounds, calculated at each of the two or three time points were used to derive linear regressions, reflecting migration rates (expressed as a percentage of closure/hour, ±95% confidence intervals). Data are representative of three independent experiments.

Mouse corneal angiogenesis assay

Hydron pellets incorporating sucralfate with vehicle alone, basic FGF (3 pmol/pellet; a gift from Scios, Inc), control IgG1 or EphB1/Fc (5.6 pmol/pellet) were made as described previously (Kenyon et al., 1996). Pellets were surgically implanted into corneal stromal micropockets that were created 1 mm medial to the lateral corneal limbus of C57L male mice (7-9 weeks old). At day 5, corneas were photographed at an incipient angle of 35-50° from the polar axis in the meridian containing the pellet using a Zeiss split lamp. Images were digitalized and processed by subtractive color filters (Adobe Photoshop 4.0): the fraction of the total corneal image that was vascularized, the ratio of pixels marking neovascular capillaries, both within the vascularized region (R) and within the total corneal image (T) were calculated using the Bioquant software (Nashville, TN). Each summary value is the mean±s.e.m. of nine corneas for each condition.

Results

Preparations of cultured human renal microvascular endothelial cells (HRMEC) include cells that express EphB1 (Daniel et al., 1996) and its counter-receptor, ephrin-B1 (Stein, 1998). Fig. 1 (upper panel) demonstrates immunoprecipitation of biotin-labeled endogenous ephrin-B1 from these cells using polyclonal antibodies that recognize two different ephrin-B1 epitopes - a C-terminal peptide common to ephrins B1-3 (P1) or an ephrin-B1 spacer-domain-specific peptide (P2). These results showed that HRMECs express ephrin-B1 (Fig. 1) but fail to express ephrin-B2 and ephrin-B3 (data not shown). Consistent with previous studies, ephrin-B1 is tyrosine

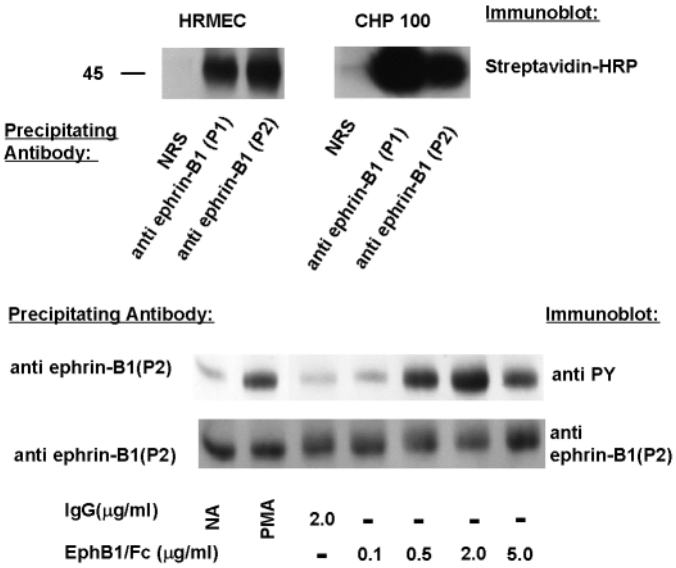


Fig. 1. EphB1/Fc stimulates endothelial ephrin-B1 tyrosine phosphorylation. Upper panel, ephrin-B1 is expressed in human renal microvascular endothelial cells (HRMEC). HRMEC or CHP100 (Davis et al., 1994) were surface biotinylated as described in the Materials and Methods, and ephrins were immunoprecipitated using non-immune rabbit serum (NRS) or rabbit polyclonal anti-ephrin-B1 antibodies recognizing C-terminal sequences (P1) or anti-ephrin-B1 recognizing an ephrin-B1-specific juxtamembrane spacer domain peptide (P2). Immunoprecipitated complexes were separated on a 10% SDS-PAGE under non-reducing conditions, transferred to PVDF membranes and detected with streptavidin-HRP using enhanced chemiluminescence (Amersham). The ephrin-B1-specific antibody (P2) was used in all subsequent experiments. Lower panel, phorbol myristate acetate and EphB1/Fc stimulate ephrin-B1 tyrosine phosphorylation. Serum-depleted HRMEC were replated on fibronectin-coated p60 tissue culture dishes for 60 minutes, then stimulated for 15 minutes at 37°C with vehicle (NA), phorbol myristate acetate (PMA, 20 ng/ml), control IgG1 (2 μg/ml) or EphB1/Fc at the indicated concentrations. Cells were lysed in RIPA buffer, immunoprecipitated with rabbit anti-ephrin-B1, and the levels of ephrin-B1 tyrosine phosphorylation were determined using the monoclonal antibody 4G10 conjugated to HRP followed by ECL detection. The results are representative of five independent experiments.

phosphorylated under basal culture conditions (Bruckner et al., 1997; Holland et al., 1996). Additional tyrosine phosphorylation was induced by exposure to EphB1/Fc at concentrations ranging from 0.5-5 μg/ml, with maximal responses at 2 μg/ml (~13 nM) and declining at higher concentrations (Fig. 1, bottom panel). Phorbol myristate acetate (PMA), an activator of protein kinase C, also stimulated ephrin-B1 tyrosine phosphorylation, which correlated with its effect on promoting oligomerization of endogenous ephrin-B1 (Stein et al., 1998).

To address whether ephrin-B1 transduces 'outside-in' signals to alter endothelial cell function, we evaluated whether EphB1/Fc stimulated endothelial cell migration. Fig. 2 shows that the rate of endothelial migration to close a circular wound in a confluent monolayer was increased in serum-free medium supplemented with EphB1/Fc, or PMA, but was unaffected by the Fc control, IgG$_1$. EphB1/Fc-stimulated endothelial cell

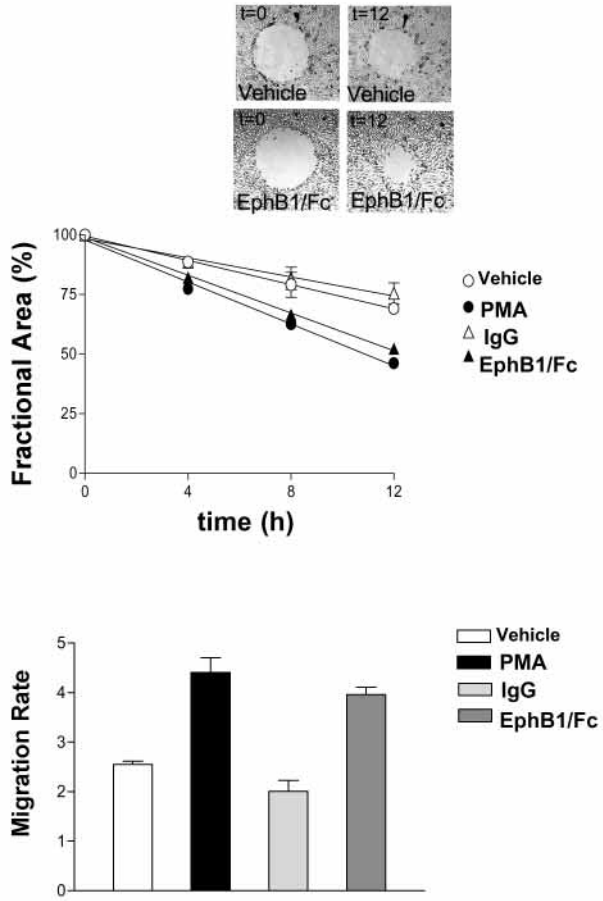


Fig. 2. EphB1/Fc promotes endothelial cell migration. Confluent HRMEC were serum depleted prior to mechanical 'wounding' to create circular defects of 600-900 μm diameter. The medium was supplemented with vehicle (NA), phorbol myristate acetate (PMA, 20 ng/ml), control IgG1 or EphB1/Fc (0.5 μg/ml each). Fractions of the areas remaining in triplicate wounds were determined by analysis of serial digital images obtained at the times indicated. Migration rates are expressed as a percentage of 'wounds' closed per hour.

migration was seen at a concentration (0.5 μg/ml) comparable to that stimulating ephrin-B1 tyrosine phosphorylation (Fig. 1). Both basal and stimulated migration were attenuated by the antibody against $\alpha_v\beta_3$, making assignment of a specific role for this integrin in EphB1-stimulated migration difficult (data not shown).

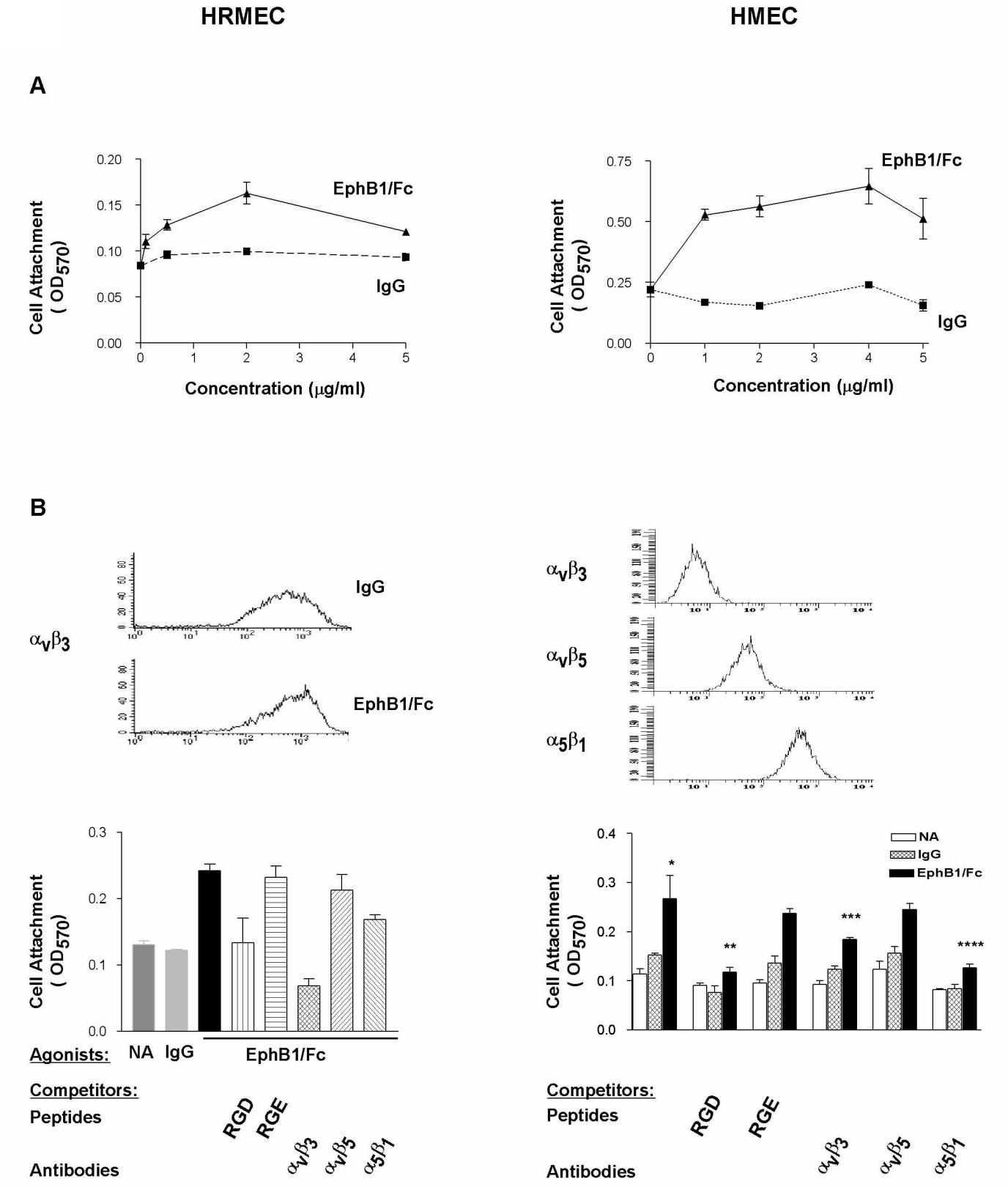
To assess whether ephrin-B1 signaling can alter endothelial cell-matrix attachment, we exposed endothelial cells to soluble EphB1/Fc and evaluated their attachment to fibrinogen-coated surfaces. Experiments were first performed in the primary HRMEC. A more extensive characterization of cell attachment was subsequently carried out in an endothelial cell line, human dermal microvascular endothelial cell (HMEC-1). EphB1/Fc stimulated cell attachment in both HRMEC and HMEC-1 in a dose-dependent manner (Fig. 3A). A class-matched human IgG_1 (Fc fusion control) was inactive at these concentrations, and anti-Fc-preclustered EphB1/Fc was functionally inactive as either a promoter or inhibitor of endothelial attachment to fibrinogen (data not shown). Thus, the dimeric form of EphB1 ectodomain (in the Fc fusion protein) promotes endothelial

Fig. 3. EphB1/Fc stimulates $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin-mediated endothelial cell attachment. (A) EphB1/Fc stimulates endothelial cell attachment to fibrinogen. 48-well plates were coated with fibrinogen (1 μg/cm^2), and endothelial cell attachment was assayed as described in the Materials and Methods. EphB1/Fc stimulated endothelial cell attachment at an optimal concentration of 2 μg/ml and 4 μg/ml for HRMEC and HMEC-1, respectively. An Fc fusion protein control, IgG, is inactive at these concentrations. (B) Upper panel, cell surface expression of integrin proteins in HRMEC and HMEC-1. Eph-B1/Fc does not increase the surface expression of endothelial $\alpha_v\beta_3$ in HRMEC cells (left panel). $\alpha_v\beta_3$ is expressed at a low level in HMEC-1 cells, whereas $\alpha_v\beta_5$ and $\alpha_5\beta_1$ are expressed at much higher levels, as assayed by FACS analysis (right panel). Lower panel, EphB1/Fc-induced endothelial cell attachment is mediated through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. Assays of endothelial cell attachment to fibrinogen-coated 48-well plates were performed as described in the Materials and Methods. Where indicated, cells were pre-incubated for 15 minutes at 22°C with blocking peptides (100 μg/ml) or anti-integrin antibodies (5 μg/ml) before plating. The data represent means±s.e.m. of three independent experiments. Group comparisons were performed using the Student's *t*-test. *P<0.05 versus IgG control; **P<0.05 versus RGE control; ***P<0.05 versus no inhibitor control, RGE control or avβ5 treated; ****P<0.05 versus no inhibitor control, RGE control or $\alpha_v\beta_5$ treated.

attachment. This effect of EphB1/Fc to promote endothelial attachment was also detected when EphB1/Fc is attached to solid phase surfaces within narrow surface densities in an alternative attachment assay (U.H.-D., unpublished) (Huynh-Do et al., 1999).

Increases in cell attachment stimulated by EphB1/Fc were sensitive to competition by a peptide containing RGD but not RGE sequences. Antibodies that block RGD engagement by $\alpha_v\beta_3$ integrin (LM609) or $\alpha_5\beta_1$ integrin (JBS5) attenuated EphB1/Fc-induced attachment to varying degrees, whereas an $\alpha_v\beta_5$ integrin blocking antibody was without effect (Fig. 3B, bottom panels). This is noteworthy as HRMEC express comparable levels of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Huynh-Do et al., 1999), whereas HMEC-1 cells express more $\alpha_v\beta_5$ than $\alpha_v\beta_3$ (Fig. 3B, top panels). As shown in Fig. 3., some of the basal endothelial attachment in this assay was also dependent upon $\alpha_v\beta_3$ integrin in HRMEC cells. The increase in $\alpha_v\beta_3$-integrin-mediated attachment elicited by EphB1/Fc occurs without changes in the abundance of surface-expressed $\alpha_v\beta_3$ integrin, as demonstrated by FACS analysis (Fig. 3B, top left panel) and recovery of surface biotinylated $\alpha_v\beta_3$ integrin by immunoprecipitation in HRMEC cells (data not shown).

To assess the potential biological activity of EphB1/Fc in a relevant in vivo assay, we implanted hydron pellets impregnated with vehicle control, bFGF (3 pmol), control IgG_1 (5.6 pmol) or EphB1/Fc (5.6 pmol) into mouse corneal micropockets. Neovascularization responses were scored by vital photography 5 days after implantation, and images were analyzed as described in the Materials and Methods. EphB1/Fc promoted consistent neovascularization responses that were not seen with the Fc fusion control IgG_1. This neovascularization response to EphB1/Fc was neutralized by coincident implantation of a pellet impregnated with ephrin-B1/Fc to interrupt EphB1/Fc interactions with endothelial ephrin-B1 (data not shown). Although EphB1-induced neovascularization responses were not as brisk as those evoked by bFGF, the fractional corneal area involved was 60%, and



the microvessel density within that area was also 60% of the bFGF response (Fig. 4). Thus, the EphB1 ectodomain is active as an agonist to promote angiogenic responses when presented in this context as a soluble Fc fusion protein in the corneal stroma. Similar neovascularization responses were evoked by ephrin-B1 and ephrin-B2 ectodomain Fc fusion proteins (H. Liu, unpublished), paralleling responses to ephrin-A1 observed previously (Pandey et al., 1995).

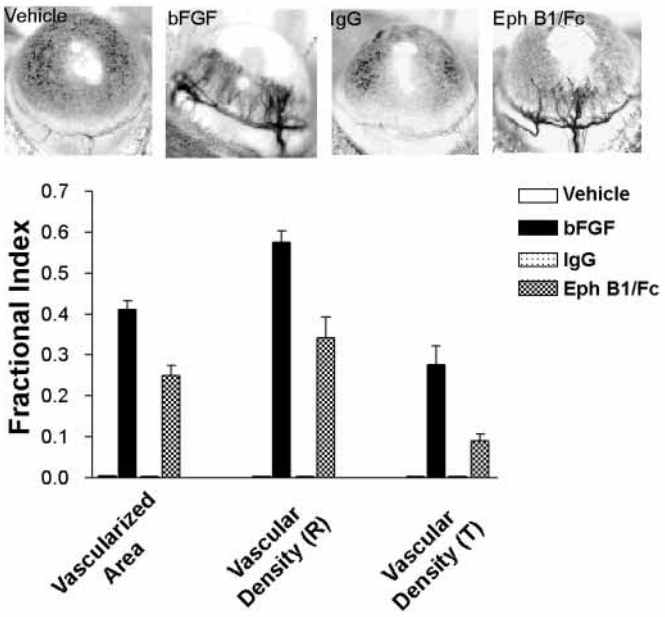


Fig. 4. EphB1/Fc stimulates mouse corneal angiogenesis. Hydron pellets impregnated with vehicle (PBS), bFGF (3.0 pmol), control IgG1 or EphB1/Fc (5.6 pmol) were implanted as described in the Materials and Methods and photographed at 5 days post-implantation. R, regional; T, total. The data are representative of four independent experiments.

We next addressed whether cytoplasmic domain sequences of Ephrin-B1 mediate EphB1/Fc-induced responses, as expected with 'outside-in' signaling processes. CHO cells do not express endogenous ephrin-Bs (1-3), as shown by FACS analysis, using EphB1/Fc as a molecular probe for ephrin-B counter-receptors (Fig. 5B, vector). Full-length ephrin-B1 (Fig. 5B, ephrin-B1) or cytoplasmic domain deletion versions lacking either the 50 (ephrin-B1ΔCy) or four C-terminal amino acids necessary for PDZ domain binding (ephrin-B1ΔPDZbd) were transiently expressed in CHO cells at comparable levels (Fig. 5B, FACS inserts). Migration responses for each CHO cell population to EphB1/Fc were evaluated using the planar wound closure assay. Cells expressing intact ephrin-B1 had increased rates of migration in response to EphB1/Fc at concentrations that are active on endothelial cells (Figs 2 and

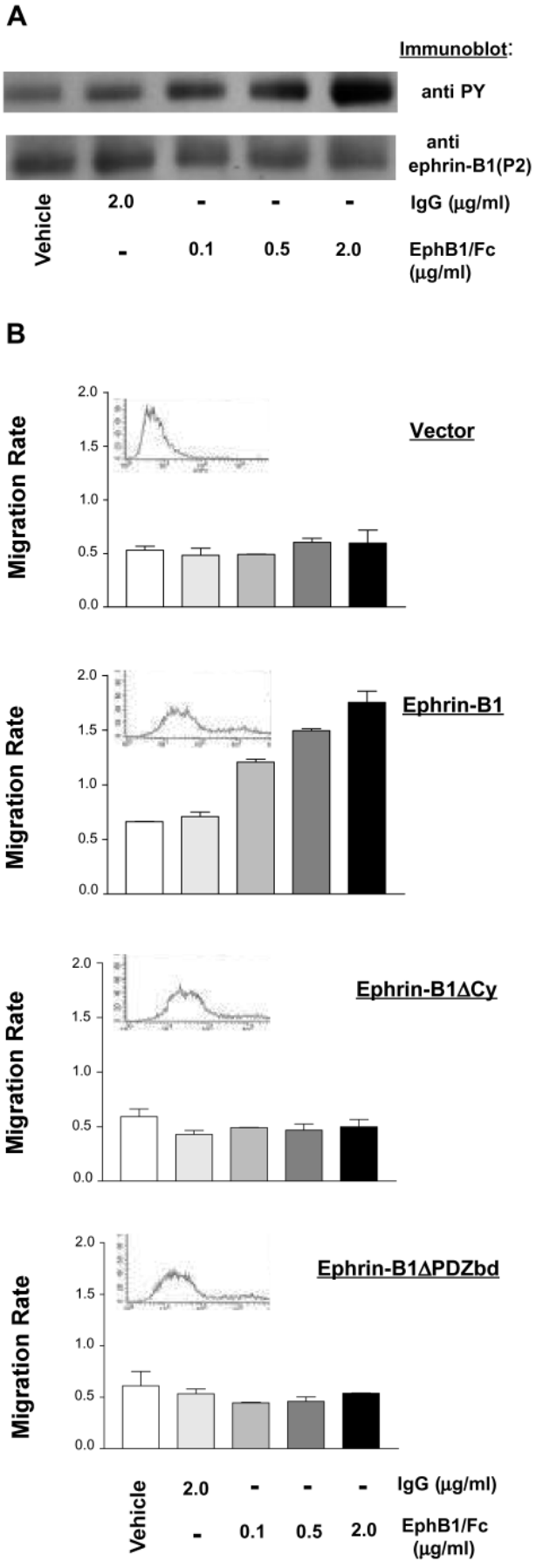


Fig. 5. EphB1/Fc stimulates migration of CHO cells transfected with ephrin-B1. (A) EphB1/Fc stimulates tyrosine phosphorylation of transfected ephrin-B1. CHO cells were transfected with plasmid expressing full-length ephrin-B1 as described in the Materials and Methods. 48 hours after transfection, cells were stimulated with the control IgG or EphB1/Fc (2 μg/ml), and ephrin-B1 was immunoprecipitated as in Fig. 1. Ephrin-B1 tyrosine phosphorylation (upper panel) was assessed by 4G10 immunoblot and recovery by anti-ephrin-B1 immunoblot (lower panel). (B) 48 hours after transfection with the plasmids indicated, wound closure assays were performed as described above. The migration rate is expressed as the percentage of closure/hour. The medium was replaced with serum-free medium (vehicle) or EphB1/Fc constructs as indicated in the bottom panel. Surface expression of ephrin-B1 ectodomain was analyzed by FACS analysis as described in the Materials and Methods. The data represent means±s.e.m of three independent experiments.

3) and that promote ephrin-B1 tyrosine phosphorylation in CHO cells (Fig. 5A). Neither of the cytoplasmic domain deletion forms of ephrin-B1, ephrin-B1ΔCy or ephrin-B1ΔPDZbd conferred EphB1/Fc responsiveness. Thus, CHO migration responses were strictly dependent upon integrity of the four most C-terminal amino acids, which are capable of interacting with PDZ domain proteins (Torres et al., 1998) and contain two tyrosine residues that are phosphorylated in vivo (Kalo et al., 2001).

Finally, we investigated signaling mechanisms that couple ephrin-B1 activation to cellular responses. As shown in Fig. 6, stimulation of CHO cells expressing wild-type ephrin-B1 with EphB1/Fc induced phosphorylation of p46 JNK in a time and dose-dependent manner, with the highest phosphorylation level at 10-45 minutes after stimulation. By contrast, activation of ephrin-B1 did not affect the phosphorylation status of ERK1/2 or p38 MAP kinases (data not shown). Furthermore, cytoplasmic domain deletion forms of ephrin-B1, ephrin-B1ΔCy or ephrin-B1ΔPDZbd failed to activate p46 JNK upon EphB1/Fc stimulation (Fig. 6C), suggesting that p46 JNK transduces signals in response to ephrin-B1 activation through the four most C-terminal amino acids of the ephrin-B1 protein.

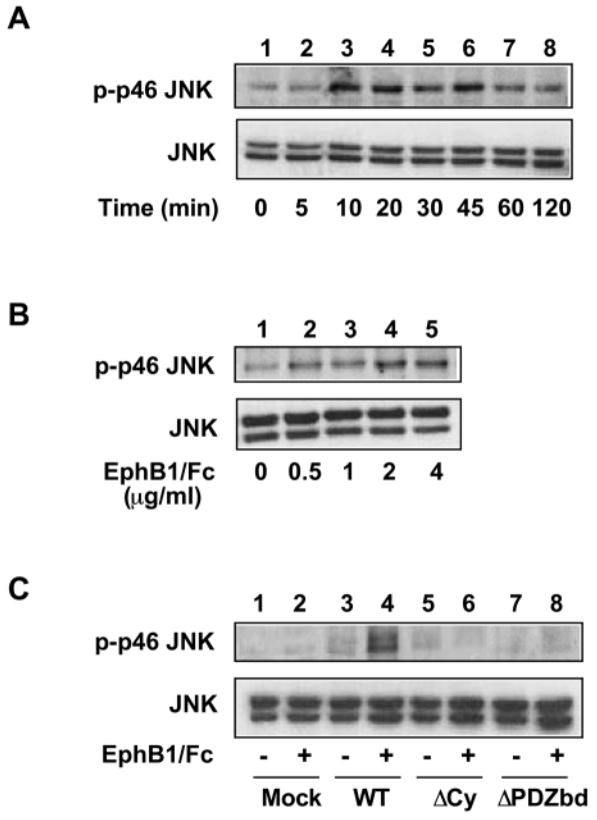


Fig. 6. EphB1/Fc stimulates phosphorylation of p46 JNK. (A) Kinetics of EphB1/Fc-induced JNK activation. CHO cells stably expressing ephrin-B1 were serum-starved overnight and stimulated with EphB1/Fc following a time course as indicated. Cells were lysed and 30 μg of protein was subjected to western blot analysis as described in the Materials and Methods. (B) Concentration dependence of EphB1/Fc-induced JNK activation. CHO cells stably expressing ephrin-B1 were treated with EphB1/Fc from 0 to 4 μg/ml for 20 minutes, and cell lysates were subjected to western blot analysis. (C) Ephrin-B1 C-terminal deletion mutants block EphB1/Fc-induced JNK activation. CHO cells transfected with vector (MOCK), full-length wild-type human ephrin-B1, ephrin-B1ΔCy or ephrin-B1ΔPDZbd were serum starved and stimulated with EphB1/Fc, and cell lysates were subjected to western blot analysis. The data are representative of three independent experiments.

Discussion

These findings provide direct evidence that ephrin-B1 transduces, through its cytoplasmic domain, signals that are coupled to JNK kinase activation and assayable integrin functions. In the systems evaluated here, ephrin-B1 functions not as a ligand but as a receptor to transduce 'outside-in' responses. In microvascular endothelial cells, EphB1/Fc induced ephrin-B1 tyrosine phosphorylation that correlated with increased endothelial attachment to fibrinogen. On the basis of the data gained using specific blocking antibodies, this attachment response is probably mediated by changes in the function of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. The abundance of surface $\alpha_v\beta_3$ was not affected, and immunoprecipitation experiments failed to demonstrate coprecipitation of ephrin-B1 with $\alpha_v\beta_3$ (data not shown), as was recently described for PDGF and insulin receptors (Schneller et al., 1997; Woodard et al., 1998).

$\alpha_v\beta_3$ integrin is implicated in tumor angiogenesis and in ocular neovascularization under conditions where its expression in endothelial cells is induced and where it probably binds to provisional extracellular matrix components, including fibrinogen and vitronectin (Brooks et al., 1994). A recent description of α_v knock-out mice provides evidence for a role in embryonic developmental vascularization (Bader et al., 1998), yet the vascular dysgenesis phenotype is less severe than that of the *ephrin-B2* gene deletion embryos (Wang et al., 1998). It seems likely that integrin responses downstream of ephrin-Bs are not limited to $\alpha_v\beta_3$, which is consistent with inhibition evoked by antibody antagonism of $\alpha_5\beta_1$ integrin (Fig. 3C). De novo ephrin-B1 expression in CHO cells conferred responsiveness to EphB1/Fc, as scored by migration, yet blocking antibodies active against the dominant integrin in these hamster cells, $\alpha_5\beta_1$, are unavailable. All these data argue for a function for ephrin-B1 cytoplasmic domain sequences in transducing a signal that is initiated by binding of EphB1 to the ephrin-B1 ectodomain and that is coupled to integrin-mediated attachment and migration. Our results are consistent with previous findings that engagement of class A ephrins, ephrinA5 and ephrinA2 by EphA-Fc reagents increased adhesion to integrin ligands in culture (Davy et al., 1999; Davy et al., 2000; Huai and Drescher, 2001).

Cell migration is a multi-step process involving lamellipodium extension, formation of new adhesions, cell body retraction and tail detachment. Cell migration requires the precise regulation of integrin-mediated adhesion and de-adhesion. Whether or not the cell migrates and the rate of the migration on a given substratum depend on several variables related to integrin-ligand interactions, including ligand levels, integrin levels and integrin-ligand binding affinities. At low ligand concentration (e.g. 1-10 μg/ml fibronectin for cells expressing α_5 integrin), increased adhesion leads to enhanced cell migration (Palecek et al., 1997). However, at high ligand concentration increased adhesion blocks cell migration (Palecek et al., 1997). Since EphB1/Fc did not affect the

expression level of integrin $\alpha_v\beta_3$ (Fig. 3B, left panel), EphB1/Fc-induced cell migration at low fibrinogen concentration (1 $\mu g/cm^2$) is probably caused by increased cell adhesion, possibly through enhanced integrin-ligand binding affinities.

The in vivo angiogenic response evoked by EphB1/Fc (Fig. 4) suggests that unliganded ephrin-B1 (or ephrin-B2 or B3) counter-receptors exist in the endothelium of the corneal limbus adjacent the cornea. Moreover, they appear to be competent to promote endothelial activation and neovascularization. In principle, EphB1/Fc acts either as an agonist for B-ephrins or as a blocking agent that antagonizes B-ephrin activity, leading to new blood vessel formation. However, B-ephrins also induce corneal angiogenesis (H.L. and T.O.D., unpublished). If EphB1/Fc antagonizes B-ephrins, it would block angiogenesis. The fact that EphB1/Fc induced, rather than inhibited, corneal neovascularization suggests that it is likely to act as an agonist for B-ephrins.

The capacity for full-length ephrin-B1, but not cytoplasmic domain deletion forms, to confer EphB1/Fc responsiveness in CHO cells provides strong evidence that cytoplasmic domain sequences participate in transducing signaling responses. The expressed ephrin-B1ΔCy lacks all of the five cytoplasmic domain tyrosine residues implicated in regulated phosphorylation, which are potential substrates for either c-src (Holland et al., 1996), FGF receptors (Jones et al., 1998) or other candidate tyrosine kinases. Further, deletion of the C-terminal four amino acids in ephrinB-1ΔPDZbd mutant removes PDZ domain protein-interacting sequences, as well as two of the five conserved tyrosine residues. This small deletion is sufficient to abrogate outside in signaling responses in the reconstituted CHO cell system, suggesting either a PDZ-domain-containing protein [such as PICK, syntenin, GRIP, or PDZ-RGS (Bruckner et al., 1999; Lu, 2001; Torres, 1998)] or other adaptor proteins capable of binding phosphorylated tyrosine residues within this region are critical to signaling process.

In summary, we provide evidence that ephrin-B1 transduces signals to activate JNK and modulate integrin-mediated cell attachment, migration and corneal angiogenesis. In the reciprocal direction, specifically oligomerized forms of ephrin-B1/Fc also engage EphB1 receptor tyrosine kinase to promote $\alpha_v\beta_3$ and $\alpha_5\beta_1$ activation (Huynh-Do et al., 1999). Interestingly, in this more classic signaling scenario, JNK also appeared to play a pivotal role linking EphB1 signaling to cytoskeletal responses (Stein et al., 1998). Such reciprocity of signaling correlates with the developmental vascularization defects shared between mice null for ephrin-B2 (Wang et al., 1998) and its binding partner, EphB4 (Gerety et al., 1999). Thus, both EphB receptors and ephrin-B counter receptors are involved in bidirectional signaling, which plays critical roles in vascular development in embryogenesis and possibly in adult angiogenesis as well.

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