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EphB4 signaling is capable of mediating ephrinB2-induced inhibition of cell migration

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Abstract

Signaling between the ligand ephrinB2 and the respective receptors of the EphB class is known to play a vital role during vascular morphogenesis and angiogenesis. The relative contribution of each EphB receptor type present on endothelial cells to these processes remains to be determined. It has been shown that ephrinB2–EphB receptor signal transduction leads to a repulsive migratory behavior of endothelial cells. It remained unclear whether this anti-migratory effect can be mediated by EphB4 signaling alone or whether other EphB receptors are necessary. It also remained unclear whether the kinase activity of EphB4 is pivotal to its action. To answer these questions, we developed a cellular migration system solely dependent on ephrinB2–EphB4 signaling. Using this system, we could show that EphB4 activation leads to the inhibition of cell migration. Furthermore we identified PP2, a known inhibitor of kinases of the Src family, and PD 153035, a known inhibitor of EGF receptor kinase, as inhibitors of EphB4 kinase activity. Using PP2, the inhibition of cell migration by ephrinB2 could be relieved, demonstrating that the kinase function of EphB4 is of prominent importance in this process. These results show that EphB4 activation is not only accompanying ephrinB2 induced repulsive behavior of cells, but is capable of directly mediating this effect.
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Keywords: EphB4; EphrinB2; Endothelial cell; MVEC; CHO; Angiogenesis; Migration; PP2; PD 153035

Members of the family of Eph receptor tyrosine kinases and their respective ligands, the ephrins, play crucial roles in pattern formation and morphogenesis during embryonal development [1–3]. Amongst others, Eph receptors and ephrins regulate cell migration, axon guidance, and angiogenesis [4–6]. In this signaling system, both the receptors and the ligands are bound to the cell membrane, restricting signal transduction to sites of close cell-to-cell contact [5,7]. Since ligand–receptor promiscuity is high in this family, the respective expression pattern of each family member ensures signaling specificity [3,7,8]. Reciprocal expression of ephrinB2 and EphB4 in arterial and venous endothelial cells, respectively, suggests that ephrinB2 and EphB4 might interact at the arterial–venous interface [9–11]. In the past few years it was shown that ephrinB2 expression on endothelial cells is essential for proper angiogenesis to occur [9,12] and that this cannot be compensated by ephrinB2 being present on surrounding mesenchymal cells [12,13]. In mice and *Xenopus*, ubiquitous and constitutive overexpression of ephrinB2 leads to abnormal growth of intersomitic veins [14–16] and it was suggested that this effect might be due to repulsive guidance mediated by ephrinB2 induced EphB receptor signaling. Recently it was shown that ephrinB2 induced signaling inhibited $VEGF_{165}$ and angiopoietin-1 mediated proliferation and migration of human umbilical vein endothelial cells [17] and possibly thereby regulates angiogenesis. EphrinB2 can bind to a variety of EphB receptors [1–3] and some of these receptors play also an important role during angiogenesis as demonstrated by double knockout of EphB2 and EphB3 [12] and the knockout of EphB4 [11]. The receptors EphB2, EphB3, and EphB4 all are expressed by endothelial cells in vivo and in vitro [3,17]. Although it is known that EphB4 plays an important role during embryonic and pathologic angiogenesis, so far it was unclear whether EphB4–ephrinB2 signaling is capable of directly regulating cell migration and whether EphB4 kinase activity is necessary for this effect.

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In this study, we first determined whether ephrinB2 has an anti-migratory effect on primary human dermal microvascular endothelial cells (MVEC). Once we demonstrated this effect, we identified inhibitors of EphB4 kinase activity, capable of reversing ephrinB2 mediated inhibition of endothelial cell migration. In order to clarify whether EphB4 can regulate cell migration, we developed a cellular migration system solely dependent on EphB4. Using this system, we show that EphB4 is capable of mediating such a cell migratory effect upon stimulation with ephrinB2.

Materials and methods

Cell culture. Primary human microvascular endothelial cells (MVEC) were prepared as described [18]. MVEC were cultured in flasks coated with 10 μg/ml collagen I (Serva) and in M199 medium (Biochrom) containing 10% fetal calf serum, 10% human serum, 2 mM glutamax, penicillin (100 μg/ml), streptomycin (100 μg/ml), ascorbic acid (1.27 mM), pyruvic acid (1 mM), 1% non-essential amino acids (Biochrom), 6 μg/ml endothelial growth factor from bovine brain (Sigma), and 7.5 μg/ml heparin (Sigma).

CHO-FRT cells were purchased from Invitrogen and maintained in uncoated culture flasks in DMEM/HAMS-F12, 10% fetal calf serum, 2 mM glutamax, penicillin (100 μg/ml), and streptomycin (100 μg/ml).

Generation of EphB4 expressing CHO cells. Full length human EphB4 was amplified from total RNA derived from MVEC using the primers EphForHind (5′-GCCAAGCTTGGCGCCATGGAGCTCC GGGT-3′) and EphRevEco (5′-CAGATATCTGTACTGCGGGGCC GGTCCT-3′). The resulting product was cloned into pcDNA3.1 myc/His A (Invitrogen) using *Hin*dIII and *Eco*RV sites resulting in an expressible fusion protein containing full length human EphB4 and a C-terminal myc- and His-tag. The resulting vector was sequenced to control for the correct EphB4 sequence. Using the sites *Hin*dIII and *Pme*I the whole fragment including the myc- and His-tag was excised and cloned into pcDNA5/FRT as part of the Flp-In System (Invitrogen). Using this EphB4-mycHis/pcDNA5/FRT construct CHO/FRT cells (Invitrogen) were stably transfected, cloned, and selected using 1200 μg/ml hygromycin B according to the Flip-In System protocol (Invitrogen). CHO/FRT cell lines expressing tagged EphB4 were stimulated with 1 μg/ml mouse ephrinB2-Fc (R&D Systems) and a CHO/FRT cell line was identified that expressed tagged human EphB4 and showed inducible autophosphorylation of EphB4 upon stimulation with mouse ephrinB2-Fc. This cell line was called CHO/EphB4.

Immunoprecipitation and Western blot. The 1×10^6 cells were seeded onto a 10 cm dish and allowed to adhere. The medium was changed to serum-free (CHO) or serum reduced (MVEC) medium and cultured overnight. The medium was changed to serum-free medium containing the respective compounds and the cells were incubated for 1 h at 4 °C. The cells were lysed in RIPA lysis buffer (50 mM Hepes, pH 7.2, 10 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% deoxycholate, 50 mM Na-pyrophosphate, 100 mM Na-fluoride, 1 mM Zn-acetate, 2 mM orthovanadate, and complete proteinase inhibitor (Roche)). The lysates were homogenized using a syringe with 18 G needle, the samples were microfuged for 1 min and the supernatants were pre-incubated with 20 μl protein G–Sepharose (Pharmacia Biotech) for 2 h at 4 °C with agitation. The Sepharose was removed by centrifugation and the samples were incubated with the respective first antibody (anti-EphB4 antibody C-16 (Santa Cruz) or anti-c-myc antibody (Roche)) for 2 h at 4 °C with agitation. The 20 μl/sample protein G–Sepharose was added and the samples were incubated overnight at 4 °C with agitation. The samples were washed three times in lysis buffer and the final pellet was resuspended in 50 μl sample buffer, boiled for 5 min, and subjected to PAGE. The gels were blotted onto Immobilon-P Transfer Membrane (Millipore) and blocked in 3% TOP BLOCK (Fluka). The membranes were incubated with the respective antibodies (anti-P-Tyr-HRP 1:20,000 (upstate), anti-EphB4 C-16 (Santa Cruz), and anti-c-myc antibody (Roche)), developed using ECL plus (Amersham), and exposed to Hyperfilm ECL (Amersham).

MVEC proliferation. The 30,000 MVEC per well were plated into a 24-well plate in the appropriate full medium. After 2 h the medium was serum reduced to 2% human serum and cells were grown overnight. Next morning medium was changed to fresh medium containing 2% human serum. Various amounts of growth factors or compounds as indicated were added ($VEGF_{165}$ (ProQinase, Freiburg), mouse ephrinB2-Fc (R&D Systems), and mouse EphB4-Fc (R&D Systems)). After 3 days in culture 1:100 (v/v) alamar Blue (Biosource) was added to the cells. The cells were incubated for 2 h and then the fluorimetric changes were measured using a Cytofluor 2350 reader (Millipore). All experiments were done at least in triplicate.

MVEC migration. Endothelial cells were passaged and maintained overnight in the appropriate full medium without growth factors supplemented. The next day cells were trypsinized and ten thousand cells in 100 μl M199 containing 2% human serum and glutamine were seeded on a culture well insert containing a porous filter bottom (8 μm, Costar) that had been washed with PBS and coated with collagen (10 μg/ml) previously. In the lower chamber is 600 μl of M199 with 2% human serum. The cells were allowed to attach for 2 h. Then $VEGF_{165}$ (10 ng/ml) and compounds were added to the lower chamber and the cells were incubated for an additional 18 h. The inserts were rinsed with PBS and stained by immersion into a PBS solution containing 1% rose bengal in 30% ethanol. Excess rose bengal was removed by washing with PBS. Cells from the upper side of the insert were removed using cotton swaps. For quantification randomized inserts were evaluated under the microscope counting three fields each. All experiments were done at least in triplicate.

ELISA-EphB4 autophosphorylation. As much as 400,000 CHO/EphB4 cells per well were seeded into a 24-well plate and cultured overnight in full medium. The cells were washed once with PBS and the medium was changed to serum-free medium containing the respective additives like mouse ephrinB2-Fc (R&D Systems) and PP2 [19]. The cells were incubated for 1 h at 4 °C and then cells were lysed in 100 μl/well lysis buffer (50 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM $Na_4P_2O_7$, 100 mM NaF, 10% glycerin (v/v), 1.5% Triton X-100, 2 mM orthovanadate, and complete proteinase inhibitor (Roche), adjusted to pH 7.4). ELISA 96-well plates (Lumitrac 600, Greiner) were coated with 100 μl/well anti-c-myc antibody (5 μg/ml; Roche) overnight at 4 °C, blocked with 250 μl/well 3% TOP BLOCK (Fluka) for 3 h at room temperature, and incubated with 100 μl/well cell lysate overnight at 4 °C. ELISA plates were washed twice with PBS and incubated with 100 μl/well anti-P-Tyr-HRP antibody (upstate) diluted 1:10.000 in 3% TOP BLOCK overnight at 4 °C. ELISA plates were washed three times with PBS and developed with 100 μl/well BM Chemiluminescence ELISA substrate POD (Roche). Luminescence was measured using a LumiCount device (Packard).

HTRF-EphB4 tyrosine kinase assay. Human recombinant GST-tagged EphB4 intracellular kinase domain (Proqinase) was incubated for 20 min at 20 °C with 2.7 μg/ml biotinylated polyGAT (CisBio) in the presence of 2 μM ATP in a buffer containing 5 mM Hepes (pH 7.3), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, 0.1 mM $NaVO_4$, 1% (v/v) glycerin, 0.02% NP40, and EDTA-free complete protease inhibitors (Roche). The reaction (30 μl) was terminated with 50 μl of a solution containing 50 mM Hepes, pH 7.0, 0.2% (w/v) BSA, 0.14 μg/ml PT66-Eu (Wallac), 3.84 μg/ml streptavidin-XL665 (CisBio), 75 mM EDTA, and incubated for 60 min. Homogeneous time-resolved fluorescence (HTRF) was measured in a Discovery instrument (Packard) by excitation at 337 nm and emission at 615 and 665 nm. The ratio of the signal at 665 nm (specific FRET signal) divided by the signal at 615 nm (internal control) was calculated. These values were normalized using positive control (with enzyme) and negative control (without enzyme). PP2 inhibition plots were evaluated using the Grafit 4 Software (Erithacus).

CHO migration. CHO cells were passaged, after adherence the medium was changed to serum-free medium, and the cells were incubated overnight. Transwell filters (8 μm, Costar) were coated with 5 μg/ml gelatine in PBS for 2 h. The 20,000 cells were seeded on top of each filter in 100 μl serum-free medium. The bottom was filled with 600 μl full medium including the respective compounds. The cells were allowed to migrate for 6 h at 37 °C, and then the filters were stained for 10 min in 1% bengal rose in 30% ethanol and washed three times in PBS. Cells from the upper side of the insert were removed using cotton swaps. For quantification randomized inserts were evaluated under the microscope counting three fields each. All experiments were done at least in triplicate.

Table 1
Sequences of primers used for RT-PCR

Primer	Sequence
EphB4_F	TTCCTGCGGCTAAACGACGG
EphB4_R	TTGCTCATGTCCCAGTACGGC
EphB3_F	CCCGATGAGAGCTTCTCGCG
EphB3_R	GCACCTCAAAGGTGTAGCGCGT
EphB2_F	GAGAAGGAGCTCAGTGAGTACAACGC
EphB2_R	TGTAGGTGGGGTCTGAGGTATCGTC
EphB1_F	CCTTGGTGGCCATCTCTATCGTC
EphB1_R	GATCCCATAGCTCCAAACGTCG

Results

MVEC express EphB4 and other EphB receptor family members

Since ephrinB2, the only known ligand for EphB4, was shown to bind to a variety of Eph receptors of class B [1–3], we elucidated the expression pattern of EphB receptors on MVEC. We designed specific primers for several members of the EphB receptor family (see Table 1 for primer sequences) and performed RT-PCR for each set with human brain cDNA as positive control. As summarized in Fig. 1, EphB1, EphB2, EphB3, and EphB4 mRNA was detectable in MVEC. This expression pattern is identical to that recently published for human umbilical vein endothelial cells [17]. In order to ensure the presence of EphB4 protein on MVEC, a Western blot experiment was performed. As shown in Fig. 2A, we detected EphB4 protein in MVEC lysate.

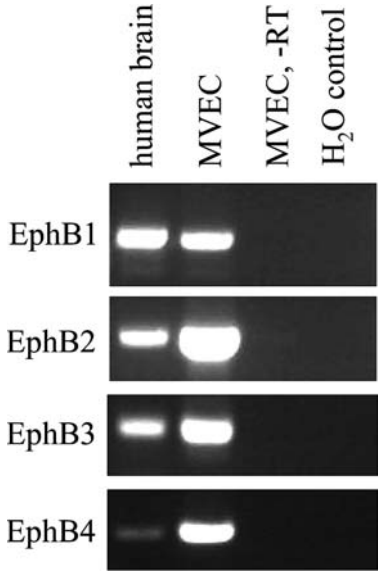

Fig. 1. MVEC express mRNA for EphB1–4. RT-PCRs were performed using primers specific for EphB1, EphB2, EphB3, and EphB4 (primers, see Table 1). Human brain cDNA was used as positive control and as negative control MVEC reverse transcription sample not treated with reverse transcriptase and H_2O control were used. EphB1–4 cDNA was present in the positive control and in MVEC, but not in negative controls.

Stimulation with mouse ephrinB2 induces autophosphorylation of human EphB4 and leads to inhibition of MVEC migration

EphrinB2 triggers autophosphorylation of EphB4 if it is present in oligomeric, at least dimerized, form, like it occurs in lipid rafts of the cell membrane [3,9,12]. In our experiments, we used the extracellular part of mouse ephrinB2 fused to the Fc portion of an IgG. As shown in Fig. 2B, we were able to induce human EphB4 autophosphorylation in MVEC in response to mouse ephrinB2-Fc. Clustering of ephrinB2-Fc by anti-Fc antibodies did not further influence the induction of EphB4 autophosphorylation (data not shown). Since mouse ephrinB2-Fc is active on human EphB4, we performed MVEC proliferation and migration assays in response to different amounts of mouse ephrinB2-Fc. EphrinB2 alone had no effect on proliferation and migration of MVEC (data not shown). Since $VEGF_{165}$ is a known inducer of endothelial cell proliferation and migration, we repeated the MVEC proliferation and migration assays in the presence of $VEGF_{165}$. As shown in Fig. 4A, ephrinB2 had no effect on $VEGF_{165}$ stimulated MVEC proliferation. EphrinB2 inhibited the $VEGF_{165}$ induced migration of MVEC in a dose-dependent manner (Fig. 4B). This inhibition can be neutralized by pre-incubating ephrinB2 with soluble EphB4-Fc protein. Oligomerization of ephrinB2-Fc by anti-Fc antibodies did not further influence the migration of MVEC. These results demonstrate that the inhibition of MVEC migration is due to ephrinB2 specific induction of signaling events in MVEC and not to any contaminants in the ephrinB2 probe.

PP2 and PD 153035 are inhibitors of recombinant soluble EphB4 kinase domain and PP2 reverses ephrinB2 effect on MVEC migration

In order to identify a potent inhibitor of the EphB4 kinase domain, we set up a homogeneous assay using

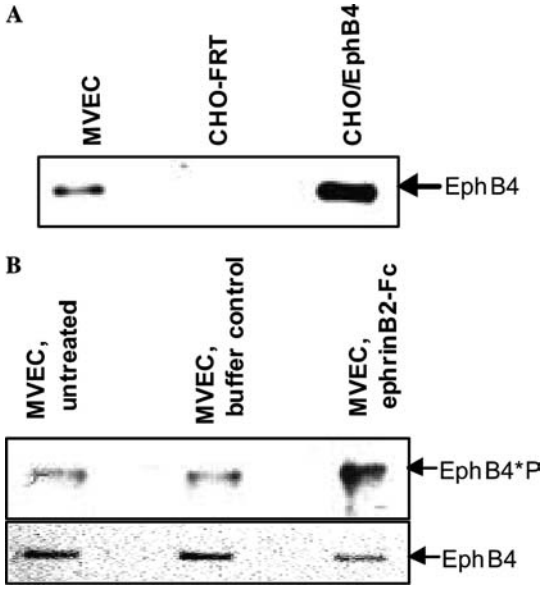

Fig. 2. Human full length EphB4 protein is present on MVEC and CHO/EphB4, EphB4 phosphorylation in MVEC is inducible via ephrinB2. (A) Cell lysates of MVEC, CHO/FRT, and CHO/EphB4 were separated by PAGE and transferred to PVDF membrane. EphB4 protein was detected using the anti-EphB4 antibody C-16 (Santa Cruz) and EphB4 protein was detectable in MVEC, absent from CHO/FRT and strongly present in CHO/EphB4. (B) MVEC remained untreated were treated with buffer control or 1 μg/ml mouse ephrinB2-Fc. The cells were lysed and subjected to EphB4 specific immunoprecipitation. Phosphorylated EphB4 protein was detected by Western blot using an anti-P-Tyr-HRP antibody. Untreated and buffer control treated MVEC show background levels of phosphorylated EphB4. MVEC treated with ephrinB2 show elevated EphB4 phosphorylation. Presence of comparable amounts of EphB4 in all samples was checked by probing the same blot with anti-EphB4 antibody (H-200).

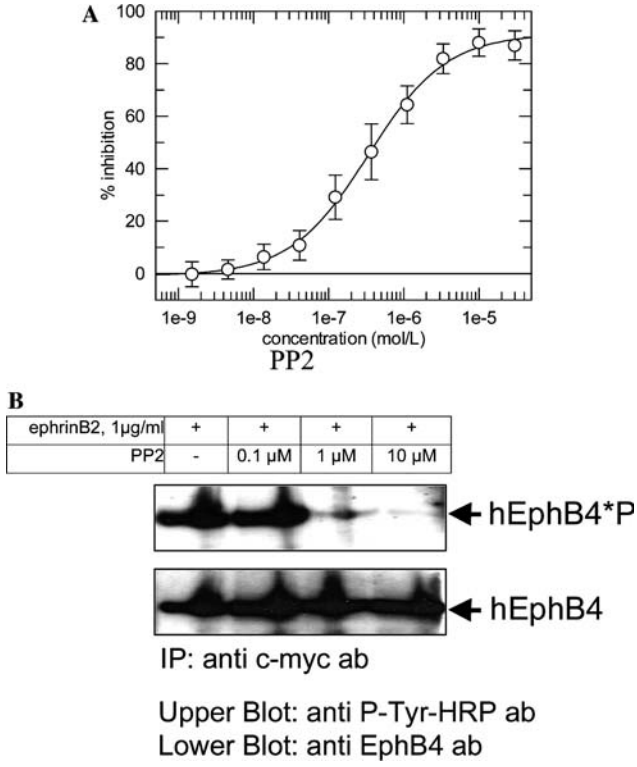

Fig. 3. PP2 inhibits EphB4 kinase in vitro and in vivo. (A) Dose-dependent inhibition of soluble recombinant human GST-EphB4 kinase domain by PP2 resulted in an IC50 of ~340 nM. (Bars represent means ± SD of four separate experiments with two datapoints per concentration.) (B) CHO/EphB4 cells were stimulated with 1 μg/ml ephrinB2-Fc and different concentrations of PP2 as indicated. The cells were lysed and phosphorylation of EphB4 was shown by immunoprecipitation (anti-c-myc antibody), subsequent Western blotting and detection using an anti-P-Tyr-HRP antibody. Equal presence of EphB4 protein was demonstrated by probing the blot with anti-EphB4 antibody. PP2 is able to enter the cells and inhibit EphB4 kinase activity in vivo with a comparable IC50 of ~0.3 μM.

recombinant soluble GST-EphB4 kinase domain and polyGAT as a substrate. We could show that PP2, a known inhibitor of the Src-family of kinases [19], inhibits GST-EphB4 kinase in this system with an IC50 of ~340 nM (Fig. 3A). As shown in Fig. 3B, PP2 is not only able to inhibit EphB4 kinase activity in vitro but also inhibits EphB4 autophosphorylation in a cellular system. Further PP2 was capable of reversing the ephrinB2 induced inhibition of VEGF stimulated MVEC migration (Fig. 4C). PP2 alone or in combination with VEGF had no additional effect on MVEC migration. Further, we identified a second inhibitor of EphB4 kinase activity, PD 153035, a known inhibitor of EGF receptor kinase [20]. As summarized in Table 2, both structurally unrelated inhibitors differ in their specificity to other kinases, e.g., to c-Src. In subsequent investigations both inhibitors were used in separate sets of experiments in order to exclude the possibility that the observed effects may be due to inhibition of unrelated kinases.

Generation of a CHO cell line expressing functional human EphB4

EphrinB2 can bind to a variety of EphB receptors. Since MVEC express EphB1–4, it is not possible to decide whether its inhibitory effect on MVEC migration is mediated by EphB4 signaling or by other EphB receptors. To address this question, we generated an unrelated, non-human, non-endothelial cell line expressing only human EphB4, but not the other human EphB receptors. Since the genomic site of integration can alter the behavior of a cell line, we chose the Flip-In System (Invitrogen). Using this system, we were able to produce a cell line that integrated the gene of interest at a defined site in the genome, where the parental Chinese hamster ovary (CHO) cell line, CHO/FRT, also contains an insert comprising a Neo resistance gene. We included a c-myc tag at the C-terminus of human EphB4 full length protein. As shown in Fig. 2A, we were able to identify a cell line that shows

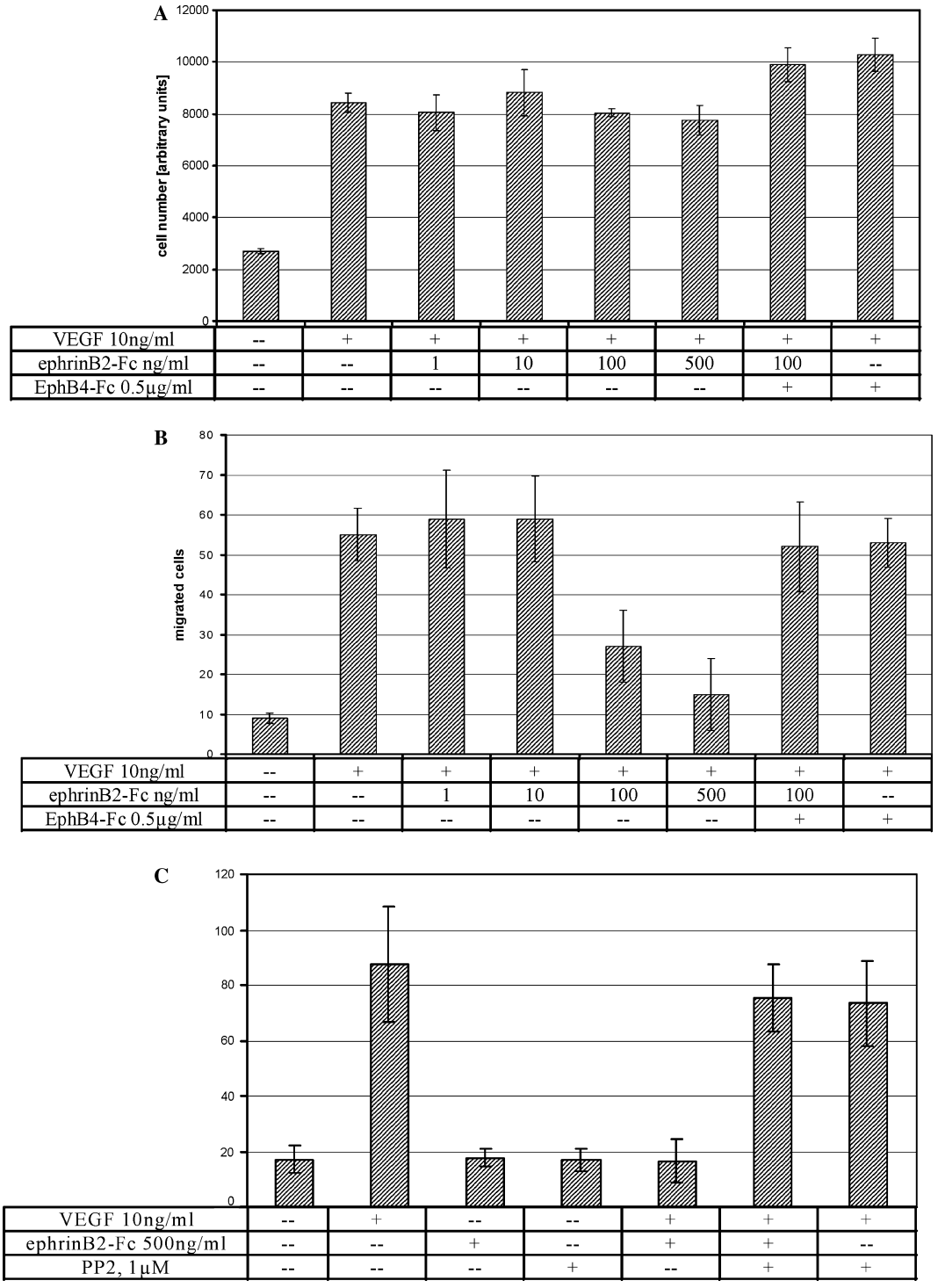

Fig. 4. EphrinB2 stimulation of MVEC does not affect VEGF induced proliferation and inhibits VEGF stimulated MVEC migration. (A) MVEC were treated as indicated and cell proliferation was measured after 3 days. EphrinB2 had no significant influence on VEGF induced proliferation of MVEC. (B) MVEC were treated as indicated and migrated cells were counted. EphrinB2 inhibited VEGF stimulated cell migration in a concentration-dependent manner. The ephrinB2 induced inhibition was neutralized by pre-incubating ephrinB2 with soluble EphB4-Fc. Soluble EphB4-Fc alone had no effect on VEGF induced MVEC migration. (C) MVEC were treated as indicated and migrated cells were counted. VEGF stimulated migration of MVEC, whereas ephrinB2 and PP2 alone had no effect on baseline migration. PP2 reversed ephrinB2 mediated inhibition of VEGF induced MVEC migration, but had no effect on MVEC migration stimulated by VEGF alone. (Bars represent means ± SD of at least three experiments.)

EphB4 expression and called it CHO/EphB4. In order to test whether EphB4 is functional in CHO/EphB4, we treated the cells with different amounts of mouse ephrinB2-Fc. As shown in Fig. 5, we could induce EphB4 autophosphorylation in CHO/EphB4 in a dose-dependent manner. Again, we showed that this

Table 2
IC50 values for PP2 and PD 153035

	PP2 (μM)	PD 153035 (μM)
c-Src, isolated kinase	~0.03	~20
EphB4, isol. kinase	~0.34	~3
ELISA, cellular EphB4	~0.3	~1
CHO/EphB4 migration	~0.3	N.A.

induction of EphB4 autophosphorylation can be inhibited by pre-incubating ephrinB2 with soluble EphB4-Fc.

PP2 and PD 153035 inhibit cellular EphB4 kinase activity dose dependently

We translated the above-mentioned immunoprecipitation assay into an ELISA format and demonstrated a clearly concentration-dependent induction of EphB4 autophosphorylation by ephrinB2, reaching a plateau at ~1 μg/ml ephrinB2 (Fig. 6A). Stimulating CHO/EphB4 with 1 μg/ml ephrinB2 pre-incubated with different amounts of soluble EphB4-Fc, we showed that a dose-dependent inhibition of EphB4 autophosphorylation is

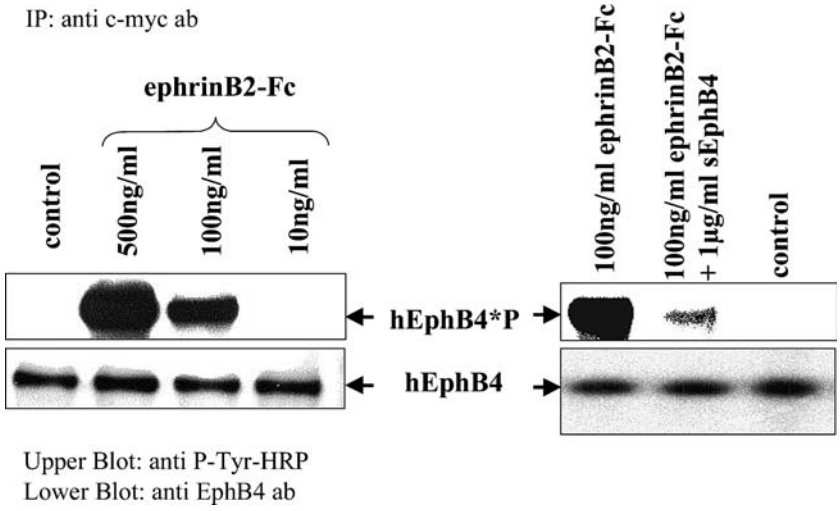

Fig. 5. CHO/EphB4 express functional human EphB4. CHO/EphB4 were treated with different amounts of ephrinB2-Fc and phosphorylation of EphB4 was shown by immunoprecipitation (anti-c-myc antibody), subsequent Western blotting and detection using an anti-P-Tyr-HRP antibody. EphB4 autophosphorylation could be induced by ephrinB2-Fc in a dose-dependent manner. The induction of EphB4 autophosphorylation was inhibited by pre-incubating ephrinB2-Fc with soluble EphB4-Fc. Presence of comparable amounts of EphB4 in each sample was checked by probing the same blot with an anti-EphB4 antibody (H-200).

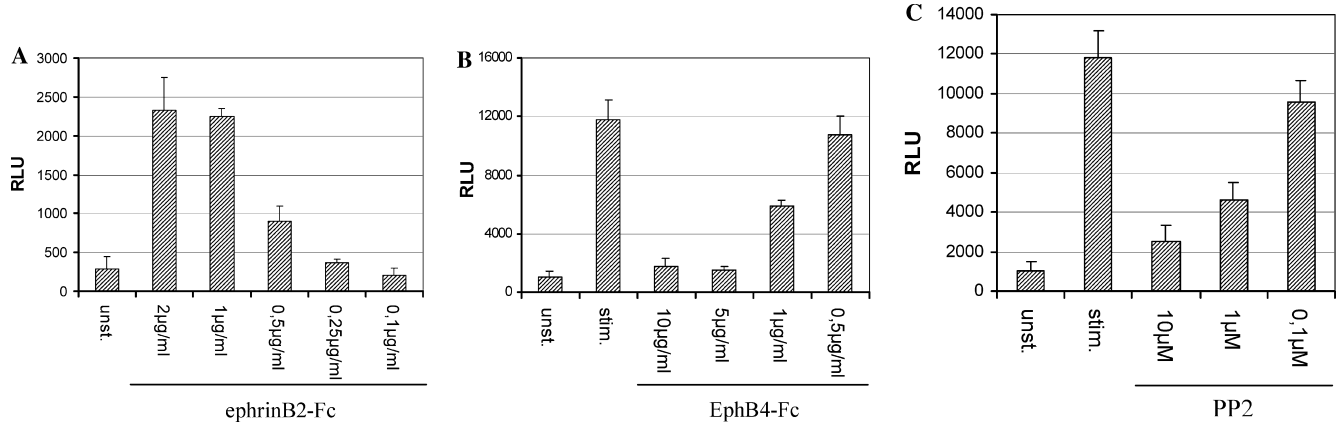

Fig. 6. Identification of PP2 as a cell penetrating EphB4 kinase inhibitor. (A) CHO/EphB4 cells were treated with raising amounts of ephrinB2, lysed, and subjected to ELISA assay as described. EphrinB2 induced EphB4 autophosphorylation was increased dose dependently and reached a plateau at 1 μg/ml ephrinB2. (B) CHO/EphB4 cells were treated with 1 μg/ml ephrinB2 pre-incubated with the given amounts of soluble EphB4-Fc and EphB4 autophosphorylation was measured using the ELISA assay. Soluble EphB4-Fc inhibited EphB4 autophosphorylation dose dependently with an IC50 of ~1 μg/ml. (C) CHO/EphB4 cells were pretreated with given amounts of PP2 for 10 min at 4 °C, and then ephrinB2-Fc was added to a final concentration of 1 μg/ml. EphB4 autophosphorylation was measured by ELISA. The isolated EphB4 kinase inhibitor, PP2, inhibited also cellular EphB4 autophosphorylation in a concentration-dependent manner with an IC50 of ~300 nM. (Bars represent means ± SD of at least three experiments.)

detectable and measurable with this system (Fig. 6B). These results were confirmed by accompanying semi-quantitative Western blot analysis to validate the ELISA format (data not shown). The newly identified inhibitors of soluble EphB4 kinase domain, PP2 and PD 153035, also inhibited cellular EphB4 receptor autophosphorylation with an IC50 of ~300 nM and ~1 μM, respectively (Fig. 6C and Table 2). The IC50 values measured in both assays, the in vitro soluble EphB4 kinase assay and the cellular EphB4 kinase assay, are comparable for each inhibitor. These results provide evidence that inhibition of EphB4 phosphorylation in the cellular assay system is facilitated by PP2 and PD 153035 mediated inhibition of EphB4 kinase activity, respectively.

EphB4 signaling in CHO cells leads to inhibition of CHO migration

EphrinB2 inhibited $VEGF_{165}$-dependent migration of MVEC. To elucidate whether ephrinB2–EphB4 signaling can lead to inhibition of cell migration, we investigated ephrinB2 mediated effects on cell migration by using CHO/EphB4 cells expressing only EphB4 but not the other human EphB receptors. As shown in Fig. 7A, full serum stimulated migration of the parental cell line, CHO/FRT, was not influenced by different amounts of ephrinB2. Full serum stimulated migration of CHO/EphB4 was inhibited in a dose-dependent manner by ephrinB2 (Fig. 7B). This inhibition could be reversed by pre-incubating ephrinB2 with soluble EphB4-Fc. To further confirm that this effect is due to EphB4 kinase activity, we treated CHO/EphB4 cells with ephrinB2 and increasing amounts of PP2. As shown in Fig. 7C, administration of PP2 neutralized the ephrinB2 induced inhibition of CHO/EphB4 migration in a dose-dependent manner with an IC50 of ~300 nM, which is comparable to those IC50 values found in the other two EphB4 kinase activity assays (see Table 2). In the concentrations used, PP2 had no effect on migration of full serum stimulated CHO/FRT (data not shown) and CHO/EphB4 in the absence of ephrinB2, respectively (Fig. 7C). These results indicate that EphB4 kinase activity is necessary for inhibition of cell migration. PD 153035 showed effects on migration of CHO/FRT and CHO/EphB4 in the absence of ephrinB2. Therefore PD 153035 was not used to measure an IC50 value for relief of ephrinB2 induced inhibition of CHO/EphB4 migration.

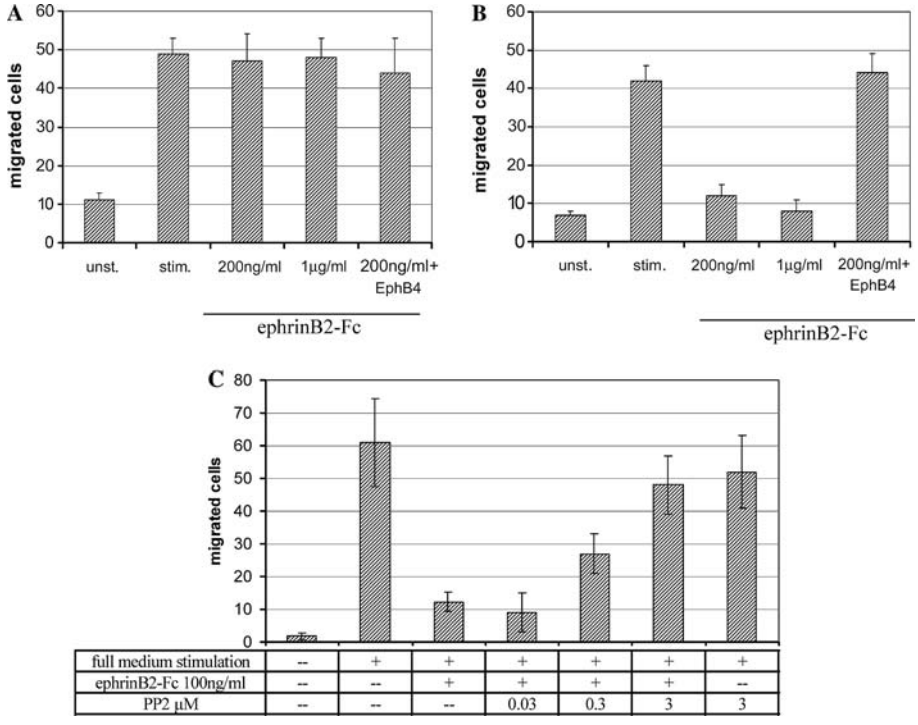

Fig. 7. EphrinB2 inhibits migration of CHO/EphB4 via EphB4 kinase activity. (A) The parental cell line CHO/FRT was stimulated with full serum containing medium and migration was measured in response to different amounts of ephrinB2-Fc. CHO/FRT migration was not influenced by ephrinB2 at all, nor by EphB4-Fc. (B) Migration of CHO/EphB4 was investigated in response to different amounts of ephrinB2-Fc. EphrinB2 inhibited full serum containing medium induced migration of CHO/EphB4 dose dependently and this inhibition was neutralized by pre-incubating ephrinB2 with 1 μg/ml soluble EphB4-Fc. (C) CHO/EphB4 cells were treated as described and migrated cells were counted. The inhibitor of EphB4 kinase activity, PP2, reversed ephrinB2 mediated inhibition of cell migration dose dependently with an IC50 of ~300 nM. (Bars represent means ± SD of at least three experiments.)

Discussion

Primary endothelial cells in culture express, like endothelial cells in vivo, several EphB receptors, all capable of binding ephrinB2 ligand [3,12,17]. MVEC used in this study were shown to express EphB1–4, so that it is not possible to attribute ephrinB2 induced effects directly to one of its receptors. Further, we demonstrated that mouse ephrinB2-Fc dimers are functional on human cells and lead to increased autophosphorylation of EphB4 in human MVEC. Interestingly, ephrinB2 stimulation alone had no effect on basal MVEC proliferation and migration. It was necessary to stimulate endothelial cells first with $VEGF_{165}$ to induce proliferation and migration. Under these conditions, stimulation with ephrinB2 led to the inhibition of $VEGF_{165}$ induced MVEC migration whereas proliferation remained unaffected. These results confirm the recent findings that describe migration inhibitory effects of ephrinB2 on endothelial cells in vitro and in vivo [14–17]. Kim et al. described anti-proliferative effects of ephrinB2 on human umbilical vein endothelial cells (HUVEC) in addition to the inhibition of $VEGF_{165}$ induced migration. Recently Maekawa et al. [21] described stimulatory effects of ephrinB2 on HUVEC migration. The reason for the differences observed is not known and might be due to the different cells used (MVEC in this study vs. HUVEC by Maekawa et al. and Kim et al.), differences in pre-conditions during cell culture or the use of VEGF that was necessary in our studies and was used by Kim et al. to demonstrate the effects described. It might be speculated that it is the growth factor activated but not the resting endothelial cell that is particularly responsive to ephrinB2–EphB4 signaling as it occurs during angiogenesis and vessel reorganization. However, these differences do not affect the conclusions reached in this manuscript.

It is known that EphB4 plays an important role during angiogenesis, but it remained unclear whether EphB4–ephrinB2 signaling is directly capable of regulating cell migration and whether EphB4 kinase activity is necessary for this effect. To answer this question, we identified two inhibitors of soluble EphB4 kinase activity, PP2 and PD 153035, that inhibited EphB4 kinase activity in vitro and in vivo with comparable IC50 values, respectively (summarized in Table 2). Both molecules inhibit a different spectrum of other kinases, providing evidence that the observed effects are due to inhibition of EphB4 kinase activity and are not mediated by inhibition of unrelated kinases like Src.

Since PD 153035 is also a potent inhibitor of VEGFR II (IC50 for EphB4 and VEGFR II is identical), a VEGF-dependent MVEC migration experiment demonstrating PD 153035 mediated relief of ephrinB2 induced inhibition of MVEC migration is not feasible. By using PP2, we showed that PP2 is able to reverse ephrinB2 induced inhibition of VEGF stimulated MVEC migration, but it remained unclear whether this was due to inhibition of EphB4 or other EphB receptors. Due to the high conservation of the kinase domain within the family of EphB receptors, PP2 may also inhibit other EphB receptors in addition to the EphB4 inhibition. A cellular migration system solely dependent on EphB4 was needed to clarify this question.

In order to investigate whether EphB4 is capable of regulating cell migration, we developed a cellular migration system solely dependent on ephrinB2–EphB4 signal transduction. Since cellular clones often show differences in behavior compared to the parental cell line, we chose CHO-FRT cells and the Flip-In System (Invitrogen) to generate an EphB4 expressing cell line bearing the expression cassette at the identical genomic localization where the parental cell line had incorporated an antibiotic resistance gene. This CHO/EphB4 cell line expresses full length human EphB4. We demonstrated that EphB4 is functional in CHO/EphB4 and that EphB4 autophosphorylation in these cells can be stimulated with ephrinB2 in a dose-dependent manner. In subsequent migration experiments, we could show that migration of CHO/EphB4 cells was inhibited by ephrinB2, whereas CHO-FRT cells did not respond to ephrinB2 stimulation at all. These results provide evidence that EphB4 alone is capable of triggering the regulation of cell migration and does not need the presence of other EphB receptors.

The relevance of the EphB4 kinase functionality in cell migration was investigated by the use of the kinase inhibitor PP2, which reversed the ephrinB2 induced inhibition of cell migration. Since PP2 inhibited EphB4 kinase activity in vitro and in vivo with an IC50 comparable to the IC50 of restoration of ephrinB2 inhibited cell migration, evidence is provided that the EphB4 kinase activity is necessary for this effect.

During creation of CHO/EphB4, a c-myc tag was introduced at the C-terminus of EphB4. This c-myc tag may influence the functionality of the PDZ domain located at the C-terminus of EphB4. Since the functionality of EphB4 with respect to regulation of cell migration remained unaffected, the PDZ domain binding motif may not be essentially required for the activity of this receptor on cell migration.

In summary our results show for the first time that ephrinB2 induced inhibition of cell migration can be mediated directly by activation of EphB4. EphB4 activation by ephrinB2 is sufficient to induce inhibition of cell migration and does not necessarily depend on other EphB receptors. Furthermore, we demonstrated that EphB4 kinase activity is necessary for this effect. The functions of the other receptors of the EphB class on endothelial cells remain to be determined, especially EphB2 and EphB3, which are known to play an important role in vessel formation [12].

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