Article

EphB1 recruits c-Src and p52^{Shc} to activate MAPK/ERK and promote chemotaxis

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Eph receptors and their ligands (ephrins) play an important role in axonal guidance, topographic mapping, and angiogenesis. The signaling pathways mediating these activities are starting to emerge and are highly cell- and receptor-type specific. Here we demonstrate that activated EphB1 recruits the adaptor proteins Grb2 and p52^{Shc} and promotes p52^{Shc} and c-Src tyrosine phosphorylation as well as MAPK/extracellular signal–regulated kinase (ERK) activation. EphB1-mediated increase of cell migration was abrogated by the MEK inhibitor PD98059 and Src inhibitor PP2. In contrast, cell adhesion, which we previously showed to be c-jun NH2-terminal kinase (JNK) dependent, was unaffected by ERK1/2 and Src inhibition. Expression of dominant-negative c-Src significantly reduced EphB1-dependent ERK1/2 activation and chemotaxis. Site-directed mutagenesis experiments demonstrate that tyrosines 600 and 778 of EphB1 are required for its interaction with c-Src and p52^{Shc}. Furthermore, phosphorylation of p52^{Shc} by c-Src is essential for its recruitment to EphB1 signaling complexes through its phosphotyrosine binding domain. Together these findings highlight a new aspect of EphB1 signaling, whereby the concerted action of c-Src and p52^{Shc} activates MAPK/ERK and regulates events involved in cell motility.

Introduction

Angiogenesis and axonal guidance are complex processes necessitating a tightly regulated temporal and spatial control. In the past years, Eph receptors and their membrane-bound ligands (ephrins) have emerged as key players in these crucial events, as demonstrated by several genetic studies in mice. Mutant animals lacking ephrinB2 or EphB4 die before day 11 of embryonic development displaying various cardiovascular defects (Wang et al., 1998; Adams et al., 1999). Targeted mutation of the EphB2 gene in mice revealed a profound defect in the pathfinding of a specific axon tract of the anterior commissure (Henkemeyer et al., 1996). Recently, Eph receptors have also been shown to regulate synapse formation and maturation by modulating NMDA receptor function (Takasu et al., 2002). As the known range of biological functions performed by this multifaceted receptor/ligand family is widening, focus is shifting to dissecting the molecular mechanisms whereby these molecules transduce such a variety of important signals into the cells.

Eph receptors, as other receptor tyrosine kinases (RTKs), become phosphorylated at specific tyrosine residues in the cytoplasmic domain after ligand binding. Phosphorylated motifs serve as sites of interaction with certain cytoplasmic signaling proteins (Songyang et al., 1993). Indeed, a variety of SH2 domain–containing proteins have been identified as EphB receptor binding partners, including the adaptor proteins Crk, Grb10, SHEP-1, and SLAP, PI-3 kinase, and the Ras GTPase-activating protein (Holland et al., 1997; Dodelet et al., 1999). In previous work, we showed that activated EphB1 recruits the adaptor protein Nck and the low molecular weight phosphatase LMW-PTP through involvement of Y594 and Y929 tyrosine residues (Stein et al., 1998a,b). We demonstrated that EphB1 could discriminate ephrinB1 surface density to direct integrin-mediated cell adhesion through "inside-out" signaling (Huynh-Do et al., 1999). Furthermore, we showed that the Ste20 kinase NIK (Nck-interacting kinase) couples EphB1 to c-jun NH$_2$-terminal kinase (JNK) and integrin activation (Becker et al., 2000). Here we provide the first evidence that ligand-activated EphB1 forms a signaling com-
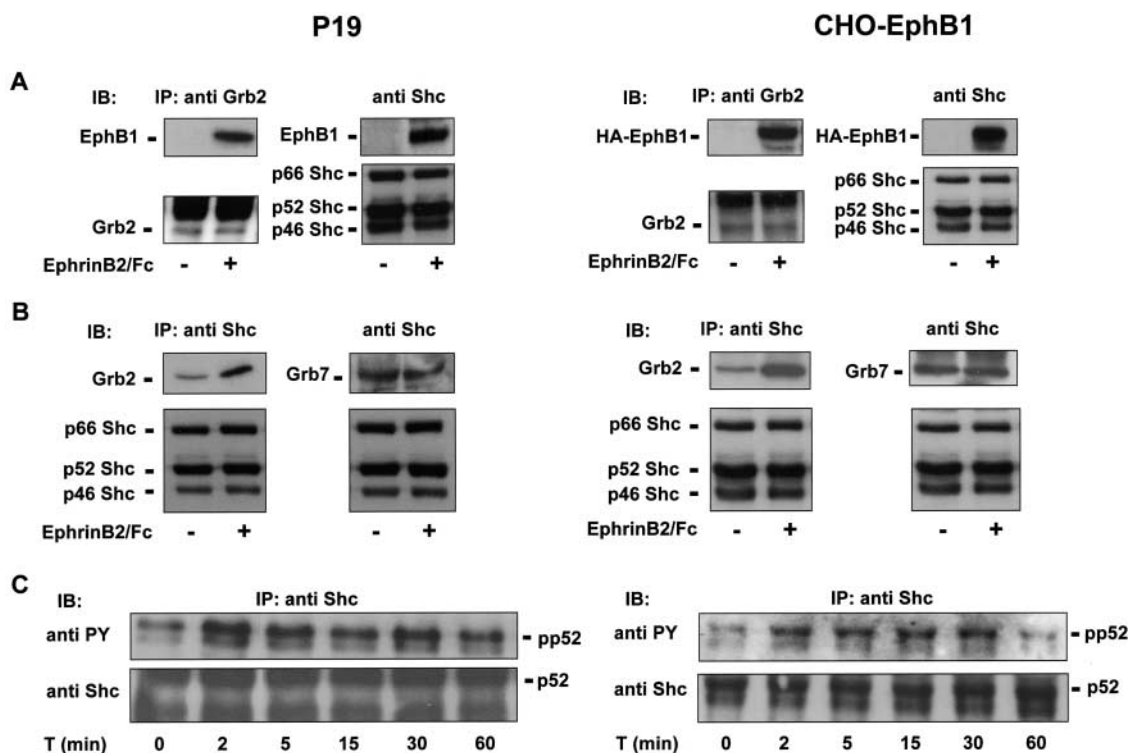
Figure 1.   **Activated EphB1 associates with Grb2 and p52^{Shc} and induces tyrosine phosphorylation of p52^{Shc}.** Serum-starved P19 (left) or CHO-EphB1 (right) were stimulated with 0.5–1 μg/ml ephrinB2/Fc, and coimmunoprecipitations were performed as described in the Materials and methods. (A) Grb2 and p52^{Shc} immunoprecipitates were immunoblotted with anti-EphB1 or anti-HA, respectively. (B) p52^{Shc} immunoprecipitates were immunoblotted with anti-Grb2 or anti-Grb7. (C) Time course of p52^{Shc} tyrosine phosphorylation. p52^{Shc} was immunoprecipitated with rabbit anti-Shc and immunoblotted with anti–PY20-HRP. Stripped blots were reprobed with anti-Shc. Blots are representative of at least three independent experiments.

plex with c-Src kinase and p52^{Shc} adaptor protein to promote extracellular signal–regulated kinase (ERK) 1/2 activation and cell migration. Specifically we report that mutation of tyrosine 600 and 778 to phenylalanine impairs EphB1's interaction with c-Src and the p52^{Shc} phosphotyrosine binding (PTB) domain and thereby the subsequent ERK activation necessary for chemotaxis.

Results

Activated EphB1 recruits the adaptor proteins Grb2 and p52^{Shc}

In the present study, we used P19, a murine cell line expressing endogenous EphB1, as well as CHO cells stably transfected with EphB1 receptor (CHO-EphB1). We showed that ligand stimulation of EphB1 resulted in its association with the adaptor proteins Grb2 and p52^{Shc} (Fig. 1 A). Concomitantly, EphB1 activation also increased Grb2 binding to p52^{Shc} (Fig. 1 B), indicating that the Grb2 complex may be recruited to EphB1 either directly or indirectly through p52^{Shc}. We also looked at the interaction between EphB1 and Grb7, an adaptor protein recently shown to play an important role in cell migration (Han et al., 2002). Grb7 also bound to ligand-activated EphB1 (unpublished data). However, in contrast to the p52^{Shc}–Grb2 interaction, we observed only a constitutive association between p52^{Shc} and Grb7, without further increase after EphB1 stimulation (Fig. 1 B).

The Src tyrosine kinase inhibitor PP2 inhibits p52^{Shc} tyrosine phosphorylation, EphB1–p52^{Shc} interaction, and p52^{Shc}–Grb2 association. As Shc proteins can be substrates either of activated RTKs or Src family kinases (SFKs), we analyzed p52^{Shc} tyrosine phosphorylation status and its interaction with Grb2 in the absence or presence of the SFK inhibitor PP2. Time course analysis showed that p52^{Shc} tyrosine phosphorylation was an early event, taking place already 2 min after EphB1 activation (Fig. 1 C). In the presence of PP2, EphB1-mediated tyrosine phosphorylation of p52^{Shc} was partly reduced (Fig. 2 A), whereas EphB1 phosphorylation was not affected (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200302073/DC1). Pretreatment with PP2 did not affect the EphB1–Grb2 association (Fig. 2 B) but prevented the EphB1–p52^{Shc} and p52^{Shc}–Grb2 interactions (Fig. 2 C). In aggregate, these results suggest that ligand-stimulated EphB1 induces tyrosine phosphorylation of p52^{Shc} through SFKs and at least another tyrosine kinase. However, Src kinase–dependent tyrosine phosphorylation of p52^{Shc} is necessary for its recruitment by EphB1 and its association with Grb2.

Ligand-stimulated EphB1 induces c-Src and ERK1/2 activation

We next asked whether c-Src itself would interact with activated EphB1. As shown in Fig. 3 A, c-Src is constitutively bound to EphB1, however, the amount of EphB1-associated
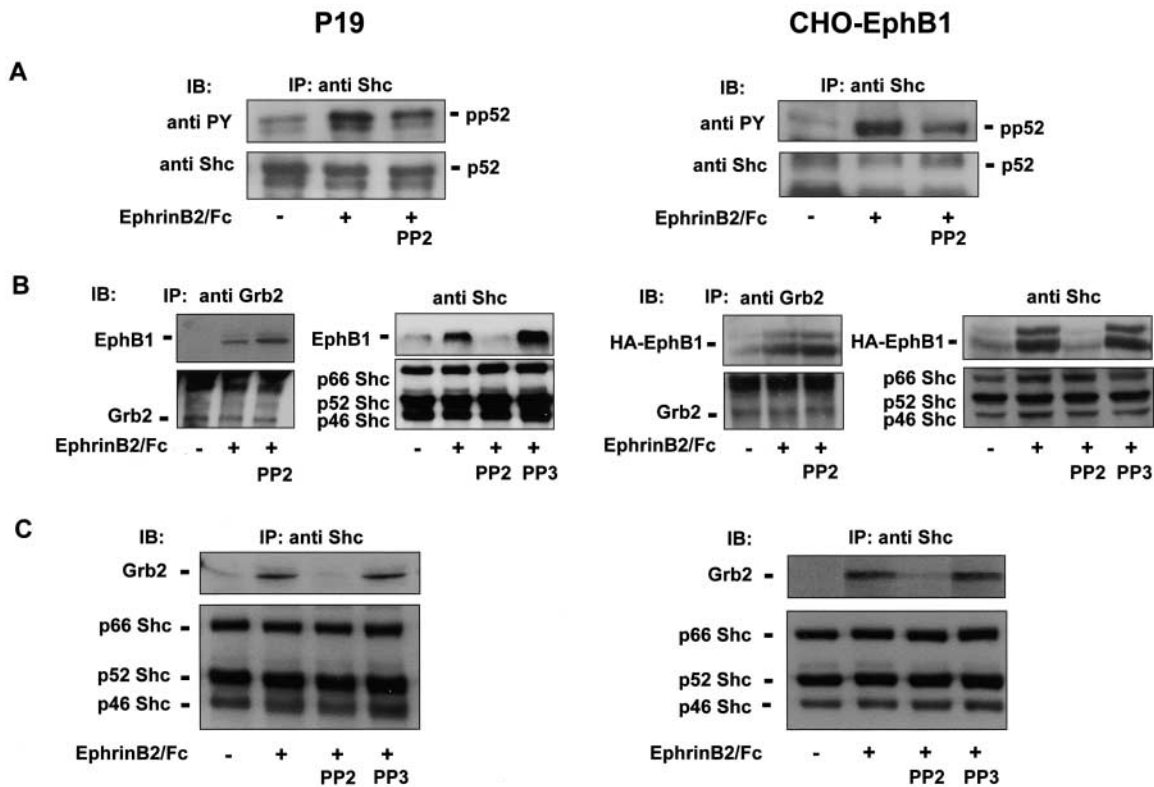
Figure 2. **The Src tyrosine kinase inhibitor PP2 inhibits p52^{Shc} tyrosine phosphorylation, EphB1–p52^{Shc} interaction, and p52^{Shc}–Grb2 association.** Serum-starved P19 (left) or CHO-EphB1 (right) were pretreated for 45 min with PP2 or PP3 (10 µM) before ephrinB2/Fc stimulation as described in the Materials and methods. (A) p52^{Shc} were immunoprecipitated with rabbit anti-Shc and immunoblotted with anti–PY20-HRP. To ensure that the same amount of protein has been immunoprecipitated, stripped blots were reprobed with anti-Shc. (B) After stimulation with 0.5–1 µg/ml ephrinB2/Fc for 10 min, cells were lysed in coimmunoprecipitation buffer (Materials and methods). Lysates were equally divised, and immunoprecipitations were performed with polyclonal anti-Shc or Grb2. Immunoprecipitates were immunoblotted with anti-EphB1 or anti-HA. (C) p52^{Shc} was immunoprecipitated with rabbit anti-Shc and immunoblotted with anti-Grb2. Blots are representative of at least three independent experiments.

Src protein clearly increases after ligand stimulation. Autophosphorylation of c-Src at Y418 induces an active conformation, whereas phosphorylation at Y527 by c-Src kinase represses its kinase activity (Abram and Courtneidge, 2000). Immunoblotting of cell lysates with an antibody specifically recognizing Y418-phosphorylated c-Src showed a positive signal within 5 min after ephrinB2/Fc stimulation (Fig. 3 B). Furthermore, Y418-phosphorylated c-Src is detected in EphB1 immunoprecipitates from CHO-EphB1 cells, indicating that this is the form of active c-Src bound to EphB1 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200302073/DC1). Together, these results identify c-Src as a binding partner and downstream target of EphB1. As SFKs have been proposed to function as intermediates between RTKs and MAPK/ERK to mediate mitogenesis and cytoskeletal changes, we next investigated the effect of ephrinB2/Fc on ERK1/2 activation. We had previously observed in P19 cells a sustained JNK activation after EphB1 stimulation (Stein et al., 1998a). Using an antibody that recognizes the dually phosphorylated, active forms of ERK1/2 (p42/p44), we demonstrated that ephrinB2/Fc induced a strong activation of ERK1/2 of 60- and 10-min duration for P19 and CHO-EphB1 cells, respectively (Fig. 3 C). In the same cellular context, EGF-dependent ERK1/2 activation lasted only 10 and 5 min for P19 and CHO-EphB1, respec-

tively (unpublished data). EphB1-mediated ERK activation was abrogated by the MEK inhibitor PD98059 and the Src inhibitor PP2 (Fig. 3 D), but not by wortmannin, a PI-3 kinase inhibitor (unpublished data). Together, these findings confirm the central role played by c-Src in mediating ERK1/2 activation by ephrinB2/Fc.

ERK1/2 and c-Src mediate EphB1-dependent cell migration

In an effort to link these molecular events to biological endpoints, we next explored the role of ERK1/2 and c-Src on cell adhesive and invasive behavior in response to EphB1 stimulation. In previous work, we used a wound closure assay to assess EphB1-dependent cellular chemokinesis (Huynh-Do et al., 1999). Here, we extended these findings using a modified Boyden chamber assay. Clustered ephrinB2/Fc added to both lower and upper chambers induced a weak chemokinetic response, whereas the most robust response was observed with clustered ephrinB2/Fc added to the lower chamber only: we showed a two- to threefold increase of chemotaxis compared with the controls with vehicle or IgG1. Dose–response experiments showed that optimal responses were reached at concentrations of 0.5–1 µg/ml for P19 cells and 1.5–3 µg/ml for CHO-EphB1 cells (unpublished data). Pretreatment with PD98059 or PP2 in-

Figure 3. **Activated EphB1 recruits c-Src and induces c-Src and ERK1/2 activation.** Serum-starved P19 (left) or CHO-EphB1 (right) were stimulated as described for the indicated times. (A) c-Src immunoprecipitates were immunoblotted with anti-EphB1 or anti-HA. (B) Time course of c-Src activation. 40 μg of lysate was immunoblotted with an antibody recognizing Y418-phosphorylated Src. Stripped blots were reprobed with anti–c-Src. (C) Time course of ERK1/2 activation. 40 μg of lysate was immunoblotted with an antibody recognizing the dually phosphorylated, active forms of ERK1/2 (p42/p44). Stripped blots were reprobed with anti-ERK1/2. (D) For inhibition experiments, cells were pretreated for 45 min with PD98509 (20 μM), PP2, or PP3 (10 μM) before stimulation with ephrinB2/Fc, and cell lysates were immunoblotted with anti–phospho-ERK1/2. Stripped blots were reprobed with anti-ERK1/2. Blots are representative of at least three independent experiments.
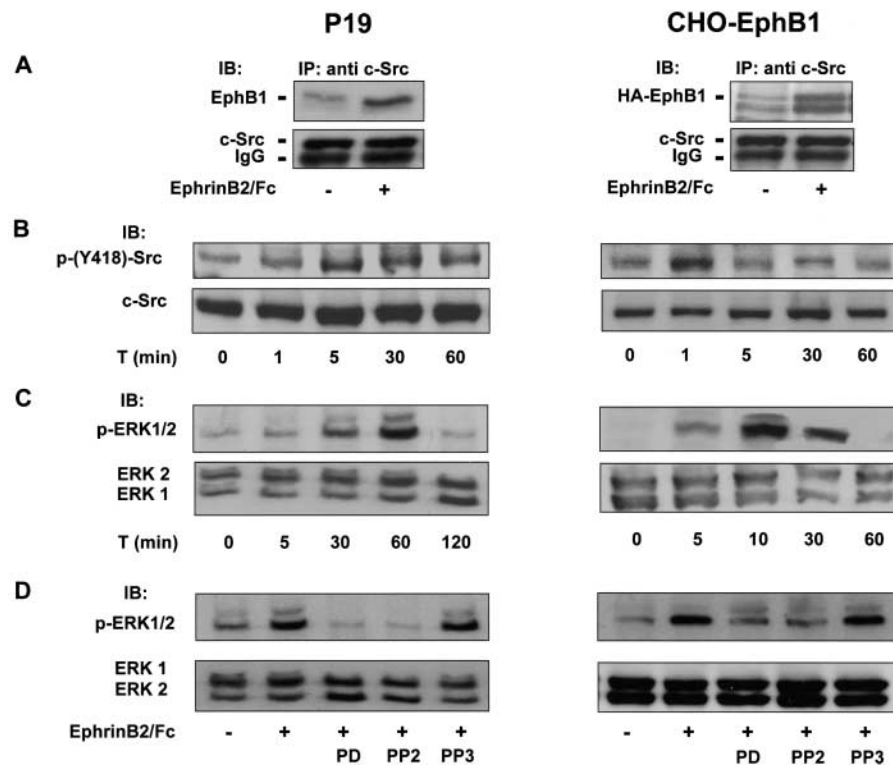
hibited ephrinB2/Fc-dependent chemotaxis (Fig. 4 A) but had no effect on cell migration after serum stimulation (unpublished data). In sharp contrast, the increase of cell adhesion in response to ephrinB2/Fc was unaffected by ERK and c-Src inhibition (Fig. 4 B). Transfecting CHO cells with dominant-negative EphB1-Y594F, which we previously showed to block EphB1–Nck interaction as well as JNK activation (Stein et al., 1998a; Becker et al., 2000), impaired not only cell adhesion but also cell migration (Fig. 4 C). These results support a model whereby EphB1-induced cell adhesion is mainly dependent on JNK, whereas cell migratory responses require the concerted action of both ERK1/2 and JNK.

Activated EphB1 recruits c-Src and p52[Shc] and stimulates ERK1/2 to promote chemotaxis in primary human renal microvascular endothelial cells

So far, all the data presented here were generated either in CHO-EphB1 cells or P19 cells expressing endogenous EphB1. Indeed, we have validated the P19 system in previous work, showing similar biochemical and functional results when compared with primary endothelial cells. For example, Stein et al. (1998b) demonstrated that in both P19 and human renal microvascular endothelial cells (HRMECs) expressing high levels of EphB1, EphB1 receptor discriminates between different oligomeric forms of ephrinB1/Fc to determine alternative signaling complexes, attachment, and assembly responses. While this manuscript was in preparation, another group (Han et al., 2002) used P19 and CHO cells transfected with EphB1 to show, independently from us, that activated EphB1 associates with the adaptor protein Grb7. Here, we further confirm the biological significance of our results by placing them into the context of a primary

cell system. Fig. 5 (A and B) shows tyrosine phosphorylation of p52[Shc] as well as coimmunoprecipitation between EphB1 and, respectively, Shc and c-Src in response to stimulation of HRMECs with ephrinB2/Fc. In agreement with our previous findings, ephrinB2/Fc induced an activation of ERK1/2, which was maximal at 15–20 min (unpublished data) and was abrogated by PD98059 or PP2 (Fig. 5 C). Pretreatment of HRMECs with the same inhibitors strongly impaired ephrinB2-dependent migration but not adhesion (Fig. 5 D).

With the exception of the recent work from Pratt and Kinch (2002), most of the studies published to date have shown a down-regulation of the ERK1/2 pathway by ligand-stimulated EphA2 or EphB2. Eph receptors have also been shown to negatively regulate the activation of ERK1/2 by some other growth factors (Elowe et al., 2001; Miao et al., 2001). We therefore asked whether in the case of EphB1, adhesion-induced activation of ERK1/2 could also be blocked by ephrinB2/Fc, as in NG108 cells stably transfected with EphB2 (Elowe et al., 2001). As shown in Fig. S3 (top; available at http://www.jcb.org/cgi/content/full/jcb.200302073/DC1), plating of CHO-EphB1 cells on fibronectin led to a weak, but constant, increase of ERK1/2, which was clearly potentiated at 5 and 60 min by low concentrations of ephrinB2/Fc. EphrinB2/Fc also increased integrin-induced ERK1/2 activation in P19, although to a lesser extent (unpublished data). This biphasic (but not sustained) potentiation of ERK1/2 by EphB1 is striking. The early peak at 5 min is most probably due to ERK1/2 stimulation by both ephrinB2/Fc and integrin activation. Presently, however, we can only speculate on the biological significance of the second peak, which could be triggered by various cytoskeletal modifications taking place during the
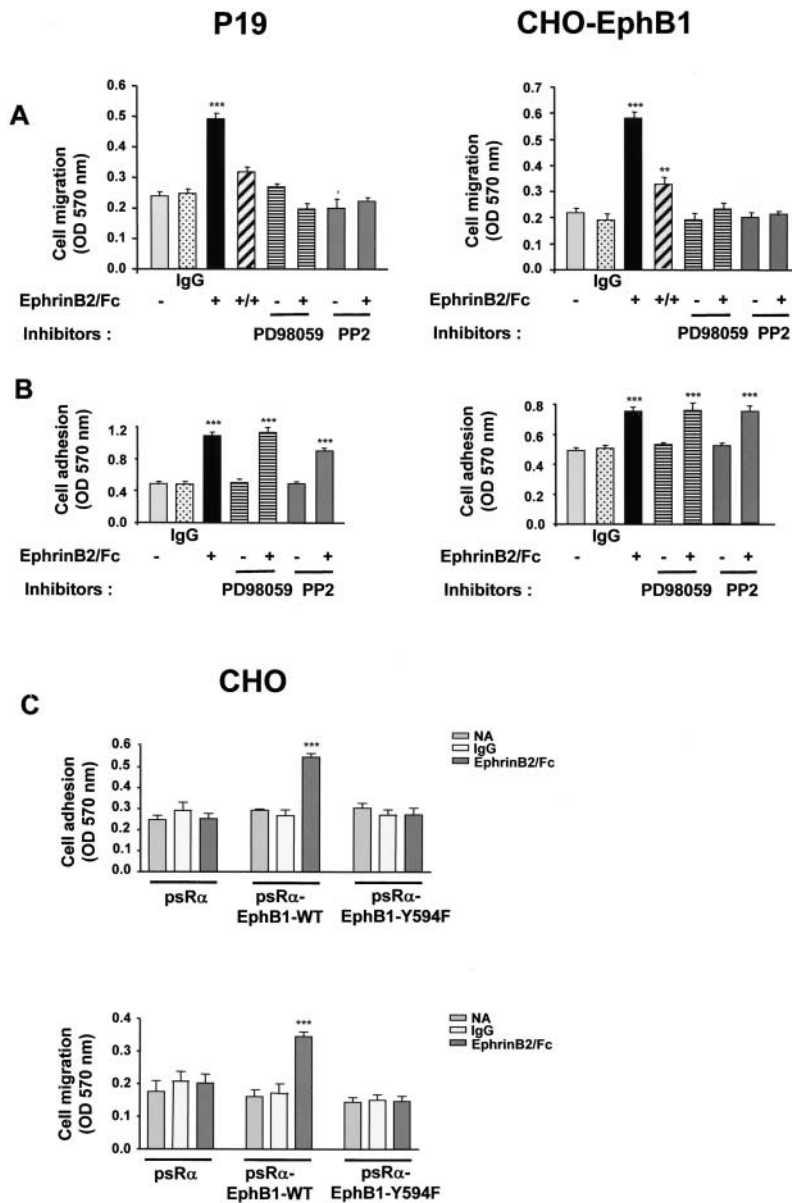
Figure 4. **ERK1/2 and c-Src mediate EphB1-dependent cell migration but not adhesion.** Cell migration and adhesion were performed on P19 (left) or CHO-EphB1 (right) as described in the Materials and methods. For migration assays (A), cells were seeded on the top chamber of fibronectin-coated Transwell inserts. 1–2 μg/ml clustered ephrinB2/Fc (+) or human IgG$_1$ was added to the lower chamber. In control experiments for random migration, clustered ephrinB2/Fc was added to the lower and upper chambers (+/+). Then cells were allowed to migrate to the underside for 6 h at 37°C. Migrated cells were fixed with glutaraldehyde, stained with crystal violet, and quantified by OD reading at 570 nm. For attachment assays (B), cells were seeded on 48-well plates coated with fibro-nectin, in the presence of either ephrinB2/Fc or human IgG$_1$ (300 ng/cm^2). After 30 min, adherent cells were fixed with glutaraldehyde, stained with crystal violet, and quantified by OD reading at 570 nm. For inhibition experiments, cells were pre-treated for 45 min with PD98509 (20 μM) or PP2 (10 μM). (C) CHO cells were transiently trans-fected with vector control (psRα), EphB1-WT, or EphB1-Y594F. Adhesion and migration assays in response to ephrinB2/Fc were performed as described 48 h after transfection. Results are repre-sentative of at least five independent experiments and represent the mean of triplicate wells ± SEM. **, $P < 0.01$; ***, $P < 0.001$.

complex process of cell migration. In primary HRMECs (Fig. S3, bottom), ephrinB2/Fc was not able to further po-tentiate the robust ERK activation induced by VEGF; how-ever, we also could not see the inhibition described by Miao et al. (2001) in bovine aortic endothelial cells.

Dominant-negative c-Src inhibits EphB1-dependent ERK activation and cell migration

To dissect the role of c-Src in EphB1 signaling, we next per-formed a series of dominant-negative experiments. As shown in Fig. 6 A, transfection of kinase-dead (K295R) or domi-nant-negative c-Src (K295R/Y527F) inhibited the recruit-ment of p52^{Shc} by ligand-stimulated EphB1. Cells trans-fected with mutant c-Src displayed a 60–70% reduction of ERK1/2 activation after exposure to ephrinB2/Fc (Fig. 6 B). CHO-EphB1 transfected with increasing amounts of mu-tant c-Src cDNA displayed a significantly decreased migra-tory response to ephrinB2/Fc (Fig. 6 C). In contrast to the abrogation of ERK activity in PP2-pretreated cells, the 60–

70% inhibition attained with mutant c-Src is consistent with the previously observed functional redundancy among different SFKs. For example, Stein et al. (1994) showed that in some cell types, these kinases are able to compensate for the loss of the other related kinases. Therefore, using two in-dependent methods (i.e., Src kinase inhibitor and domi-nant-negative c-Src), we demonstrated a requirement for c-Src in EphB1-mediated ERK activation and cell migration.

EphB1 associates with c-Src and the Shc PTB domain through tyrosines Y600 and Y778

The cytoplasmic region of all Eph receptors contains several conserved phosphorylated tyrosine residues. Sequence analy-sis of EphB1 revealed that Y594 and Y600 are located in a region highly conserved in the Eph receptor family. The se-quence motifs surrounding these tyrosines, Y$_{594}$IDP and Y$_{600}$EDP, correspond to high affinity Src family binding motifs (Songyang et al., 1993; Ellis et al., 1996). We previ-ously showed that Y594 in EphB1 binds to the SH2 domain
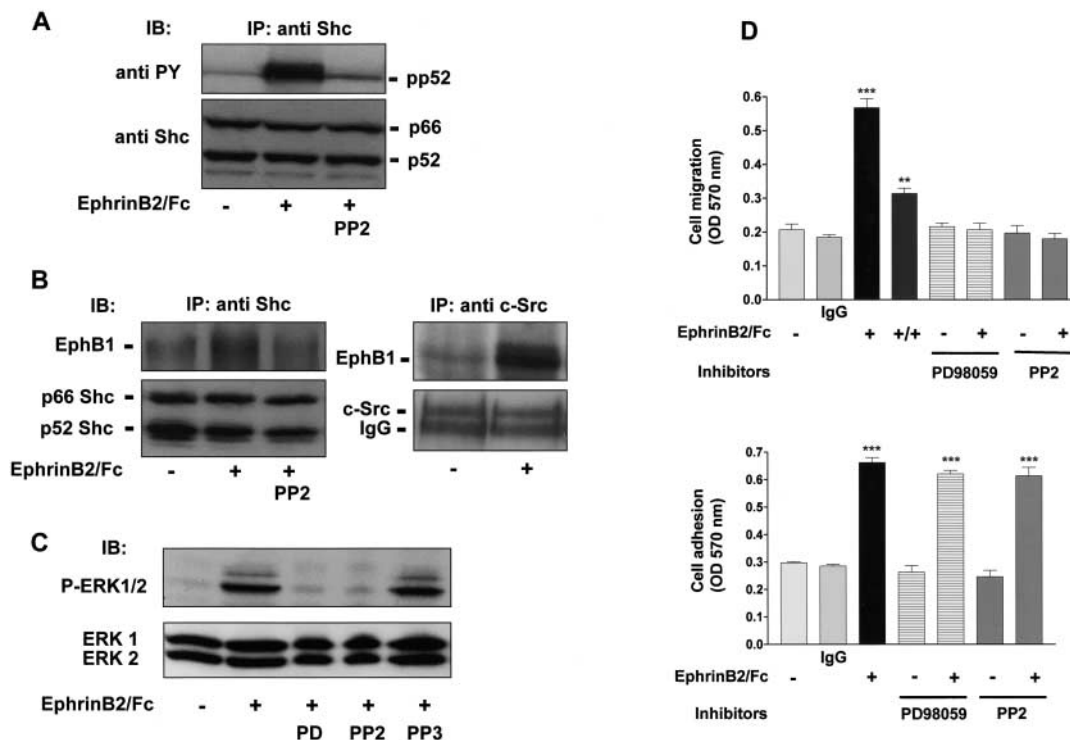
HRMEC

Figure 5.   **Activated EphB1 recruits c-Src and p52^{Shc} and stimulates ERK1/2 to promote chemotaxis in primary HRMECs.** Serum-starved HRMECs were incubated with 0.5 mM suramin for 6–8 h. After suramin washout, cells were stimulated with 0.5–1 μg/ml ephrinB2/Fc for 15 min. (A) p52^{Shc} was immunoprecipitated with rabbit anti-Shc and immunoblotted with anti–PY20-HRP. Stripped blots were reprobed with anti-Shc. (B, left) p52^{Shc} was immunoprecipitated with rabbit anti-Shc and immunoblotted with anti-EphB1. Stripped blots were reprobed with anti-Shc. (B, right) c-Src immunoprecipitates were immunoblotted with anti-EphB1. Stripped blots were reprobed with anti–c-Src. (C) Cells were pretreated for 45 min with PD98509 (20 μM), PP2, or PP3 (10 μM) before stimulation with ephrinB2/Fc. 40 μg of lysate was immunoblotted with an antibody recognizing the dually phosphorylated, active forms of ERK1/2 (p42/p44). Stripped blots were reprobed with anti-ERK1/2. (D) Cell migration and adhesion were performed on HRMECs as described in the Materials and methods. For inhibition experiments, cells were pretreated for 45 min with PD98509 (20 μM) or PP2 (10 μM). Results are representative of at least three independent experiments and represent the mean of triplicate wells ± SEM. **, $P < 0.01$; ***, $P < 0.001$.

of Nck and is essential for JNK and integrin activation by EphB1 (Stein et al., 1998a; Huynh-Do et al., 1999). To evaluate the role of Y594 and Y600 in Src binding, we preformed site-directed mutagenesis experiments. A single substitution, Y600F, disrupted the interaction between EphB1 and c-Src (Fig. 7 A) in transiently transfected CHO cells, whereas Y594F appears not be required for the interaction (Fig. S4, top, available at http://www.jcb.org/cgi/content/full/jcb.200302073/DC1). Furthermore, EphB1-Y600F also loses the ability to associate with p52^{Shc}, a finding corroborating our inhibition experiments with PP2 and implying that phosphorylation of p52^{Shc} by c-Src is necessary for its recruitment to EphB1 signaling complexes (Fig. 7 B).

The adaptor protein Shc contains an SH2 domain at its COOH terminus and a PTB domain at its NH$_2$ terminus. The PTB domain binds phosphotyrosine-containing proteins, including activated receptor kinases. In vitro binding experiments have indicated that the Shc PTB domain associates with activated EGF and NGF receptors (for review see van der Geer and Pawson, 1995). Using a phosphotyrosine peptide library, Songyang et al. (1995) demonstrated that the Shc PTB domain recognizes preferentially pTyr in the context NPXpY. Although this motif showed the strongest

selectivity for Asn at the pTyr^{-3}, according to their peptide library prediction, Asp at pTyr^{-3} ranked second behind Asn. Examination of the cytoplasmic domain of EphB1 allowed us to identify tyrosine 778, located in the sequence DPTY, as a putative Shc PTB domain binding site. Indeed we observed that the mutation Y778F inhibited the recruitment of p52^{Shc} by activated EphB1 (Fig. 7 C). To confirm the involvement of the Shc PTB domain, we performed in vitro binding assays: GST fusion proteins containing the PTB domain of Shc (GST–Shc PTB) bound to activated EphB1 but not to EphB1-Y778F (Fig. 7 D).

EphB1-dependent ERK activation and chemotaxis require Y600 and Y778

In view of these findings, we next assessed the roles of Y600 and Y778 in mediating ERK activation and cell migration. Indeed, EphB1-induced ERK activation was strongly reduced in CHO cells expressing mutant EphB1-Y600F and EphB1-Y778F (Fig. 8 A, bottom), whereas the expression level or the tyrosine phosphorylation status of EphB1 remained unchanged (Fig. 8 A, top). In contrast, EphB1-Y594F, which was capable to recruit c-Src, retained the ability to induce ERK activation upon ephrinB2/Fc stimulation
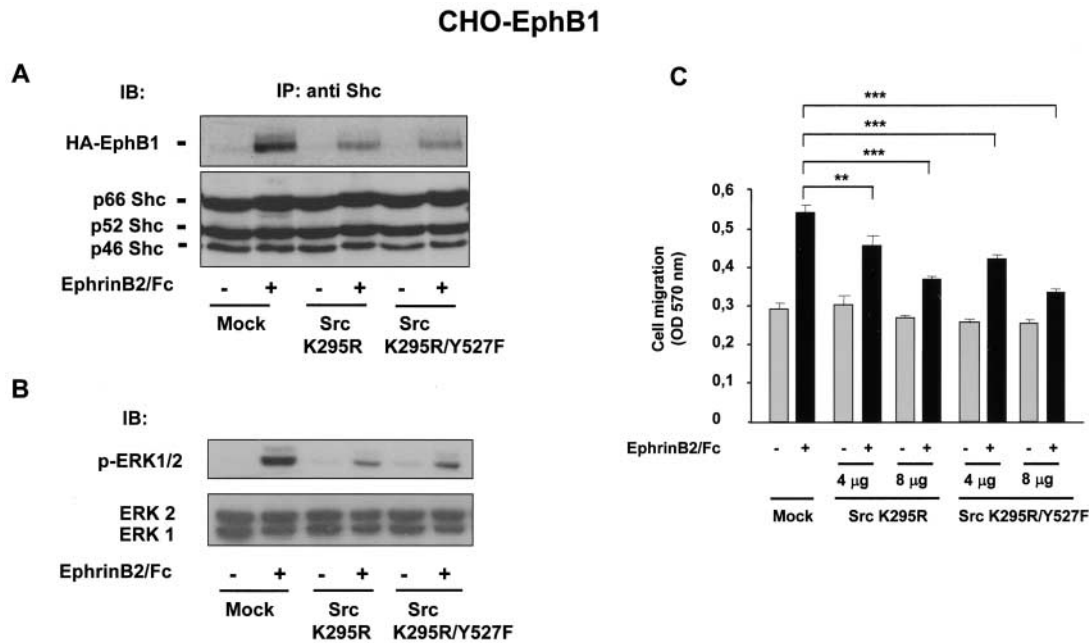
CHO-EphB1

Figure 6. **Dominant-negative c-Src significantly reduces EphB1-dependent ERK1/2 activation and cell migration.** CHO-EphB1 transiently transfected with kinase-dead (K295R) or dominant-negative (K295R/Y527F) c-Src were stimulated as described with 1 µg/ml ephrinB2/Fc. (A) p52^{Shc} immunoprecipitates were immunoblotted with anti-HA. (B) Cell lysates were immunoblotted with anti–phospho-ERK1/2. Stripped blots were reprobed with anti-ERK1/2 and, to confirm similar transfection efficiency, with anti–c-Src (not depicted). Blots are representative of at least three independent experiments. (C) CHO-EphB1 were transiently transfected with either 4 or 8 µg of kinase-dead or dominant-negative c-Src, and migration assays were performed 48 h after transfection as described in the Materials and methods. Results are representative of at least three independent experiments and represent the mean of triplicate wells ± SEM. **, P < 0.01; ***, P < 0.001.

(Fig. S4, bottom). Finally, in CHO cells transiently transfected with mutant EphB1-Y600F and EphB1-Y778F, we observed a clear inhibition of migratory responses after ephrinB2/Fc stimulation (Fig. 8 B). Taken together, these results demonstrate that Y600 and Y778 are indispensable for the recruitment of c-Src and p52^{Shc} to EphB1 signaling complexes, and thus are important regulators of EphB1-dependent ERK activation and cell migration.

Discussion

The intracellular signaling pathways mediating the biological effects of Eph receptors and ephrins are only starting to emerge. Although a plethora of proteins binding to the cytoplasmic domains of Eph receptors and ephrins has been identified, their function in regulating angiogenesis, axonal guidance, and topographic mapping in the developing embryo are still poorly understood. MAPKs are major signaling enzymes by which cells transduce extracellular stimuli to regulate intricate intracellular processes; however, the actual roles of each MAPK module are highly cell type and context specific. In previous studies, we showed that the Nck–Nck-interacting kinase (NIK)–JNK cascade modulates cell adhesion in response to EphB1 activation (Stein et al., 1998a; Becker et al., 2000). In the present work, using three cellular systems expressing endogenous or stably transfected EphB1, we provide evidences that the MAPK/ERK cascade plays a crucial role in EphB1-dependent cell migration. We found a strong activation of ERK1/2 in response to EphB1 stimulation and identified key molecules linking EphB1 to ERK1/2

activation. First we showed that activated EphB1 recruits Grb2 either directly or through the intermediary of the adaptor protein p52^{Shc}. We demonstrated that tyrosine phosphorylation of p52^{Shc} by SFKs is a prerequisite for its association with EphB1, on the one hand, and for recruitment of Grb2, on the other hand. Finally, we identified c-Src itself as an important target of EphB1, as ligand-stimulated EphB1 recruits c-Src and induces its active conformation by phosphorylation of tyrosine Y418.

In recent years, Src has emerged as a key molecule mediating specific angiogenic processes. Eliceiri et al. (1999) demonstrated that dominant-negative Src mutants blocked VEGF- but not bFGF-induced angiogenesis. Recently, Palmer et al. (2002) nicely showed that SFKs are positive regulators of ephrinB tyrosine phosphorylation and ephrinB-mediated angiogenic responses in endothelial cells. An association between Eph receptors and SFKs has also been reported in a number of studies. For example, Zisch et al. (1998) showed in vitro binding of c-Src to Y611 of EphB2 and found tyrosine-phosphorylated EphB2 in Src immunoprecipitates from COS cells transfected with both EphB2 and Src. Using the same approach, i.e., in vitro binding assays and coimmunoprecipitation from transfected cells, Hock et al. (1998) showed that Fyn associates with activated EphB3 in an SH2-dependent manner. So far, however, these studies have not clearly defined the precise role of SFKs in mediating angiogenic processes downstream of Eph receptors. The data presented here not only substantiate these findings by placing them into the physiological context of endogenous EphB1 and c-Src proteins, but also link c-Src
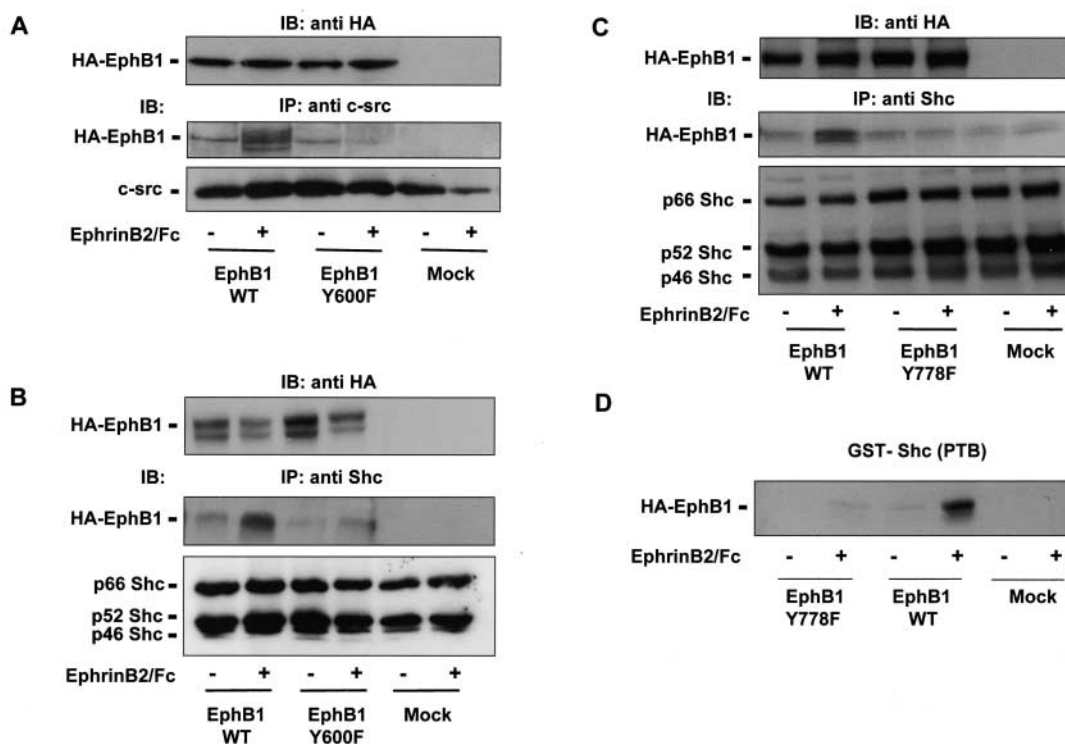
CHO-EphB1

Figure 7. **Functional analysis of EphB1-Y600F and EphB1-Y778F mutants in CHO cells.** Cells were transiently transfected with EphB1-WT, EphB1-Y600F, or EphB1-Y778F as indicated and stimulated with ephrinB2/Fc, and coimmunoprecipitations were performed as described in the Materials and methods. Similar transfection efficiency was confirmed by probing lysates with anti-HA. (A) c-Src immunoprecipitates were immunoblotted with anti-HA. Stripped blots were reprobed with anti-c-Src. (B) $p52^{Shc}$ immunoprecipitates were immunoblotted with anti-HA. Stripped blots were reprobed with anti-Shc. (C) $p52^{Shc}$ immunoprecipitates were immunoblotted with anti-HA. Stripped blots were reprobed with anti-Shc. (D) GST pull-down assays. Cells were transfected and stimulated as described in the Materials and methods. 500 μg of cleared lysate was incubated with purified GST (not depicted) or GST–Shc PTB for 2 h at 4°C. Pellets were washed three times in coimmunoprecipitation buffer, resuspended in SDS sample buffer, and resolved by 8% SDS-PAGE for HA–EphB1 expression. Results are representative of at least three independent experiments.

activation to two important biological endpoints: EphB1-dependent ERK activation and chemotaxis. In addition, our mutational analysis strongly suggests that Y600 in EphB1 is an important autophosphorylation site and the predominant binding site for c-Src.

Activated c-Src can phosphorylate Shc on tyrosines to provide docking sites for downstream signaling proteins. In particular tyrosine-phosphorylated Shc is able to recruit Grb2/SOS through association between the Grb2 SH2 domain and Shc phosphotyrosine residues (Pelicci et al., 1992; Rozakis-Adcok et al., 1992), ultimately resulting in activation of the Ras–MAPK pathway. Our results demonstrate that $p52^{Shc}$ is a key substrate for c-Src after EphB1 ligand stimulation and that its interaction with EphB1 is mediated by phosphorylated Y778 of EphB1. Y778 is located within a DPTY sequence, a motif that has been predicted by Song-yang et al. (1995) as a putative Shc PTB domain binding sequence. To our knowledge, however, all the RTKs shown to date to interact with Shc did so through the canonical sequence NPXpY. Our data thus provide new insight into the binding specificities of the PTB domain of Shc. EphB1-Y778F mutant was normally phosphorylated in response to ephrinB2/Fc stimulation but failed to activate MAPK/ERK

and cell migration. These findings are consistent with the experiments of Collins et al. (1999), which demonstrated by mutational analysis that the PTB domain of Shc transduces migratory signals while the SH2 domain is required for mitogenesis.

Interestingly, in the cellular systems we used, cell adhesion was dependent on JNK (Stein et al., 1998a) but not on ERK1/2 activity. On the other hand, our results showed clearly that cell migration in response to EphB1 stimulation required both the ERK1/2 and JNK pathways. To date, studies on the role of Eph and ephrins on ERK activation have yielded disparate results. We demonstrated that receptor (EphB1) stimulation resulted in activation of both the ERK and JNK pathways, whereas stimulation of its cognate ligand (ephrinB1) activated only JNK (Huynh-Do et al., 2002). Miao et al. (2000, 2001) found suppression of integrin function and down-regulation of ERK1/2 after activation of EphA2 in prostate carcinoma cells. On the contrary, in mouse fibroblastic cell lines, the ephrinA1–EphA2 system promotes adhesion and actin cytoskeletal assembly in a FAK- and p130cas-dependent manner (Carter et al., 2002). Also, very recently, Pratt and Kinch (2002) found a stimulation of the MAPK pathway by activated EphA2 in various
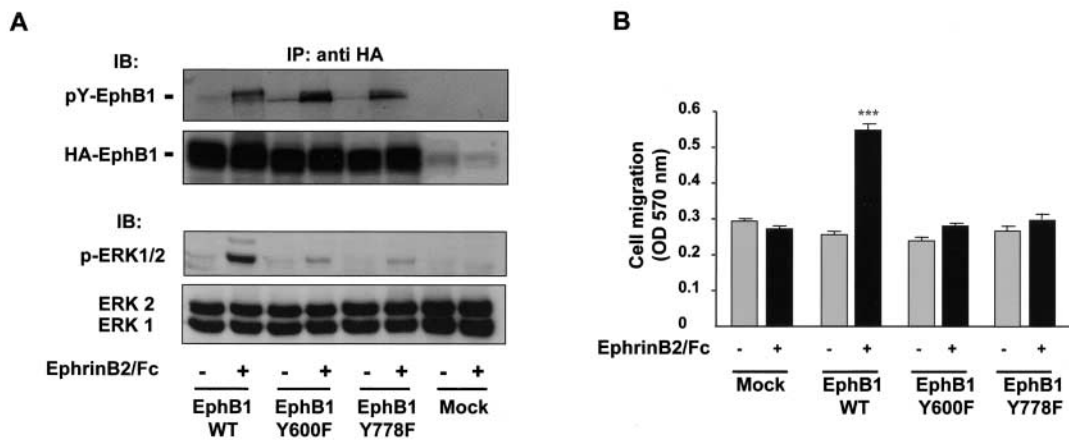
CHO-EphB1

Figure 8. **EphB1-Y600F and EphB1-Y778F mutants inhibit ERK activation and cell migration.** (A) Cells were transiently transfected with EphB1-WT, EphB1-Y600F, or EphB1-Y778F and stimulated as described in the Materials and methods. (Top) EphB1 was immunoprecipitated with monoclonal anti-HA, and samples were divided and separated on two different gels. Immunoblotting was performed using 4G10–HRP (pY-EphB1) and anti-HA. (Bottom) Cell lysates were immunoblotted with anti–phospho-ERK1/2. Stripped blots were reprobed with anti-ERK1/2. (B) Cells were transiently transfected with EphB1-WT, EphB1-Y600F, or EphB1-Y778F. Migration assays in response to 1 µg/ml clustered ephrinB2/Fc were performed 48 h after transfection. Results are representative of at least three independent experiments and represent the mean of triplicate wells ± SEM. ***, P < 0.001.

cell lines. Interestingly, this was also accompanied by the formation of an EphA2–Shc–Grb2 molecular complex, as in the case of EphB1. However the authors did not further identify the amino acid residues involved in this process.

Recently Elowe et al. (2001) showed that down-regulation of MAPK by ephrinB1/Fc was required for neurite retraction of NG108 cells stably transfected with EphB2. Inversely, in a study by Davy and Robbins (2000), activation of ephrinA5 induced sustained phosphorylation of ERK1/2 as well as increased cell adhesion.

These apparently contradictory findings could be explained by several, not mutually exclusive, scenarios. First, the observed differences could result from the variable expression of each individual receptor–ligand pair on the surface of a cell. We previously demonstrated that cellular adhesion in response to EphB1 stimulation follows a biphasic curve, depending on the surface density of encountered ephrinB1 (Huynh-Do et al., 1999). In light of these results, we propose that the cellular context (i.e., the different levels of protein expression) determines the extent of receptor or ligand clustering, which in turn would initiate either activation or inactivation of integrins. As the findings presented here were essentially based on endogenous EphB1, we expect them to reflect real signaling processes initiated upon juxtacrine contact.

In recent years, interactions between Eph and other cell surface receptors have emerged as an important issue (Miao et al., 2001; Takasu et al., 2002). In contrast to EphA2 and EphB2, which have been shown to negatively regulate the ERK1/2 activation induced by some other growth factors, ligand stimulation of EphB1 receptor expressed in primary HRMECs did not interfere with the robust ERK activation in response to VEGF. Furthermore, ephrinB2/Fc clearly potentiated the integrin-induced ERK1/2 activa-

tion in CHO-EphB1 or P19. At the present, we can only speculate about possible mechanisms underlying these discrepancies. On the one hand, endothelial cells from different vascular lineages have been shown to display different biological behaviors. For example, although HRMECs and human umbilical venous endothelial cells (HUVECs) express both EphA2 and EphB1, HRMECs assemble into capillary-like structures in response to ephrinB1/Fc, but not ephrinA1/Fc, under conditions that promote HUVECs to assemble in response to ephrinA1/Fc but not ephrinB1/Fc (Daniel et al., 1996). Furthermore it appears that distinct Eph receptors can transmit opposite signals to the MAPK pathways through their ability to engage distinct targets; thus, the variation between systems may well reflect receptor cross-talk and/or different feedback loops. Finally, as SFKs have now emerged as major regulators of both Eph and ephrin signaling, we anticipate that the identification of the kinases and phosphatases responsible for the phosphorylation of the Eph–ephrin system will be an important step in molecularly dissecting these multiple signaling pathways.

In summary, the observations reported here represent, to our knowledge, the first demonstration of an activation of the ERK1/2 pathway by a member of the EphB receptor family. In addition, they shed new light on the precise mechanism of c-Src activation downstream of EphB1 receptor. Based on these data, we propose the following model (Fig. 9). EphB1 engagement with its cognate ephrin ligands induces rapid recruitment of c-Src through Y600 of EphB1. Autophosphorylated c-Src then phosphorylates p52[Shc], allowing binding of the Shc PTB domain to Y778 of EphB1. The resulting activation of the MAPK cascade, in turn, triggers all cellular events necessary for cytoskeletal rearrangement and cell migration.
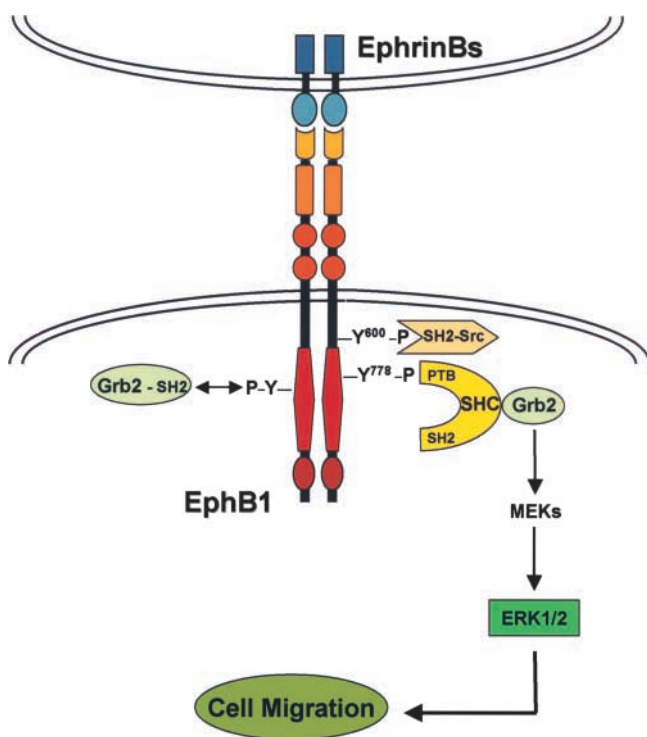
Figure 9. **Proposed model of EphB1-dependent chemotaxis.** Shown is a proposed model of the biochemical mechanisms of EphB1-dependent activation of the Src–ERK pathway. Ligand-activated EphB1 recruits c-Src through Y600 of EphB1. Autophosphorylated c-Src (Y418-c-Src), in turn, phosphorylates p52^{Shc}, allowing binding of the Shc PTB domain to Y778 of EphB1. On the other hand, Grb2 is recruited either directly to EphB1 or through the intermediary of p52^{Shc}. The resulting activation of the ERK cascade triggers cell migration.

Our findings add one more piece to the Eph–ephrin puzzle: they provide a good model to study the dichotomy of the JNK and ERK modules, confirming that multiple, interconnected signaling pathways underlie Eph receptor–mediated changes in cytoskeletal organization and chemotaxis. As both axonal guidance and angiogenesis are complex processes that are crucial for survival, this versatility could be interpreted as an important tool allowing fine tuning of the cellular responses to a particular environment. As the list of functions that involve Eph receptors and ephrins is growing, further delineation of the roles of their effector molecules will be of considerable value to the understanding of the molecular mechanisms whereby neural or endothelial cells regulate shape changes, movements, cell–matrix interactions, and ultimately survival.

Materials and methods

Reagents

Cell culture reagents and fibronectin were from Life Technologies, ephrinB2/Fc was from Immunex, human IgG$_1$, suramin, and VEGF were from Sigma-Aldrich, and PD98059, PP2, and PP3 were from Calbiochem. The following antibodies were used for immunoprecipitation: rabbit anti-Grb2 (Santa Cruz Biotechnology, Inc.) and rabbit anti-Shc and monoclonal anti-Src (Upstate Biotechnology). The following antibodies were used for immunoblotting: goat anti-EphB1 (R&D Systems), monoclonal anti-HA (12CA5) (Boehringer), polyclonal anti–active MAPK (Promega), anti-ERK1/2

and anti–phosphotyrosine 4G10-HRP (Upstate Biotechnology), phospho-specific Y418 anti-Src (Biosource International), and monoclonal anti-Grb2 (Transduction Laboratories). Rabbit anti-Grb7 was a gift from B. Margolis (University of Michigan, Ann Arbor, MI). The ECL chemiluminescence kit and ECL phosphorylation module anti–phosphotyrosine-HRP conjugate were from Amersham Biosciences.

Generation of a cell line stably expressing EphB1–HA receptor

The previously described pSRα-hEphB1-HA construct (Stein et al., 1998a) was used to generate stably expressing CHO cell lines. Cells were transfected with 4 μg of the pSRα-hEphB1-HA plasmid using LipoFECTAMINE Plus (Life Technologies), following the manufacturer's guidelines. CHO cells expressing the hEphB1–HA construct were identified by flow cytometry (FACSCalibur®; Becton Dickinson), using biotinylated ephrinB2/Fc and streptavidin–FITC. Fluorescence-activated cell sorting was performed to isolate different populations of cells expressing low, middle, or high levels of hEphB1–HA. CHO-EphB1 cells used here expressed high levels of EphB1–HA.

Site-directed mutagenesis

Mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene). PCRs were performed using an overlap extension by Pfu DNA polymerase with pSRα-hEphB1-HA plasmid as a template. The oligonucleotides used to generate point mutations converting Y600 to F and Y778 to F were 5'-GACCCCTTCACTTTTGAGGATCCCAAC-GAAGCTGTCCGG-3' (sense) and 5'-CCGGACAGCTTCGTTGGGATCCT-CAAAAGTGAAAGGGGTC-3' (antisense), and 5'-CCAGGATGACACCT-CAGATCCCACCTTCACCAGCTCCTT-3' (sense) and 5'-AAGGAGCTGGT-GAAGGTGGGATCTGAGGTGTTCAT-3' (antisense), respectively. Mutations were confirmed by nucleotide sequencing analysis. The pSRα-hEphB1-Y594F-HA plasmid has been previously described by Stein et al. (1998a).

Cell culture and transfection

P19 cells were cultured in α-MEM with 10% FCS, and CHO-EphB1 cells were cultured in DME–F12 with 10% FCS containing Zeocin (Invitrogen). Primary HRMECs were isolated and cultured as previously described (Stein et al., 1998b) and used up to passage 7 or 8. For transient transfections, CHO-EphB1 cells were seeded into 100-mm plates and incubated in culture medium for 24 h before transfection. Cells were transfected using LipoFECTAMINE Plus with 4 or 8 μg of the indicated plasmids. Cotransfection of the plasmid pEGFP was used at 1 μg/100-mm plate as a marker of transfection efficiency (∼70%). Cells were used for biochemical or functional assays 48 h later.

Western blots and immunoprecipitation

Cells were serum starved for 24 h (P19 and HRMEC) or 48 h (CHO-EphB1) in Opti-MEM and then treated with 0.5 mM suramin for 3–8 h. Cells were then rinsed twice with PBS and incubated in 1% BSA for 45–60 min before stimulation for the indicated times at 37°C with 0.5–1 μg/ml of ephrinB2/Fc, control IgG1, or VEGF (10 ng/ml). For assessment of ERK1/2 activation and c-Src tyrosine phosphorylation, cells were lysed in RIPA buffer (Huynh-Do et al., 1999), and 40 μg of protein was loaded on a 10% SDS-PAGE. After transfer to Immobilon/PVDF membranes (Millipore), phosphorylated ERK1/2 or c-Src was detected with phosphospecific anti-ERK1/2 or anti–phosphoY418-Src. Membranes were then stripped and reprobed with anti-ERK1/2 or anti–c-Src. For coimmunoprecipitations, cells were lysed in buffer containing 1% Triton X-100, 20 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM NaF, 5 mM EDTA, 60 mM n-octyl-glucoside, supplemented with 1 mM orthovanadate and protease inhibitors. Immunoprecipitations were performed with the indicated antibodies for 2 h at 4°C as described previously (Stein et al., 1998a).

Cell adhesion and migration

Attachment assays were performed as described previously (Huynh-Do et al., 1999). For migration assays, a modified Boyden chamber system was used. In brief, serum-starved cells were resuspended in Opti-MEM and seeded on fibronectin-coated Transwell inserts with 8-μM pore size membranes (Costar). 1–2 μg/ml clustered ephrinB2/Fc was added to either the lower chamber alone (+, chemotaxis assay) or the lower and upper chambers (+/+, chemokinetic control), and then cells were left to migrate for 6 h at 37°C. Nonmigrating cells were removed with a cotton swab, and migrated cells were fixed with glutaraldehyde, stained with 0.5% crystal violet, and then eluted with acetic acid. Cell migration was quantified by spectrophotometric reading at OD$_{570}$.

GST fusion protein pull-down assay

GST fusion proteins constructed in pGEX vectors were purified as previously described (Stein et al., 1998a). CHO cells transfected with EphB1-WT or EphB1-Y778F were stimulated with ephrinB2/Fc and lysed as described above. 500 μg of cleared lysate was incubated with purified GST–Shc PTB or GST control (not depicted) for 2 h at 4°C. Pellets were washed three times in coimmunoprecipitation buffer, resuspended in SDS sample buffer, and resolved by 8% SDS-PAGE for HA–EphB1 expression.

Statistical analysis

All values are presented as mean ± SEM. Analysis of variance (ANOVA) *t* test was used for statistical analysis, and differences were considered significant when $P < 0.05$. Unless indicated otherwise, data are from at least three independent experiments.

Online supplemental material

The supplemental material (Figs. S1–S4) is available at http://www.jcb.org/cgi/content/full/jcb.200302073/DC1. Fig. S1 shows that the Src tyrosine kinase inhibitor PP2 does not inhibit tyrosine phosphorylation of EphB1. Fig. S2 shows that ligand-stimulated EphB1 recruits Y418-phosphorylated c-Src. Fig. S3 (top) shows that ephrinB2/Fc stimulation increases integrin-induced ERK1/2 activation and (bottom) that ephrinB2/Fc stimulation does not inhibit VEGF-induced ERK activation. Fig. S4 shows that EphB1-Y594 associates with c-Src (top) and stimulates MAPK/ERK (bottom).

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