EphrinB Phosphorylation and Reverse Signaling: Regulation by Src Kinases and PTP-BL Phosphatase

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Summary

Ephrins are cell surface-associated ligands for Eph receptors and are important regulators of morphogenic processes such as axon guidance and angiogenesis. Transmembrane ephrinB ligands act as "receptor-like" signaling molecules, in part mediated by tyrosine phosphorylation and by engagement with PDZ domain proteins. However, the underlying cell biology and signaling mechanisms are poorly understood. Here we show that Src family kinases (SFKs) are positive regulators of ephrinB phosphorylation and phosphotyrosine-mediated reverse signaling. EphB receptor engagement of ephrinB causes rapid recruitment of SFKs to ephrinB expression domains and transient SFK activation. With delayed kinetics, ephrinB ligands recruit the cytoplasmic PDZ domain containing protein tyrosine phosphatase PTP-BL and are dephosphorylated. Our data suggest the presence of a switch mechanism that allows a shift from phosphotyrosine/SFK-dependent signaling to PDZ-dependent signaling.

Introduction

Eph receptor tyrosine kinases regulate an amazing variety of developmental processes, including cell migration, segmentation and compartment boundary formation, axon guidance, topographic mapping, synaptogenesis, and angiogenesis, and may also be involved in adult functions such as synaptic plasticity and proliferation of neural stem cells (Grundwald et al., 2001; Henderson et al., 2001; Takasu et al., 2002; Klein, 2001; Wilkinson, 2001 and references within). The Eph receptor subfamily is the largest within the family of receptor tyrosine kinases and is subdivided into two structurally distinct subclasses, termed EphA and EphB. EphA receptors (EphA1-A8) bind glycosylphosphatidyl-anchored ephrinA ligands (ephrinA1-A5), whereas EphB receptors (EphB1-B6) bind transmembrane ephrinB ligands (ephrinB1-B3). Binding across subclasses does not occur with the exception of EphA4, which binds ephrinA and ephrinB ligands. Binding specificity within a subclass is low (Wilkinson, 2000).

A peculiarity of this ligand/receptor system is the potential to engage in bidirectional signaling. The cytoplasmic tail of ephrinB ligands undergoes rapid phosphorylation on highly conserved tyrosine residues upon engagement of the ephrinB extracellular domain with its cognate EphB receptor in *trans*; i.e., when the EphB receptor is presented by neighboring cells or as soluble fusion protein (Brückner et al., 1997; Holland et al., 1996). By an alternative pathway, the ephrinB cytoplasmic domain becomes phosphorylated downstream of activated receptor tyrosine kinases, such as the platelet-derived growth factor (PDGF) receptor, in *cis*; i.e., when the receptor is endogenously expressed by the ephrinB-expressing cell (Brückner et al., 1997). Moreover, ephrinB ligands have been shown to be phosphorylated in vivo in the mouse embryo (Brückner et al., 1997; Holland et al., 1996). Three of the five tyrosines conserved in the ephrinB ligands have been characterized as the major phosphorylation sites in ephrinB1, both in vitro and in vivo (Kalo et al., 2001). Tyrosine-phosphorylated ephrinB molecules engage the SH2/SH3 domain adaptor protein Grb4, which activates a biochemical pathway that leads to disassembly of F-actin-containing stress fibers (Cowan and Henkemeyer, 2001).

The C terminus of all B ephrins contains an identical binding motif for PDZ domains. PDZ domain proteins are thought to serve as multiple adaptors and scaffolds for signaling complexes (Sheng and Sala, 2001), and the interaction of ephrinB ligands with several PDZ domain proteins has been demonstrated (reviewed in Wilkinson, 2000). The PDZ domain protein PDZ-RGS3 links ephrinB reverse signaling to G protein-coupled receptor signaling, which is implicated in the migration of cerebellar granule cells (Lu et al., 2001). This and indirect genetic evidence points to an active signaling role for ephrinB ligands in which these transmembrane proteins would act as "receptor-like" molecules transducing a signal to the interior of the cell. Although phosphorylation is thought to be an important event in such a process, the identity of the kinases and phosphatases that regulate phosphorylation of the carboxy-terminal domain of ephrinB ligands and their mechanism of activation remains to be elucidated.

Here we identify Src family kinases (SFKs) and the PDZ domain-containing phosphatase PTP-BL as regulators of ephrinB phosphorylation and reverse signaling. SFKs are activated upon binding to ephrinB ligands and

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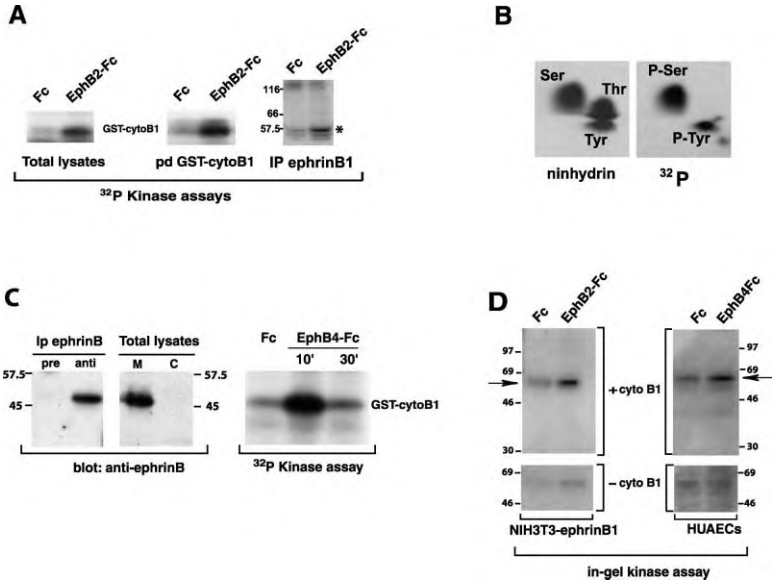

Figure 1. A 60 kDa Kinase Phosphorylates the Cytoplasmic Domain of EphrinB1 in an EphB Receptor-Dependent Manner

(A) NIH3T3 cells ectopically expressing ephrinB1 (NIH3T3-ephrinB1) were stimulated with either Fc or EphB2-Fc receptor bodies for 10 min. Total lysates (left panel) and pull-downs (pd) (middle panel) were used in an in vitro kinase assay using GST-cytoB1 as a substrate. Immunoprecipitates of ephrinB1 from lysates of Fc- and EphB2-Fc-stimulated NIH3T3-ephrinB1 cells were used in an in vitro kinase assay to detect phosphorylation of full-length ephrinB1 (right panel). The asterisk indicates phosphorylated ephrinB1.

(B) Phosphoamino acid analysis of in vitro phosphorylated GST-cytoB1 in pull-down experiments from NIH3T3-ephrinB1 stimulated with EphB2-Fc.

(C) Immunoprecipitation with preimmune serum (pre) or anti-ephrinB polyclonal antiserum (anti) to detect endogenous ephrinB ligand levels in extracts from HUAECs. The levels of ephrinB in HUAECs were also detected by directly immunoblotting membrane [M] and cytosolic [C] fractions of cell lysates. Total lysates from cells stimulated with Fc or EphB4-Fc were used in GST-cytoB1 kinase assays (right panel).

(D) In-gel kinase assays using as a substrate either GST-cytoB1 (upper panels) or GST alone (lower panels) were performed with lysates from Fc-stimulated and EphB-Fc-stimulated NIH3T3-ephrinB1 and HUAECs. Arrows in the upper panel indicate the kinase that phosphorylated GST-cytoB1.

are required for ephrinB tyrosine phosphorylation and ephrinB-mediated angiogenic sprouting of primary endothelial cells. Although SFKs are rapidly and stably recruited to ephrinB-containing membrane clusters, their activation is transient. At later time points, ephrinB ligands recruit PTP-BL to the membrane via PDZ domain interactions and become dephosphorylated. Our findings suggest the presence of a switch mechanism that allows ephrinB ligands to shift from phosphotyrosine/SFK-dependent signaling to PDZ domain-dependent signaling.

Results

A 60 kDa Kinase Phosphorylates the Cytoplasmic Domain of EphrinB1 in an EphB Receptor-Dependent Manner

To identify the kinase(s) responsible for ephrinB phosphorylation, we established an in vitro kinase assay using as the substrate the purified ephrinB1 cytoplasmic domain fused to GST (GST-cytoB1). GST-cytoB1 contains all five tyrosine residues, which are conserved in the three known ephrinB ligands (ephrinB1-B3). NIH3T3 fibroblasts stably expressing ephrinB1 (NIH3T3-ephrinB1), which were acutely stimulated with the EphB2 receptor ectodomain fused to the Fc portion of human IgG (EphB2-Fc), contained a kinase activity that phosphorylated GST-cytoB1 (Figure 1A). The kinase(s) activated by EphB2-Fc bound to GST-cytoB1 as they were also detected in pull-down experiments with GST-cytoB1 (Figure 1A). No phosphorylation of GST-cytoB1 was observed when the cells were stimulated with unfused Fc or when GST-cytoB1 was exogenously added to GST pull-downs (Figure 1A and data not shown). A similar kinase activity was bound to and phosphorylated full-length ephrinB1 immunoprecipitated from cells stimulated with EphB2-Fc (Figure 1A), showing that kinase(s) were able to bind ephrinB1 ligands in intact cells. Phosphoamino acid analysis of GST-cytoB1, which was phosphorylated in vitro in a pull-down assay in the presence of [^{32}P]γ-ATP, revealed the presence of phosphoserine and phosphotyrosine, but not phosphothreonine residues (Figure 1B).

The related ephrinB2 ligand is an essential regulator of early cardiovascular development and an angiogenic factor for endothelial cells in vitro (reviewed in Adams and Klein, 2000; Gale and Yancopoulos, 1999). To test if the putative ephrinB carboxy-terminal kinase is also activated by engagement of ephrinB2 with its EphB receptor, we used primary human umbilical aortic endothelial cells (HUAECs) in this assay. HUAECs endogenously express ephrinB ligands (Figure 1C), including ephrinB2, which is specifically enriched on arteries. Acute stimulation of HUAECs with the ephrinB2-specific receptor EphB4 (reviewed in Wilkinson, 2000) activated a kinase that specifically bound and phosphorylated GST-cytoB1 (data not shown and Figure 1C). The kinase was active 10 min after stimulation with the EphB4 receptor and later downregulated. These findings suggested that the observed in vitro kinase activity represented that of the physiological "ephrinB carboxy-terminal kinase," which is activated by ephrinB/EphB interaction.

Next, we performed in-gel kinase assays to determine the apparent molecular mass of the putative ephrinB carboxy-terminal kinase. Total cell lysates of EphB-Fc-activated NIH3T3-ephrinB1 cells and HUAECs were separated using SDS-PAGE polymerized in the presence of the substrate GST-cytoB1. In-gel kinase assays re-

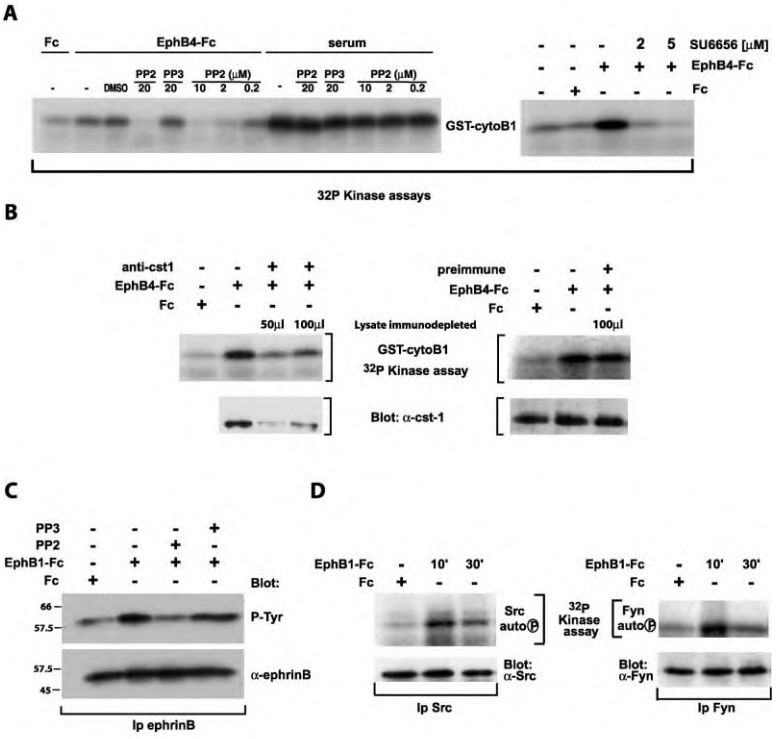

Figure 2. SFKs Are Required for Phosphorylation of EphrinB Ligands

(A) HUAECs were stimulated with Fc, EphB4-Fc, or serum for 10 min in the absence or presence of decreasing concentrations of the SFK inhibitors, PP2 (left panel) and SU6656 (right panel). DMSO and PP3, an inactive analog of PP2, were used as controls. Lysates were used for GST-cytoB1 kinase assays.

(B) Lysates of EphB4-Fc-stimulated HUAECs were immunodepleted from SFKs. GST-cytoB1 kinase assays were performed before and after immunodepletion (upper left panel). Two different amounts (50 and 100 μl) of stimulated cell lysate were immunodepleted with the same amount of antibody. The amount of SFKs remaining in the lysates was monitored by immunoblotting with anti-cst1 antibody (lower left panel). Control immunodepletion with preimmune serum was done in parallel (right panels).

(C) SFK activation is required for endogenous ephrinB ligand phosphorylation in primary cortical neurons. Primary cortical neurons from E14.5 mouse embryos were stimulated with either Fc or EphB1-Fc in the presence or absence of PP2 or the control compound PP3. Cell lysates were immunoprecipitated with anti-ephrinB polyclonal antiserum #23 and analyzed by immunoblotting with 4G10 (upper panel) and anti-ephrinB polyclonal antiserum #200 (lower panel).

(D) Src and Fyn are activated in cortical neurons upon stimulation with EphB-Fc. Neurons were stimulated as above for the indicated time. Cell lysates were immunoprecipitated with monoclonal antibodies anti-Src (2-17) or anti-Fyn (Fyn-14), and in vitro kinase assays (upper panels) or immunoblotting (lower panels) were performed. Autophosphorylation (auto P) is used as an indication of Src and Fyn kinase activities.

vealed the presence of a kinase that specifically phosphorylated GST-cytoB1 in an EphB-Fc-dependent manner and migrated with an apparent molecular mass of 60 kDa (Figure 1D). Identical results were obtained in both NIH3T3-ephrinB1 cells and HUAECs. When a duplicate of the samples was analyzed in gels polymerized with GST alone, we could detect faint signals which were likely to be autophosphorylated 60 kDa kinase (Figure 1D). Among the protein tyrosine kinases, Src family kinases (SFKs) have a molecular mass of ~60 kDa and were previously shown to phosphorylate the carboxy-terminal tail of ephrinB ligands in vitro (Holland et al., 1996), suggesting that the receptor-stimulated kinase could be a SFK.

SFKs Are Required for Phosphorylation of EphrinB Ligands

To investigate the requirement for SFKs in ephrinB phosphorylation, we first used specific SFK inhibitors in the in vitro ephrinB kinase assay. PP2 inhibitor completely neutralized the ephrinB carboxy-terminal kinase in EphB4-stimulated HUAECs (Figure 2A), whereas the structurally nearly identical control compound PP3 was ineffective. The inhibition was dose dependent with half maximal inhibition at 1 μM. Interestingly, ephrinB phosphorylation induced by calf serum was unaffected by PP2, showing the specificity of the inhibitor for the receptor-stimulated kinase and indicating that kinases other than SFKs can mediate the serum response (Figure 2A). We next independently confirmed these data by using the recently developed SFK inhibitor SU6656 (Blake et al., 2000). SU6656 abolished EphB4-Fc-induced phosphorylation of GST-cytoB1 in HUAECs, as did the PP2 inhibitor (Figure 2A)

To obtain biochemical evidence for a requirement of SFKs in ephrinB phosphorylation, we used an anti-SFK specific antibody (anti-cst1) (Kypta et al., 1990) to immunodeplete this kinase activity from lysates of EphB4-stimulated HUAECs. Anti-cst1 immunoprecipitation of SFKs depleted the amount of SFK protein in a dose-dependent manner (Figure 2B). Concomitantly, ephrinB carboxy-terminal kinase activity was reduced proportionally to the reduction of SFKs from the lysates (Figure 2B). As a control, immunoprecipitation of EphB4-stimulated HUAECs with preimmune serum did not deplete the "ephrinB kinase" present in these cells (Figure 2B). Thus, two independent methods (i.e., use of specific SFK inhibitors and anti-SFK immunodepletion) demonstrated a requirement for SFKs in ephrinB phosphorylation in vitro.

In Vivo EphrinB Phosphorylation in Primary Cortical Neurons Requires SFKs

We next asked if SFKs are required for ephrinB phosphorylation in vivo and whether this pathway is active in other cell types as well. EphrinB reverse signaling is also thought to occur in developing cortical neurons of the forbrain, in particular during commissural axon tract formation (Kullander et al., 2001; reviewed in Wilkinson, 2000). Most of the evidence came from genetic studies; however, Eph receptor-induced ephrinB phosphorylation has never been demonstrated in neurons. As shown

in Figure 2C, stimulation of primary mouse cortical neurons with preclustered EphB1-Fc led to a marked increase in phosphorylation of the endogenous ephrinB ligand, compared to the stimulation with preclustered, unfused Fc control protein. In the presence of the PP2 inhibitor, but not of the control compound PP3, this increase in phosphorylation was completely abolished. These observations strongly suggest that in living neurons, EphB receptor-induced ephrinB tyrosine phosphorylation required the activity of SFKs. Moreover, short-term stimulation of neurons with EphB1-Fc significantly increased in vitro autophosphorylation of both Src and Fyn kinases (Figure 2D). Increased Src and Fyn autophosphorylation was used as an indication of kinase activity. The increase was transient and returned to baseline by 30 min, thus correlating with the in vitro activity of the ephrinB carboxy-terminal kinase (see Figure 1C). Cortical neurons did not show detectable levels of Yes kinase (data not shown).

EphrinB Ligands and Src Colocalize in Membrane Rafts and Are Coclustered by EphB Receptors

Membrane raft microdomains are characterized by detergent insolubility at low temperatures and low buoyant density. In order to investigate if ephrinB ligands and Src colocalize in membrane rafts, Triton X-100 flotation gradients were prepared from E12.5 mouse embryo heads. A large fraction of ephrinB was found in the top fractions of the flotation gradient, indicating raft localization (Figure 3A). Probing the same fractions with an anti-Src antibody revealed the colocalization of Src in the rafts fraction.

We next attempted to visualize if ephrinB1 and Src colocalize in living cells. EphB2-Fc, but not unfused Fc, stimulation of NIH3T3-ephrinB1 cells induced the formation of receptor clusters in the membrane (Figures 3B and 3C). Immunofluorescence using anti-ephrinB antibodies showed the redistribution in vivo of ephrinB1 into these receptor patches (Figure 3B). Double immunostainings with anti-human Fc and anti-Src monoclonal antibodies revealed a redistribution of endogenous Src protein into patches that essentially overlapped with EphB-ephrinB complexes (Figure 3C), indicating regulated colocalization of these proteins in the living cell. EphB-induced recruitment of Src into membrane patches was also confirmed in primary cultures of cortical neurons expressing endogenous ephrinB ligands and Src kinases (Figure 3D).

We next asked if raft localization was necessary for ephrinB/Src coclustering. Cholesterol is the key component in maintaining the integrity of raft microdomains, and methyl-β-cyclodextrin (MβCD) is a membrane-impermeable oligosaccharide that selectively and rapidly extracts cholesterol from the plasma membrane (Awasthi-Kalia et al., 2001 and references within). Surprisingly, cholesterol depletion in NIH3T3-ephrinB1 cells did not prevent the formation of ephrinB/Src coclusters when cells were stimulated with EphB2-Fc (Figure 3C). However, treatment with MβCD dramatically elevated the phosphotyrosine content of ephrinB ligands, even in unstimulated cells (Figure 3E). These findings indicate that coclustering of ephrinB and Src occurs independently of rafts, but that raft localization of ephrinB and SFKs is required for proper regulation of ephrinB phosphorylation.

SFKs Are Required for Sprouting Angiogenesis Mediated by EphrinB Ligands

Having established this strong causal link between SFKs and ephrinB phosphorylation, we asked if SFKs were also required for ephrinB reverse signaling. Adrenal cortex-derived microvascular endothelial (ACE) cells coexpress ephrinB ligands and EphB receptors and can be induced in vitro, by various angiogenic factors, to form small capillary sprouts (Koblizek et al., 1998). Interestingly, sprout formation could also be induced by soluble, preclustered EphB4-Fc, suggesting that ephrinB ligands can trigger this response (Figure 4). EphB4-Fc induced a 2- to 5-fold increase in the number of sprouts, depending on the experiment (Figure 4A). The activity of EphB4-Fc was comparable to potent angiogenic factors such as vascular-endothelial growth factor (VEGF) (Figure 4A, Exp1). We examined whether in this assay SFKs are required for the angiogenic response mediated by ephrinB ligands. The PP2 inhibitor (0.5 μM) completely neutralized EphB4-Fc-induced sprouting, but did not reduce basal sprouting activity (Figures 4A and 4B). Basal sprouting activity of ACE cells was only inhibited at higher concentrations of PP2 (data not shown). The control compound PP3 was not inhibitory in this assay at concentrations up to 40-fold above (0.5–20 μM) the effective concentrations of PP2 (Figure 4A, Exp4). The effect of PP2 in the angiogenic response mediated by ephrinB ligands was specific, since PP2 was unable to block the angiogenic response of the chemokine stromal cell-derived factor-1 (SDF-1) and Angiopoietin-1, the ligand of the Tie-2 receptor tyrosine kinase (Gale and Yancopoulos, 1999; Nagasawa et al., 1998) (Figure 4A, Exp2 and Exp3).

PTP-BL Tyrosine Phosphatase, a Negative Regulator of EphrinB Phosphorylation

We next attempted to identify a protein tyrosine phosphatase (PTPase) that could regulate SFK-mediated ephrinB phosphorylation. The murine cytoplasmic PTPase PTP-BL and its human homolog FAP-1/PTP-BAS/PTPL1 (Erdmann et al., 2000 and references within) are 250 kDa proteins containing an amino-terminal FERM domain (*f*our point one, *e*zrin, *r*adixin, *m*oesin domain) common to cytoskeleton-associated proteins (Chishti et al., 1988), five PDZ domains, and a carboxy-terminal PTP domain. PTP-BL is expressed prominently in the developing peripheral nervous system, including the dorsal root ganglia (DRGs) (Thomas et al., 1998), and the PTP-BL PDZ4 domain binds ephrinB1. Since ephrinB ligands have also been shown to be expressed during development in the DRGs (Brückner et al., 1999), we asked if PTP-BL would colocalize with ephrinB in the growth cones of DRG neurons and if PTP-BL could regulate the phosphorylation state of ephrinB and Src.

Up to now, the interaction of ephrinB1 and PTP-BL was only demonstrated in vitro by pull-down assays from COS-1 cells overexpressing ephrinB1 using a PDZ domain of PTP-BL fused to GST (Lin et al., 1999). In order to verify interaction in vivo, we first performed pull-down assays with GST-cytoB1 on lysates prepared

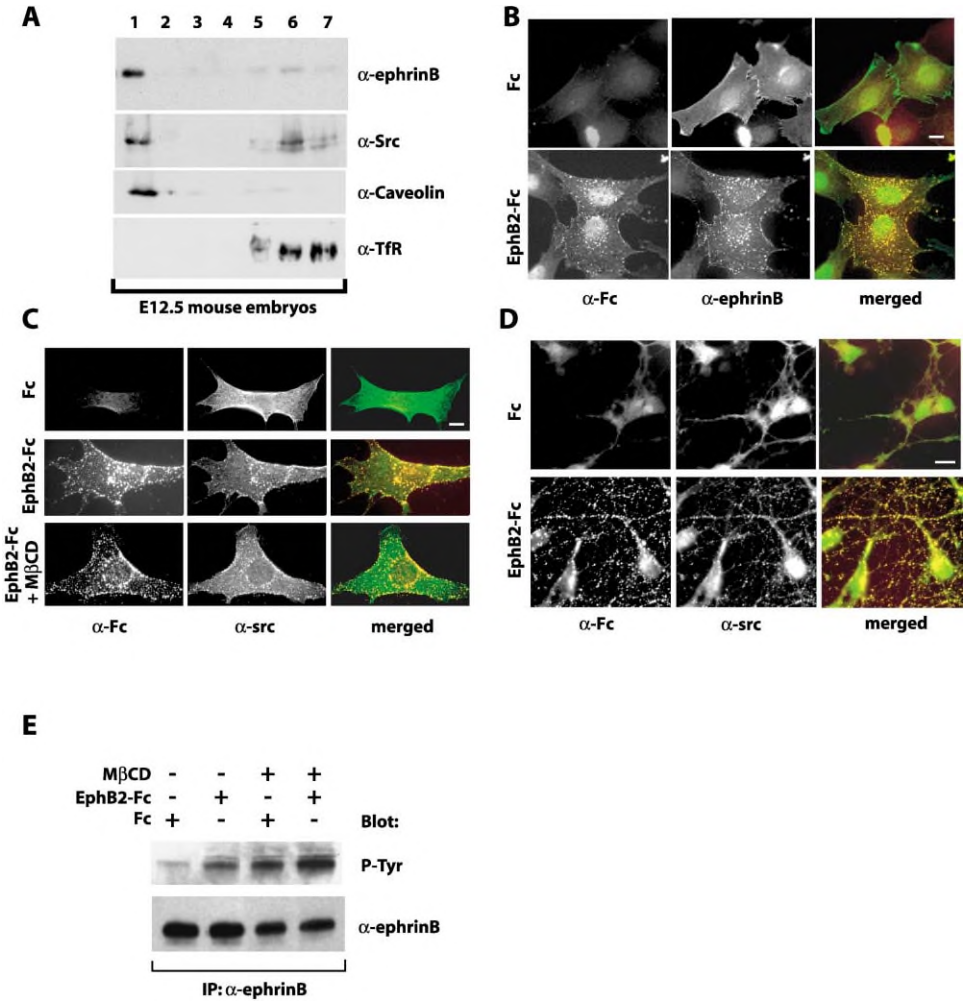

Figure 3. Stimulation with EphB Receptors Induces Formation of Large Membrane Patches Containing EphrinB Ligands and Src

(A) EphrinB ligands and Src colocalize in membrane rafts in the mouse embryo. A membrane preparation of heads from E12.5 mouse embryos was subjected to Triton X-100 extraction followed by Optiprep flotation gradients. Fractions were analyzed by Western blotting for the presence of ephrinB and Src. Caveolin and transferrin receptor (TfR) were used as raft and nonraft markers, respectively.

(B) EphB2-Fc stimulation of NIH3T3-ephrinB1 cells causes a redistribution of ephrinB1 into large membrane patches. Cells were stimulated with Fc (upper panels) or EphB2-Fc (lower panels). Receptor bodies were visualized with an anti-human Fc antibody conjugated to Texas-Red (left panels). EphrinB1 was detected using anti-ephrinB1 antiserum #200 (middle panel). The merge of the two signals is shown in the right panel.

(C) Src is recruited into ephrinB-containing patches induced by EphB-Fc. NIH3T3-ephrinB1 cells were stimulated with Fc (upper panels) or EphB2-Fc (horizontal-middle and -lower panels). Receptor bodies were visualized as above (left panels). The vertical-middle panels show distribution of endogenous Src detected with an anti-Src monoclonal antibody (2-17), and panels on the right show the merge of the two signals. In lower panels, cells were treated with 10 mM MβCD.

(D) Src is recruited to EphB receptor-induced patches in cortical neurons. Primary cortical neurons from E14.5 mouse embryos were stimulated with either Fc (upper panels) or EphB2-Fc (lower panels). Immunofluorescence to detect EphB2-Fc patches and endogenous Src was performed as described above.

(E) Cholesterol rafts are required for regulation of ephrinB phosphorylation. NIH3T3-ephrinB1 cells were pretreated for 20 min with or without 10 mM MβCD and subsequently stimulated for 10 min with Fc or EphB2-Fc in the presence or absence of MβCD. Immunoprecipitates of ephrinB1 were analyzed by Western blotting for phosphotyrosine (upper panel) and ephrinB1 (lower panel). Scale bars in (B), (C), and (D) represent 10 μm.

from E12.5 mouse embryos. GST-cytoB1, but not GST alone, specifically pulled down endogenous PTP-BL (Figure 5A). Moreover, we confirmed the interaction between ephrinB1 and PTP-BL in living cells by coimmunoprecipitating PTP-BL (PDZ1-5-EGFP) with full-length ephrinB1, but not ephrinB1 lacking the PDZ domain target site (Figure 5B). Interestingly, we found a proportion of PTP-BL phosphatase localized in rafts prepared from membranes of E12.5 mouse embryos and cultured cortical neurons (Figure 5C), indicating the presence of PTP-BL in the same subcellular compartment as ephrinB and SFKs. Colocalization of endogenous ephrinB and PTP-BL was also detected in patches in the growth cones of dissociated DRG neurons (Figure 5D).

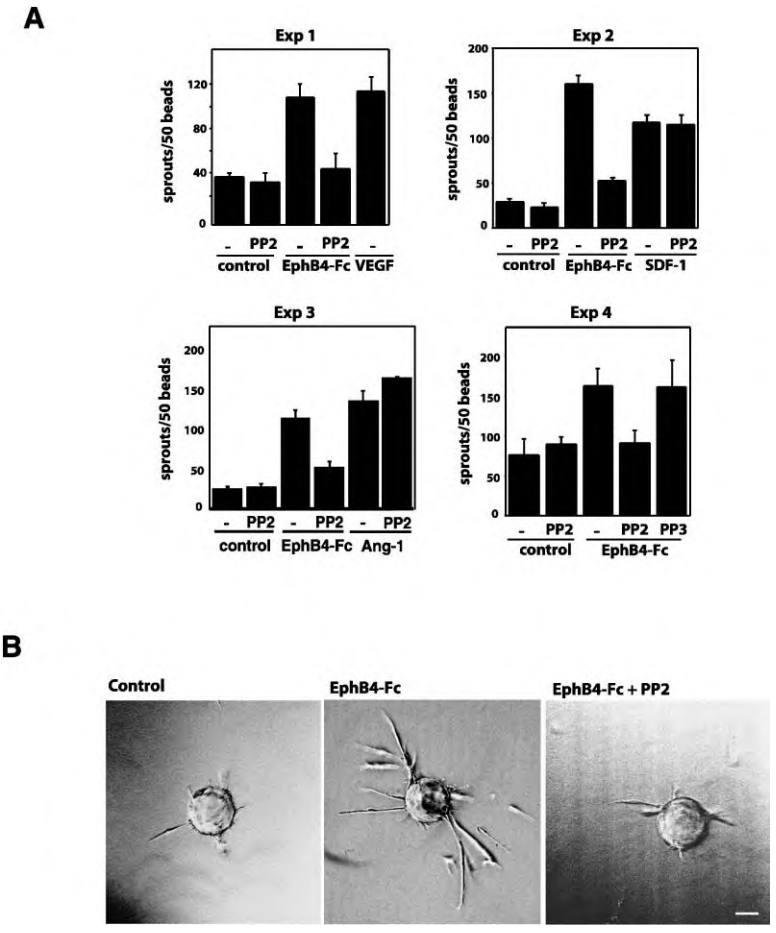

Figure 4. Src Family Kinases Are Required for the Angiogenic Sprouting Response Mediated by EphrinB Ligands

Adrenal cortex-derived microvascular endothelial (ACE) cells were seeded on MC beads and incubated in three-dimensional fibrin gels with different angiogenic factors in the presence or absence of the Src inhibitor PP2. (A) Quantitative analysis of sprout formation expressed as the number of capillary sprouts with lengths exceeding the diameter of the MC bead for every 50 MC beads counted. (B) Phase-contrast photomicrographs of angiogenic sprouts induced by EphB4-Fc in the presence and absence of PP2. Scale bars represent 25 μm.

We next investigated whether Src and ephrinB might serve as substrates for PTP-BL. Bacterially expressed wild-type PTP-BL phosphatase domain, but not a catalytically inactive point mutant of PTP-BL (PTP-BL-CS), dephosphorylated Src specifically on Y418, a tyrosine autophosphorylation site required for Src activation (reviewed in Bjorge et al., 2000). In contrast, Y529, whose phosphorylation inhibits Src kinase activity, was not dephosphorylated by PTP-BL (Figure 5E), suggesting that dephosphorylation of Src by PTP-BL would lead to inactivation of Src. Moreover, wild-type PTP-BL tyrosine phosphatase domain, but not the Cys→Ser inactive mutant, led to the complete dephosphorylation of ephrinB immunoprecipitated from mouse embryo lysates, suggesting its specificity for ephrinB's in vivo phosphorylation sites (Figure 5E). We further investigated if ephrinB ligands could serve as substrates for PTP-BL in intact cells. HeLa cells were transiently transfected with an ephrinB1 expression construct either alone or together with expression constructs encoding PTP-BL or PTP-BL-CS. EphB2-Fc-induced tyrosine phosphorylation on ephrinB1 could be detected in ephrinB1-transfected cells and in double transfections together with the mutant PTPBL-CS (Figure 5F, lanes 2 and 6). When cotransfected with wild-type PTP-BL, tyrosine phosphorylation of ephrinB1 was reduced (Figure 5F, compare lanes 2 and 4), indicating that ephrinB ligands are in vivo substrates of the phosphatase PTP-BL.

A Switch Mechanism Shifts Phosphotyrosine/SFK-Dependent to PDZ-Dependent Signaling

The data so far indicated that SFK activation was required for ephrinB phosphorylation and that PTP-BL turned this pathway off. Such a model would suggest that SFKs are recruited to ephrin clusters and activated more rapidly than PTP-BL. Moreover, because PTP-BL carries multiple PDZ domains and interacts with other cytoplasmic effectors (Erdmann et al., 2000 and references within), prolonged association of PTP-BL with ephrins could indicate a signaling role of PTP-BL in addition to its function as a phosphatase.

To follow the kinetics of coclustering and phosphorylation/dephosphorylation, we modified our stimulation protocol such that we gave a 10 min pulse of EphB2-Fc. Src recruitment into ephrinB/EphB-containing patches was observed already at 2–5 min after EphB2-Fc addition and was maintained until 30 min to 1 hr after EphB2-Fc removal from the cell medium (Figure 6A). SFK activity was highest at 10 min (see also Figure 1C) and decreased at later time points (even when EphB2-Fc continued to be present in the cell medium; Figure 6B). The kinetics of ephrinB phosphorylation correlated very well with SFK activity, was detected after 10 min stimulation, and decreased progressively after removal of EphB2-Fc (Figure 7B). PTP-BL recruitment to receptor-bound ephrins was clearly observed at 30 min after EphB2-Fc removal (rarely already at 15 min) and was maintained

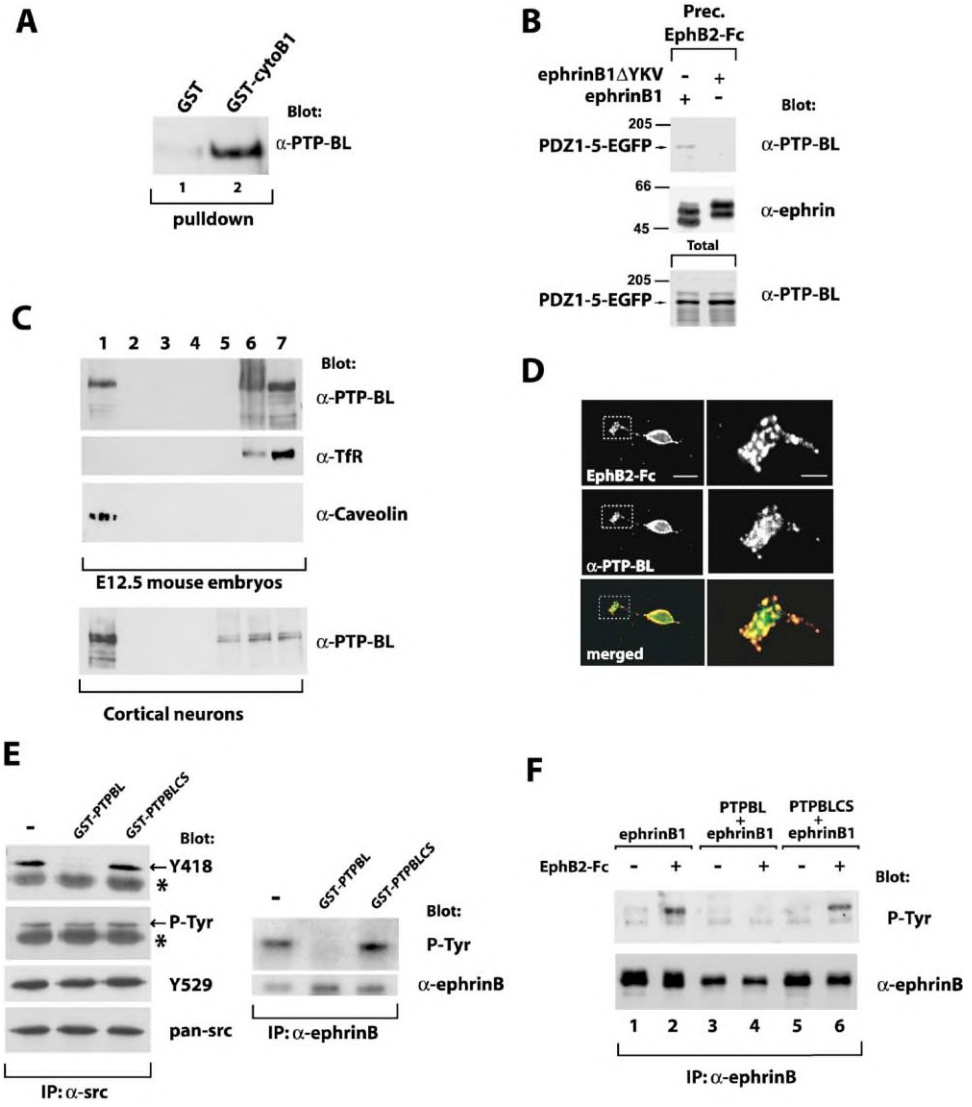

Figure 5. Regulation of EphrinB- and Src-Phosphorylation by PTP-BL Tyrosine Phosphatase

(A) PTP-BL binds to the carboxy terminus of ephrinB ligands. GST or GST-cytoB1 pull-downs were prepared from E12.5 embryo head lysates, and the presence of PTP-BL was analyzed by immunoblotting.

(B) Interaction of PTP-BL with ephrinB1 in HeLa cells. An expression construct of PTP-BL containing the PDZ domains 1–5 fused to EGFP was coexpressed in HeLa cells with full-length ephrinB1 or ephrinB1 lacking its PDZ binding motif (ephrinB1ΔYKV). EphB2-Fc precipitates were analyzed by Western blotting for PTP-BL (upper panel) and ephrinB1 (middle panel). Lower panels show levels of PTP-BL PDZ1-5 in total lysates.

(C) PTP-BL is located in rafts in the mouse embryo and in cortical neurons. A membrane preparation of heads from E12.5 mouse embryos and from primary cortical neurons was subjected to Triton X-100 extraction followed by Optiprep flotation gradients. Seven fractions were collected from the top to the bottom and analyzed by Western blotting for the presence of PTP-BL. Caveolin and transferrin receptor (TfR) were used as raft and nonraft markers.

(D) PTP-BL colocalizes with ephrinB-containing patches in the growth cones of primary dorsal root ganglion neurons. DRG neurons, dissected from E9 chick embryos, were fixed, stained with EphB2-Fc, permeabilized, and then stained for PTP-BL. Scale bars represent 10 μm, and 2μm for the enlargements.

(E) EphrinB and Src are dephosphorylated in vitro by PTP-BL. Phosphorylated ephrinB and Src proteins were immunoprecipitated from E12.5 mouse embryos and incubated with GST-PTP-BL or the catalytically inactive GST-PTP-BL CS. Phosphorylation and total levels of ephrinB ligands were detected using anti-phosphotyrosine antibodies (4G10) and anti-ephrinB polyclonal antiserum #23, respectively (right panel). The phosphorylation state of specific tyrosine residues in Src was monitored using anti-Y418 and anti-Y529 polyclonal antibodies. Total levels of Src phosphorylation and Src were detected using 4G10 and pan-src polyclonal antibody, respectively. Asterisks indicate IgG bands (left panel).

(F) EphrinB ligands are in vivo substrates of PTP-BL phosphatase. HeLa cells were transiently transfected with expression constructs encoding ephrinB1 alone (lanes 1 and 2) or cotransfected with PTP-BL (lanes 3 and 4) or the corresponding inactive mutant PTP-BL CS (lanes 5 and 6). After 30 min stimulation with EphB2-Fc, ephrinB was precipitated using EphB2-Fc, and phosphorylation was monitored using 4G10 antibody (upper panel). Total levels of ephrinB1 in the precipitates were detected by Western blotting with antibodies to anti-ephrinB (lower panels).

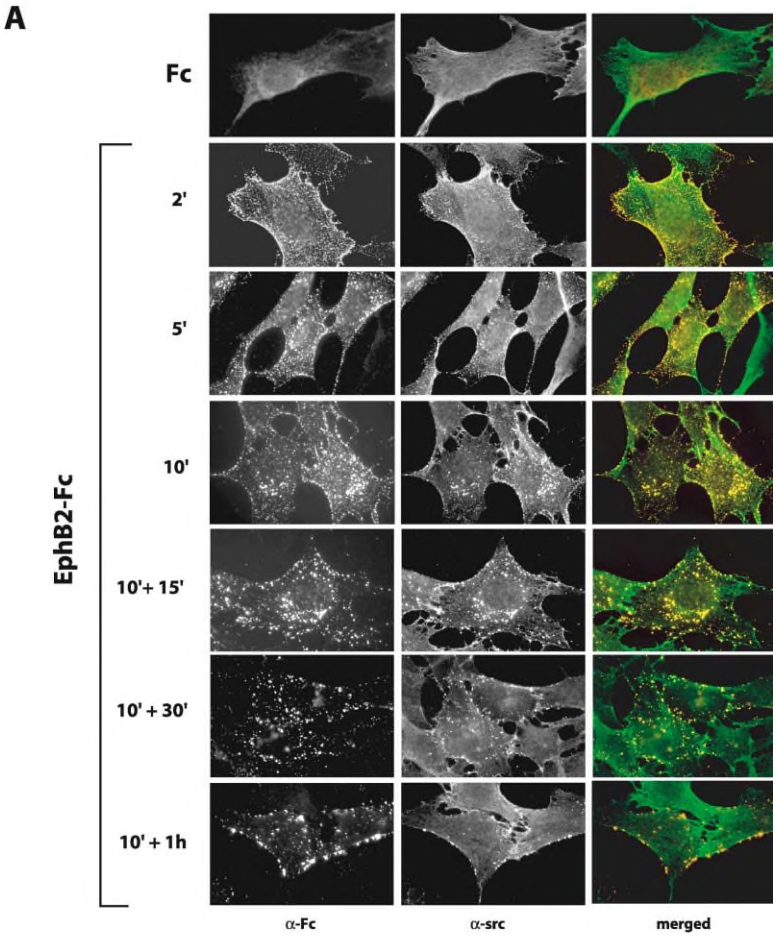

Figure 6. Src Kinase Is Recruited Rapidly to Ephrin-Containing Patches and Activated Transiently after Stimulation with EphB-Fc

(A) Rapid and prolonged Src-recruitment to EphB2-Fc-induced patches. NIH3T3-B1 cells were stimulated and fixed at the indicated time points (upper four panels). After 10 min of stimulation, cells were washed and left in medium without EphB2-Fc for 15, 30, and 60 min prior to fixation (lower three panels). Immunostaining was performed as described above.

(B) Rapid and transient SFK activation after stimulation with EphB-Fc. NIH3T3-ephrinB1 cells were stimulated with EphB2-Fc for the indicated time points, and in vitro kinase assays were performed as described above.

for at least another 30 min (Figure 7A). These findings therefore support a model in which EphB2 stimulation causes rapid recruitment and activation of SFKs, which will phosphorylate ephrinB on tyrosine and allow reverse signaling via SH2-containing effectors. This signaling is then terminated by delayed recruitment of PTP-BL and subsequent silencing of SFKs and ephrinB dephosphorylation.

Discussion

Here we show that Src family kinases are required for EphB receptor-induced ephrinB tyrosine phosphorylation and propose a mechanism for SFKs' activation by recruitment into ephrinB-containing membrane patches. Functionally, SFKs are required for ephrinB-mediated angiogenic responses in endothelial cells. We further implicate the tyrosine phosphatase PTP-BL as negative regulator of SFK activity and ephrinB phosphorylation. The kinetics of SFK and PTP-BL recruitment suggest the presence of a switch mechanism that allows a shift from phosphotyrosine-dependent signaling to PDZ-dependent signaling.

Early steps in ephrinB reverse signaling involve phosphorylation on tyrosine residues and interaction with PDZ proteins via its carboxy-terminal tail. At least in vitro, PDZ binding may in certain cases also be modulated by phosphorylation of ephrinB carboxy-terminal tyrosine residues (Lin et al., 1999). Our identification of SFKs as mediators of ephrinB phosphorylation in primary endothelial cells and neurons is an important step in molecularly dissecting this signaling pathway. Our

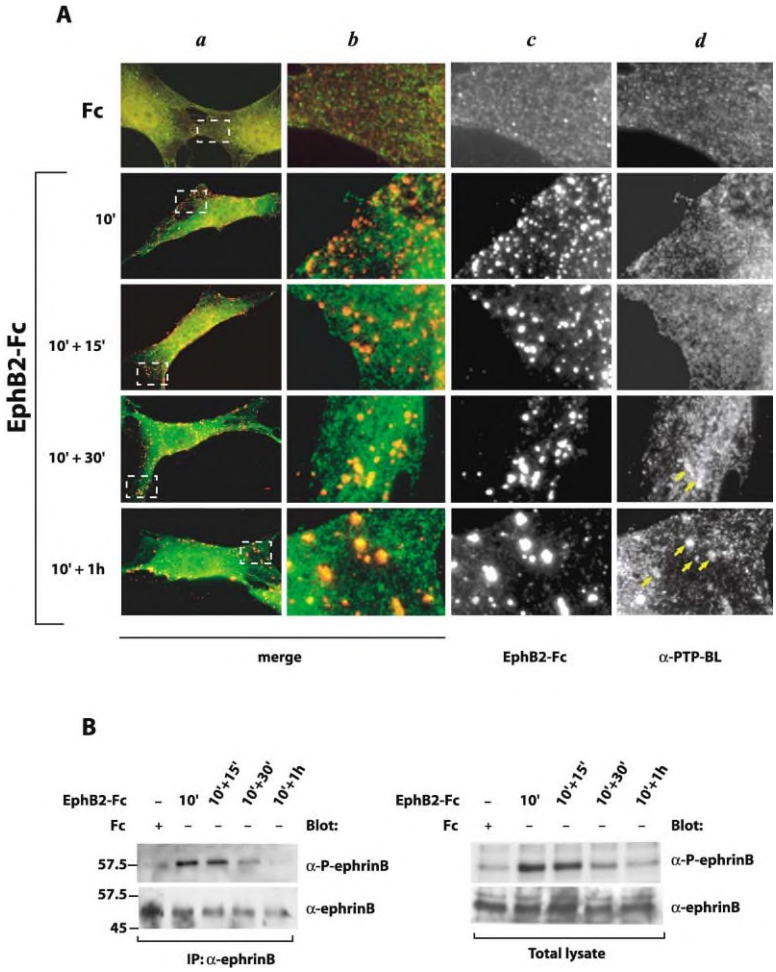

Figure 7. Downregulation of EphrinB Phosphorylation Coincides with Delayed Kinetics of PTP-BL Recruitment

(A) PTP-BL is recruited to EphB2-Fc-induced patches with delayed kinetics. NIH3T3-B1 cells were stimulated for the indicated time points (upper two panels). After 10 min of stimulation, cells were washed and left in medium without EphB2-Fc for the indicated time points (lower three panels). Column *a* shows the merge of the two signals with anti-Fc staining in red and anti-PTP-BL staining in green. Columns *b–d* show the zoom of the dashed box in column *a* (*b*, merge; *c*, anti-Fc; *d*, anti-PTP-BL). Arrows indicate places of accumulation and colocalization of PTP-BL with EphB2-Fc-induced patches. (Scale of dashed box, 28 × 19 μm).

(B) Downregulation of ephrinB phosphorylation. NIH3T3-ephrinB1 cells were stimulated as described above. Phosphospecific antibodies for ephrinB ligands were used to detect phosphorylated ephrin in ephrinB1 immunoprecipitates (left panel) and total cell lysates (right panel).

data shed light on the precise mechanism of SFK activation downstream of ephrinB ligands. EphrinB ligands and SFKs are both localized in lipid rafts, and stimulation with soluble EphB receptors leads to reorganization of ephrinB- and Src-containing rafts into larger membrane patches. SFKs may get activated due to the increased focal concentration into these patches, a mechanism that could enhance the autotransphosphorylation on Tyr418 and the consequent kinase activation. Interestingly, raft disruption by cholesterol depletion in NIH3T3-ephrinB1 cells does not affect the clustering of ephrinB ligands induced by the receptor and the subsequent recruitment of Src into these clusters. However, ephrinB phosphorylation was increased even in the absence of EphB-Fc, suggesting that Src could be already activated under such conditions. Indeed, regulation of SFKs activity has been shown to occur in rafts and be dependent on their integrity (Kawabuchi et al., 2000; Awasthi-Kalia et al., 2001). The reorganization occurs with rapid kinetics, suggesting that SFK recruitment and activation represents one of the first events in ephrinB reverse signaling. Both phosphorylated ephrins and SFKs may activate separate signaling pathways leading to actin cytoskeleton rearrangements (Abram and Courtneidge, 2000; Cowan and Henkemeyer, 2001). GPI-anchored ephrinA ligands have been shown to be recruited to rafts, and stimulation with EphA-Fc led to Fyn kinase activation (Davy et al., 1999). Interestingly, SFKs are also activated downstream of Eph receptors (reviewed in Wilkinson, 2000), therefore suggesting, together with our results, that SFKs may be important mediators of ephrin/Eph bidirectional signaling.

The in vivo phosphorylated tyrosines in ephrinB ligands involved in the process of reverse signaling are currently unknown. In a recent study, Kalo et al. (2001) identified three tyrosines of ephrinB1 as in vivo phosphorylation sites in transfected 293 cells stimulated with soluble EphB2-Fc, and the same residues are in vivo phosphorylated in the embryonic retina. Using phospho-specific anti-ephrinB antibodies on lysates of cultured cells, we have confirmed the phosphorylation of the tyrosines at positions −18 and/or −23 from the C terminus.

We have recently shown that ephrinB2 requires its cytoplasmic domain for angiogenic remodeling in vivo (Adams et al., 2001), suggesting that ephrinB2 reverse signaling is crucial for endothelial cell communication. Here we demonstrated that SFKs are required for the in vitro angiogenic response induced by soluble EphB4-Fc. The data suggest that EphB4-Fc binding to endogenous ephrinBs activates SFKs, which phosphorylate the cytoplasmic domain of ephrinB, thereby creating docking sites for SH2 adaptor proteins such as Grb4. Further, EphB4-mediated coclustering of ephrins and SFKs may

engage SFKs with other substrates, which mediate sprouting independently of or in concert with the tyrosine-phophorylated cytoplasmic domain of ephrinB. In addition to their established functions in cell proliferation, adhesion, and differentiation, SFKs have recently been shown to play important roles in angiogenesis. Dominant interfering mutants of Src blocked VEGF- but not bFGF-induced angiogenesis in chick embryos, and studies in mutant mice showed that SFKs are required for VEGF-induced blood vessel permeability (Eliceiri et al., 1999).

However, SFKs are not required for all angiogenic stimuli. The responses of endothelial cells to other potent angiogenic factors such as the chemokine SDF-1, via its receptor CXCR4, and Ang-1, via its receptor Tie-2, were not affected by inhibition of SFKs in our assay. This suggests that distinct downstream signaling cascades are involved in the angiogenic process regulated by these different molecules.

SFKs are probably not the only kinases involved in ephrinB phosphorylation. Serum stimulation of endothelial cells also led to robust ephrinB phosphorylation that was not affected by the Src inhibitor PP2. Since stimulation of HUAECs with PDGF did not activate any kinase able to phosphorylate GST-cytoB1 in our kinase assay (data not shown), we assume that PDGF receptors are not expressed in these cells, and therefore, the identity of the serum-activated ephrinB kinase is unclear. EphrinB1 has been shown to interact and be phosphorylated by FGF receptors when coexpressed in *Xenopus* embryos (Chong et al., 2000). Therefore, FGF receptors in endothelial cells could be candidates for the serum-activated kinase in our system. Moreover, phosphoamino acid analysis of in vitro phosphorylated ephrinB1 revealed the presence of a serine/threonine kinase capable of phosphorylating ephrinB on serine residues. The functional consequences of serine phosphorylation will have to be further explored. It is possible that the sequences surrounding phosphoserine residues could serve as docking sites for cytoplasmic effectors carrying interaction modules similar to 14-3-3 proteins (Pawson and Scott, 1997).

Our findings implicate the PDZ domain-containing PTPase PTP-BL as an important component of the signaling apparatus downstream of ephrinB ligands. Our coexpression studies in growth cones, together with previously published in situ data (Thomas et al., 1998; Brückner et al., 1999), support the idea that PTP-BL and ephrinB ligands act in concert in guiding peripheral axons during development. Further, PTP-BL is coexpressed with ephrinB2 in endothelial cells (data not shown), is targeted to membrane rafts in embryonic neurons, and interacts with the cytoplasmic domain of ephrinB1 (see also Lin et al., 1999).

In cells stably expressing physiological levels of ephrinB1, the interaction between endogenous PTP-BL and ephrinB1 is not constitutive, but rather is induced by EphB2 receptor engagement, possibly due to the clustering of ephrins. Regulation of PDZ domain interaction suggests functional relevance. The coclustering of PTP-BL and ephrinB happens with delayed kinetics, in contrast to the rapid copatching of ephrinB and Src that occurs only 2 min after stimulation with the receptor.

In vitro phosphatase assays on ephrinB immunoprecipitated from mouse embryos showed that PTP-BL is able to dephosphorylate the native phosphorylation sites of ephrinB ligands. By coexpression studies in HeLa cells, we could demonstrate that PTP-BL interacts with and dephosphorylates ephrinB in living cells. We have also shown that in vitro PTP-BL dephosphorylates Src at Y418, the autophosphorylation site required for Src activation, but not at Y529, the negative regulatory site phosphorylated by Csk. Together with the known role of PTP-BL a as Src antagonizer in a functional yeast growth assay (Superti-Furga et al., 1996), our data suggest that PTP-BL is a negative regulator of Src downstream of ephrinB ligands.

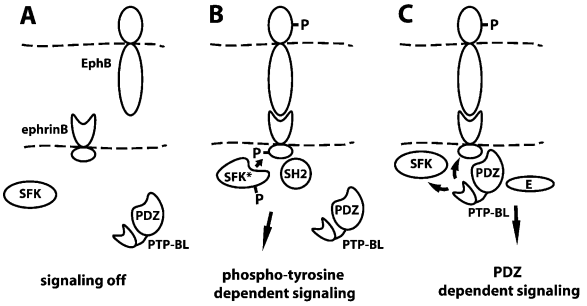

Figure 8. Switch Model for EphrinB Reverse Signaling

(A) EphrinB ligand in the unbound state. EphrinB, SFKs, and PTB-BL are localized to different compartments in the cell. The reverse signaling pathways are switched off.

(B) Binding of EphB receptors to ephrinB ligands recruits SFKs and ephrinB to the same compartments in the membrane. SFKs are activated and subsequently phosphorylate ephrinB ligands on tyrosine. The cytoplasmic domain of ephrinB now serves as a docking site for adaptor molecules like SH2 domain-containing proteins, which transduce a phosphotyrosine-dependent signal to the interior of the cell.

(C) PTP-BL is recruited with delayed kinetics to the signaling complexes containing ephrinB and SFKs. PTP-BL dephosphorylates ephrinB ligands and inactivates Src. Phosphotyrosine-dependent signaling is switched off, and the multiple PDZ domains allow interaction with other cytoplasmic effectors [E]. PDZ-dependent signaling is prolonged and supersedes the phosphotyrosine-dependent signaling.

Based on these data, we suggest the following model (Figure 8). EphrinB engagement with its EphB receptors induces the rapid coclustering of ephrinB and SFKs, causing SFK activation and ephrinB phosphorylation. Both active SFKs and phosphorylated ephrinB activate signaling pathways, either independently or in concert with each other, involving phosphotyrosine/SH2 interactions. With delayed kinetics, ephrinB clusters recruit PTP-BL, which dephosphorylates both Src and ephrinB, effectively turning off signaling by ephrinB and Src via phosphotyrosine. The recruitment of PTP-BL to ephrinB may not terminate ephrinB signaling completely, but rather shifts signaling from phosphotyrosine-dependent to PDZ domain-dependent signaling.

A first example of ephrinB signaling via PDZ domain proteins was shown for cerebellar granule cells, which require the PDZ-RGS3 protein to respond to chemoattractants signaling through heterotrimeric G proteins (Lu et al., 2001). PTP-BL contains five PDZ domains, which interact with other cytoplasmic proteins including a GTPase-activating protein (GAP) with specificity for the

Ras-like GTPase Rho (Saras et al., 1997). Rho GTPases are important regulators of actin cytoskeleton dynamics in response to external stimuli (Hall and Nobes, 2000), and ephrinB-mediated axon guidance is thought to involve rapid changes in actin filament organization. Moreover, SFKs are known to regulate cell morphology, adhesion, and migration by association with and phosphorylation of focal adhesion kinase (FAK) and p190 RhoGAP. FAK is a downstream phosphorylation target of ephrinB reverse signaling (Cowan and Henkemeyer, 2001). Mice deficient in p190 RhoGAP exhibit a lack of the anterior commissure (Brouns et al., 2001), a phenotype associated with a lack of ephrinB reverse signaling in ephB2$^{-/-}$ mice (reviewed in Wilkinson, 2000). Thus, it will be very interesting for future studies to analyze the role of the Src/p190 RhoGAP pathway and how these proteins contribute to both phosphotyrosine-dependent and PDZ domain-dependent signaling linked to rearrangements of the actin cytoskeleton downstream of ephrinB ligands.

Experimental Procedures

Cell Culture

NIH3T3-ephrinB1 cells were as previously described (Brambilla et al., 1995). Transient cotransfections were done with full-length ephrinB1, ephrinB1ΔYKV, a truncated version of PTP-BL or PTPBL-CS (Cys2285→Ser) lacking the N terminus, and the FERM domain and the PDZ domains 1–5 from PTPBL fused to EGFP. HUAECs isolated from human umbilical cords were grown in endothelial cell growth medium plus bovine pituitary brain extract (Clonetics, Verviers, Belgium) and 1 ng/ml bFGF (Sigma, Munich, Germany).

For cultures of cortical neurons, forebrain hemispheres from E14.5 mouse embryos were dissected, dissociated, and plated as described (de Hoop et al., 1998).

All cell types were stimulated with EphB-Fc (R+D systems, Wiesbaden, Germany) or Fc (Dianova, Hamburg, Germany) preclustered for 1 hr at room temperature using goat anti-human IgG (Jackson Immunoresearch, West Grove, PA). Before stimulation, all cell types except cortical neurons were starved for 16 hr in medium containing 0.5% serum. Transient stimulation was carried out as above, but after 10 min of EphB2-Fc addition, cells were washed and left in medium containing 0.5% serum.

PP2, or its negative control PP3 (Calbiochem, Schwalbach, Germany), were added to the cells together with EphB-Fc. SU6656 was added 1 hr prior stimulation.

Antibodies and Reagents

Antibodies include anti-ephrin (polyclonal antisera #23 and #200, previously described as anti-Lerk2A and B, respectively [Brückner et al., 1997]; goat anti-ephrinB1 [R+D Systems]; and anti-phospho-ephrinB, developed by Cell Signaling Technology [Beverly, MA], directed against a peptide epitope corresponding to ephrinB tyrosine phosphorylated at −18 and −23 positions from the C terminus); anti-Fyn (fyn-14; Santa Cruz Biotechnology, Heidelberg, Germany); anti-pan-src and anti-src Y529 (Biosource Deutschland, Solingen, Germany); anti-SrcY418 (Cell Signaling Technology); α-PTP-BL (Erdmann et al., 2000); Alexa Fluor488 goat anti-mouse and goat anti-rabbit conjugates (Molecular Probes, Leiden, Netherlands); Texas Red-conjugated goat anti-human Fc (Jackson Immunoresearch); and Texas Red-conjugated donkey anti-goat (Dianova) and goat anti-human Fc antibody (Dianova). Protein A Sepharose and protein G Sepharose were from Pharmacia Biotech (Freiburg, Germany).

Kinase Assays

Recombinant GST-cytoB1 expressed in *Escherichia coli* was purified as described (Palmer et al., 1998). In vitro kinase assays with total lysates were performed in a final volume of 20 μl of kinase buffer (Palmer et al., 1998) containing 4 μg total protein from the lysate, 50 μM unlabeled ATP, 10 μCi of [^{32}P]γ-ATP (5000 Ci/mmol), and 3 μg of GST-cytoB1. After 30 min at room temperature, the phosphorylation reactions were terminated by addition of sample buffer and were analyzed by SDS-PAGE and autoradiography.

For kinase assays in pull-down experiments and immunoprecipitates, washed beads were incubated in a final volume of 12 μl of kinase buffer containing 50 μM cold ATP and 10 μCi of [^{32}P]γ-ATP. In-gel kinase assays were performed following the protocol described (Palmer et al., 1998). Phosphoamino acid analysis was performed using standard procedures (Kamps, 1991).

Phosphatase Assays

GST-PTPBL and GST-PTPBL CS were expressed in bacteria and purified, and phosphatase assays were performed as described (Erdmann et al., 2000).

Immunoblotting, Immunoprecipitation, and Pull-Down Experiments

For immunoblotting, protein samples were separated by 6%, 7.5%, or 10% SDS-PAGE or 15% Anderson PAGE and transferred to 0.2 μm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). For immunoprecipitation, cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 0.5%–1% Triton X-100, 150 mM NaCl, 10 mM NaPPi, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 2.5 mM benzamidine, and 10 μg/ml each of leupeptin and aprotinin) and centrifuged at 10,000 × g for 10 min. For immunodepletion of SFKs, lysates from HUAECs were incubated with 2 μg of anti-cst1 prebound to 20 μl of protein A Sepharose. For EphB2-Fc precipitation, cells were lysed in NP40 lysis buffer (0.5% NP40, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium orthovanadate, 20 mM Tris-HCl [pH 7.4], and protease inhibitors), and 2.5 μg of EphB2-Fc, prebound to protein G beads, was used per precipitation. For GST pull-down experiments, 3–5 μg of bacterially produced GST or GST-cytoB1 was prebound to 20 μl of glutathione-sepharose beads and then incubated with lysates (1 mg of protein).

Purification of Membranes and Rafts from Mouse Embryos and Cortical Neurons

Cell membranes were prepared from homogenates of E12.5 mouse embryo heads or cultured cortical neurons by centrifugation in 40%/30%/5% Optiprep (Nycomed, Oslo, Norway) discontinuous gradients (4 hr, 24,000 rpm, 4°C; SW40 rotor, Beckman). Collected membrane fractions were extracted for 30 min on ice with 0.5% Triton X-100 for ephrinB1 and Src and 0.1% Triton X-100 for PTP-BL flotations. Rafts were prepared by centrifugation on 35%–40%/30%–35%/5% Optiprep discontinuous gradients (4 hr, 40,000 rpm, 4°C; SW60 rotor, Beckman). Collected fractions were incubated with 1% Triton X-100 at room temperature and immunoprecipitated with anti-ephrinB polyclonal antiserum (#23) for the detection of ephrinB ligands, or total protein was precipitated with tricarboxylic acid (TCA) and analyzed by SDS-PAGE and Western blotting for detection of Src and PTP-BL.

Membrane Patching of EphrinB Ligands and Immunocytochemistry

NIH3T3-ephrinB1 cells and cortical neurons were seeded on glass coverslips and stimulated with 4 μg ml^{-1} of EphB-Fc. After stimulation, cells were fixed for 20 min at 4°C with 4% PFA/4% sucrose in PBS and permeabilized for 5 min at 4°C with 0.2% Triton X-100 in PBS. Chick embryonic sensory DRG neurons (E9) were cultured on polyornithine/laminin-coated glass coverslips in low serum for at least 4 hr before fixation. Cells were fixed in warm 4% PFA for 20 min, blocked with 2% BSA, and incubated with EphB2-Fc (10 μg/ml) for 1 hr. After permeabilization with 0.2% NP-40 for 10 min at room temperature, the samples were processed for PTP-BL and anti-Fc staining.

Sprouting Assays

ACE cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL, Karlsruhe, Germany) on microcarrier (MC) Cytodex 3 beads (Sigma). The in vitro sprouting angiogenesis assay was done as described (Koblizek et al., 1998). The number of capillary sprouts with length exceeding the diameter of the MC bead was determined for every 50 MC beads counted.

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References

Abram, C.L., and Courtneidge, S. (2000). Src family tyrosine kinases and growth factor signaling. Exp. Cell Res. *254*, 1–13.

Adams, R.H., and Klein, R. (2000). Eph receptors and ephrin ligands: essential mediators of vascular development. Trends Cardiovasc. Med. *10*, 183–188.

Adams, R.H., Diella, F., Hennig, S., Helmbacher, F., Deutsch, U., and Klein, R. (2001). The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. Cell *104*, 57–69.

Awasthi-Kalia, M., Schnetkamp, P.P., and Deans, J.P. (2001). Differential effects of filipin and methyl-beta-cyclodextrin on B cell receptor signaling. Biochem. Biophys. Res. Commun. *287*, 77–82.

Bjorge, J.D., Jakymiw, A., and Fujita, D.J. (2000). Selected glimpses into the activation and function of Src kinase. Oncogene *19*, 5620–5635.

Blake, R.A., Broome, M.A., Liu, X., Wu, J., Gishizky, M., Sun, L., and Courtneidge, S.A. (2000). SU6656, a selective Src family kinase inhibitor, used to probe growth factor signaling. Mol. Cell. Biol. *20*, 9018–9027.

Brambilla, R., Schnapp, A., Casagranda, F., Labrador, J.P., Bergemann, A.D., Flanagan, J.G., Pasquale, E.B., and Klein, R. (1995). Membrane-bound LERK2 ligand can signal through three different Eph-related receptor tyrosine kinases. EMBO J. *14*, 3116–3126.

Brouns, M.R., Matheson, S.F., and Settleman, J. (2001). p190 RhoGAP is the principal Src substrate in brain and regulates axon outgrowth, guidance and fasciculation. Nat. Cell Biol. *3*, 361–367.

Brückner, K., Pasquale, E.B., and Klein, R. (1997). Tyrosine phosphorylation of transmembrane ligands for Eph receptors. Science *275*, 1640–1643.

Brückner, K., Labrador, J.P., Scheiffele, P., Herb, A., Bradke, F., Seeburg, P.H., and Klein, R. (1999). EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains. Neuron *22*, 511–524.

Chishti, A.H., Kim, A.C., Marfatia, S.M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S.C., Low, P.S., Rouleau, G.A., Mohandas, N., et al. (1988). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. Trends Biochem. Sci. *23*, 281–282.

Chong, L.D., Park, E.K., Latimer, E., Friesel, R., and Daar, I.O. (2000). Fibroblast growth factor receptor-mediated rescue of x-ephrin B1-induced cell dissociation in Xenopus embryos. Mol. Cell. Biol. *20*, 724–734.

Cowan, C.A., and Henkemeyer, M. (2001). The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals. Nature *413*, 174–179.

Davy, A., Gale, N.W., Murray, E.W., Klinghoffer, R.A., Soriano, P., Feuerstein, C., and Robbins, S.M. (1999). Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. Genes Dev. *13*, 3125–3135.

de Hoop, M.J., Meyn, L., and Dotti, C.G. (1998). Culturing hippocampal neurons and astrocytes from fetal rodent brain. In Cell Biology: A Laboratory Handbook, J.E. Celis, ed. (London: Academic Press), pp. 154–163.

Eliceiri, B.P., Paul, R., Schwartzberg, P.L., Hood, J.D., Leng, J., and Cheresh, D.A. (1999). Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol. Cell *4*, 915–924.

Erdmann, K.S., Kuhlmann, J., Lessmann, V., Herrmann, L., Eulenburg, V., Muller, O., and Heumann, R. (2000). The adenomatous polyposis coli-protein (APC) interacts with the protein tyrosine phosphatase PTP-BL via an alternatively spliced PDZ domain. Oncogene *19*, 3894–3901.

Gale, N.W., and Yancopoulos, G.D. (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev. *13*, 1055–1066.

Grundwald, I.C., Korte, M., Wolfer, D., Wilkinson, G.A., Unsicker, K., Lipp, H.-S., Bonhoeffer, T., and Klein, R. (2001). Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity. Neuron *32*, 1027–1040.

Hall, A., and Nobes, C.D. (2000). Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos. Trans. R. Soc. Lond. B Biol. Sci. *355*, 965–970.

Henderson, J.T., Georgiou, J., Jia, Z., Robertson, J., Elowe, S., Roder, J.C., and Pawson, T. (2001). The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function. Neuron *32*, 1041–1056.

Holland, S.J., Gale, N.W., Mbamalu, G., Yancopoulos, G.D., Henkemeyer, M., and Pawson, T. (1996). Bidirectional signaling through the EPH-family receptor Nuk and its transmembrane ligands. Nature *383*, 722–725.

Kamps, M.P. (1991). Determination of phosphoamino acids composition by acid hydrolysis of protein blotted to immobilon. Methods Enzymol. *201*, 21–27.

Kalo, M.S., Yu, H.H., and Pasquale, E.B. (2001). In vivo tyrosine phosphorylation sites of activated ephrin-b1 and ephb2 from neural tissue. J. Biol. Chem. *276*, 38940–38948.

Kawabuchi, M., Satomi, Y., Takao, T., Shimonishi, Y., Nada, S., Nagai, K., Tarakhovsky, A., and Okada, M. (2000). Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature *404*, 999–1003.

Klein, R. (2001). Excitatory Eph receptors and adhesive ephrin ligands. Curr. Opin. Cell Biol. *2*, 196–203.

Koblizek, T.I., Weiss, C., Yancopoulos, G.D., Deutsch, U., and Risau, W. (1998). Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr. Biol. *8*, 529–532.

Kullander, K., Mather, N.K., Diella, F., Dottori, M., Boyd, A.W., and Klein, R. (2001). Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. Neuron *29*, 73–84.

Kypta, R.M., Goldberg, Y., Ulug, E.T., and Courtneidge, S.A. (1990). Association between the PDGF receptor and members of the src family of tyrosine kinases. Cell *62*, 481–492.

Lin, D., Gish, G.D., Songyang, Z., and Pawson, T. (1999). The carboxyl terminus of B class ephrins constitutes a PDZ domain binding motif. J. Biol. Chem. *274*, 3726–3733.

Lu, Q., Sun, E.E., Klein, R.S., and Flanagan, J.G. (2001). Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. Cell *105*, 69–79.

Nagasawa, T., Tachibana, K., and Kishimoto, T. (1998). A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. Semin. Immunol. *10*, 179–185.

Palmer, A., Gavin, A.-C., and Nebreda, A.R. (1998). A link between MAP kinase and $p34^{cdc2}$/cyclin B during oocyte maturation: $p90^{rsk}$ phosphorylates and inactivates the $p34^{cdc2}$ inhibitory kinase Myt1. EMBO J. *17*, 5037–5047.

Pawson, T., and Scott, J.D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science *278*, 2075–2080.

Saras, J., Franzen, P., Aspenstrom, P., Hellman, U., Gonez, L.J., and Heldin, C.H. (1997). A novel GTPase-activating protein for Rho

interacts with a PDZ domain of the protein-tyrosine phosphatase PTPL1. J. Biol. Chem. *272*, 24333–24338.

Sheng, M., and Sala, C. (2001). PDZ domains and the organization of supramolecular complexes. Annu. Rev. Neurosci. *24*, 1–29.

Superti-Furga, G., Jonsson, K., and Courtneidge, S.A. (1996). A functional screen in yeast for regulators and antagonizers of heterologous protein tyrosine kinases. Nat. Biotechnol. *14*, 600–605.

Takasu, M.A., Dalva, M.B., Zigmond, R.E., and Greenberg, M.E. (2002). Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. Science *295*, 491–495.

Thomas, T., Voss, A.K., and Gruss, P. (1998). Distribution of a murine protein tyrosine phosphatase BL-beta-galactosidase fusion protein suggests a role in neurite outgrowth. Dev. Dyn. *212*, 250–257.

Wilkinson, D.G. (2000). Eph receptors and ephrins: regulators of guidance and assembly. Int. Rev. Cytol. *196*, 177–244.

Wilkinson, D.G. (2001). Multiple roles of EPH receptors and ephrins in neural development. Nat. Rev. Neurosci. *3*, 155–164.