Biphasic Functions of the Kinase-defective EphB6 Receptor in Cell Adhesion and Migration*

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EphB6 is a unique member in the Eph family of receptor tyrosine kinases in that its kinase domain contains several alterations in conserved amino acids and is catalytically inactive. Although EphB6 is expressed both in a variety of embryonic and adult tissues, biological functions of this receptor are largely unknown. In the present study, we examined the function of EphB6 in cell adhesion and migration. We demonstrated that EphB6 exerted biphasic effects in response to different concentrations of the ephrin-B2 ligand; EphB6 promoted cell adhesion and migration when stimulated with low concentrations of ephrin-B2, whereas it induced repulsion and inhibited migration upon stimulation with high concentrations of ephrin-B2. A truncated EphB6 receptor lacking the cytoplasmic domain showed monophasic-positive effects on cell adhesion and migration, indicating that the cytoplasmic domain is essential for the negative effects. EphB6 is constitutively associated with the Src family kinase Fyn. High concentrations of ephrin-B2 induced tyrosine phosphorylation of EphB6 through an Src family kinase activity. These results indicate that EphB6 can both positively and negatively regulate cell adhesion and migration, and suggest that tyrosine phosphorylation of the receptor by an Src family kinase acts as the molecular switch for the functional transition.

Interaction between cell surface receptors that contain tyrosine kinase activity and their cognate ligands is one of the fundamental mechanisms by which metazoan cells communicate. Generally, ligand binding to receptor tyrosine kinases induces dimerization or oligomerization of receptor monomers, which leads to activation and potentiation of their intrinsic catalytic activity and phosphorylation of tyrosine residues. These phosphorytrosines serve as binding sites for cytoplasmic signaling proteins containing Src homology 2 and phosphotyrosine-binding domains (1). The catalytic domain of both receptor and non-receptor tyrosine kinases has been highly conserved throughout evolution and includes a set of invariant amino acid residues and consensus motifs that participate in the phosphotransfer reaction (2, 3).

Through the identification of novel receptor tyrosine kinases, it has become apparent that some receptor tyrosine kinases have diverged to incorporate alterations in conserved amino acid residues in the kinase domain and to function as signal-transducing molecules that lack catalytic activity. These include ErbB3 (4–6), the Ryk subfamily (Ryk, Derailed, and Dnghnut) (7–10), and the CCK-4 (colon carcinoma kinase-4) subfamily (CCK-4/PTK7 (protein tyrosine kinase 7), Klg, Dtrk, and Lem) (11–15). Despite the lack of kinase activity, these molecules can transduce extracellular signals across the plasma membrane and mediate biological functions. The best-studied example is the ErbB3 receptor of the epidermal growth factor receptor family, which plays crucial roles in development by heterodimerizing with kinase-active ErbB receptors and then modulating their ligand affinities and functions (1). More recently, it has been reported that a targeted gene disruption of Ryk in mouse causes cleft palate (16). In Drosophila, the homolog of Ryk, Derailed, acts as a repulsive axon guidance receptor by interacting with Wnt5 (17, 18). These findings indicate that kinase-defective receptor molecules have been conserved in evolution and play crucial roles in signaling for normal development.

Eph receptors form the largest known subfamily of receptor tyrosine kinases, and to date, the Eph subfamily contains 16 members in vertebrates (19, 20) (see Eph nomenclature web site (cbweb.med.harvard.edu/eph-nomenclature/) for update). The Eph receptors interact with a family of ligands, ephrins. All the ephrins are membrane-attached, either via a glycosylphosphatidylinositol anchor (ephrin-A1–A6) or a transmembrane domain (ephrin-B1–B3). The Eph receptors are also grouped into two classes, EphA receptors (EphA1–A10) and EphB receptors (EphB1–B6). These groupings roughly correspond to the receptor-ligand interaction (i.e. ephrin-As show binding preference for EphA receptors, and ephrin-Bs for EphB receptors), although there is a wide variation in affinity within each group, and some interactions cross the group boundary. Eph receptors and ephrins have been implicated in many developmental processes, including axon guidance, synaptogenesis, embryonic compartmentation, and vascular development (21–24). The Eph-ephrin interaction has also been shown to regulate cell adhesion and migration. Although in most cases the Eph-ephrin interaction exerts repulsive or inhibitory functions, ephrins have been reported to have attractive or adhesive effects in several biological systems (25–27). However, the molecular mechanisms by which they can mediate differential functions remain to be elucidated.
EphB6 was identified in efforts to clone novel receptor tyrosine kinases in mouse (28) and in human (29), by a PCR-based screening and a low stringency library screening, respectively. Although the extracellular region of the EphB6 receptor shares all the structural features of other Eph family members, the cytoplasmic “kinase domain” has several critical amino acid substitutions and fails to show catalytic activity in vitro (28, 29). EphB6 is highly expressed in the developing and adult nervous system and thymus and is expressed at low levels in other tissues, including heart, kidney, and liver. Several reports have demonstrated that EphB6 transduces signals and plays roles in T cell functions (30–34), but its biological functions in cellular behavior are still largely unknown. In the present study, we examined functions of EphB6 in cell adhesion and migration. We demonstrated that, upon stimulation with ephrin-B2, the kinase-defective EphB6 receptor exerts biphasic functions: EphB6 promotes cell adhesion and migration in response to low concentrations of ephrin-B2, whereas EphB6 induces repulsion and suppresses migration when stimulated with high concentrations of ephrin-B2. The cytoplasmic domain is essential for repulsion and inhibition of migration, because a truncated EphB6 that lacks the cytoplasmic domain promotes cell adhesion and migration in monophasic and dose-dependent manners. In addition, when exposed to high concentrations of ephrin-B2, EphB6 is tyrosine phosphorylated by an Src family kinase, which constitutively associates with the receptor. Our results indicate that the kinase-defective EphB6 receptor can regulate cell adhesion and migration by exerting both positive and negative effects and suggest that tyrosine phosphorylation of the receptor may act as a switch on the functional transition from adhesion/attraction to de-adhesion/repulsion.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The coding region of human EphB6 cDNA (nucleotide numbers 754–3819, GenBank™ accession number D83492 (29)) was subcloned into the pcDNA3.1 expression vector (Invitrogen) (pcDNA-EphB6). To express a truncated form of EphB6 (EphB6ΔC), the sequence that corresponds to most of the intracellular domain (amino acids 625–1021 (29)) was deleted and replaced with a termination codon and was subcloned into pcDNA3.1 (pcDNA-EphB6ΔC).

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). To establish cell lines that stably express the full-length or a truncated EphB6 receptor, cells were transfected with EphB6 expression constructs (pcDNA-EphB6 or pcDNA-EphB6ΔC) using FuGENE transfection reagent (Roche Applied Science) and cultured in the presence of 1 mg/ml G418 (Sigma). G418-resistant clones were isolated by limited dilution and expanded. Multiple clones that show high expression of the receptors were established for each construct and used in the experiments described here. Establishment and maintenance of Chinese hamster ovary (CHO) cell lines that express EphB6 (CHO-HEK cells) have been previously described (35).

**Affinity Probe in Situ**—Affinity probe in situ was performed as previously described (36). Briefly, unfixed cells were incubated with ephrin-B2-AP or AP for 30 min at room temperature. After washing, cells were briefly fixed with 8% formaldehyde (3 min), washed, and heated for 60 min at 65 °C to inactivate endogenous APs. Ligand binding was detected by incubation with 5-bromo-4-chloro-3-indolyl phosphate/5-bromo-4-chloro-3-indolyl phosphate.

**Cell Adhesion Assays**—Twenty-four-well plates were coated with serial dilutions of ephrin-B2-Fc or Fc in phosphate-buffered saline (PBS) (containing 5 mg/ml bovine serum albumin (BSA)) at 4 °C overnight, incubated with 2% BSA in PBS for 60 min, and then washed with PBS, boiled for 3 min, passed through a 0.2 μm polyvinylidene fluoride membrane, and blocked with 5% skim milk in PBS for 1 h. Filters were incubated with ephrin-B2-Fc (R&D Systems) or anti-pan-Src antibody (Santa Cruz Biotechnology) at 4 °C overnight. After washing, membranes were incubated with 1:1000 antibody for 30 min at 4 °C, followed by 1:1000 horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. The filters were blocked with 5% skim milk in PBS containing 0.05% Tween 20, and treated with anti-EphB6 antibody (Santa Cruz Biotechnology, 1 μg/ml), or anti-pan-Src antibody (Santa Cruz Biotechnology, 0.2 μg/ml). For treatment with anti-Fyn antibody and the anti- phosphotyrosine antibody 4G10, the filters were blocked with 5% BSA, 0.05% Tween 20 in Tris-buffered saline. Detection was performed using the ECL Plus system (Amersham Biosciences). For inhibition of Src family kinases, cells were serum-starved for 20 h and pretreated with PP2 or PP3 (Calbiochem) or Me2SO for 1 h, and then incubated in the culture medium containing ephrin-B2-Fc (12.5 μg/ml) for 30 min.

**RESULTS**

**Stable Expression of EphB6 in HEK293T Cells**—To investigate the function of EphB6 in cellular behavior, we established HEK293T cells that stably express the full-length human EphB6 cDNA (HEK-EphB6 cells). High expression of EphB6 was detected by immunoblotting using antibody against the extracellular domain of EphB6 (Fig. 1A). HEK293T cells transfected with the pcDNA3.1 vector did not express detectable levels of EphB6 (Fig. 1A). In the studies described below, several independent clones that express a high level of EphB6 protein were used, and similar results were obtained.

We first tested whether the EphB6 receptor expressed on HEK293T cells could interact with a cognate ligand. Although the Eph-ephrin interaction generally shows a high degree of promiscuity, ephrin-B2 has been shown to be the major ligand that binds to EphB6 with a significant affinity (37). We therefore performed a ligand-binding assay using the extracellular domain of ephrin-B2 fused to an alkaline phosphatase (AP) tag (ephrin-B2-AP) as a probe (affinity probe in situ) (36). Strong binding was observed on the surface of HEK-EphB6 cells (Fig. 1B), whereas neither parental HEK293T cells (data not shown)
nor vector-transfected HEK293T cells (Fig. 1B) showed detectable binding activity. Control AP did not bind to either HEK-EphB6 cells or vector-transfected HEK293T cells (Fig. 1B). These results confirmed that in HEK-EphB6 cells the EphB6 receptor is expressed on cell surface and can interact with ephrin-B2.

Immobilized Ephrin-B2 Exerts Dual Effects on HEK-EphB6 Cell Adhesion and Morphology—Previous studies have shown that interactions between kinase-active Eph receptors and ephrins can regulate cell adhesion (25, 27, 38–41). Because EphB6 is catalytically inactive, we examined whether this receptor also has the ability to modulate cell adhesion.

HEK-EphB6 cells were plated onto a substratum coated with different concentrations of ephrin-B2-Fc, and cell adhesion to substratum was measured 30 min later. As shown in Fig. 2A, HEK-EphB6 cells showed enhanced adhesion to the substratum coated with low concentrations of ephrin-B2-Fc. A dose-dependent promotion of cell adhesion was observed between 0.04 and 1.0 μg/ml ephrin-B2-Fc. When the ligand concentration was further increased (5.0–12.5 μg/ml), however, adhesion of HEK-EphB6 cells decreased to background levels. Immobilized Fc showed no effects on adhesion of HEK-EphB6 cells. Adhesion of vector-transfected control cells was not significantly affected by the ephrin-B2-Fc-containing substratum. In a separate set of experiments, ephrin-B2-Fc was pre-clustered with anti-human IgG-Fc and used for the assay. Pre-clustered ephrin-B2-Fc showed similar dual effects on adhesion of HEK-EphB6 cells (data not shown).

This cell adhesion assay was performed under stringent conditions, in which the wells were pre-coated (blocked) with a high concentration of BSA, and the cells were allowed to attach to substratum for 30 min, so there was essentially no basal level of cell adhesion. We therefore could not determine whether high concentrations of ephrin-B2 in fact actively induced repulsion of HEK-EphB6 cells, or had no effect on cell adhesion. To verify this, HEK-EphB6 cells were incubated on ephrin-B2-Fc-coated substratum for 48 h, and the morphology of cells was observed (Fig. 2B). When the substratum was coated with 0.2 μg/ml ephrin-B2, HEK-EphB6 cells showed a slight spreading morphology compared with the cells on the control substratum. In contrast, the substratum coated with higher concentrations of ephrin-B2 caused dramatic changes to cell morphology: the cells became rounded and formed aggregates that protruded from the substratum, indicating that high concentrations of ephrin-B2 induced cell repulsion and de-adhesion from the substratum. Control cells plated on ephrin-B2-Fc-coated substratum showed no morphological changes. Ephrin-B2-Fc did not affect the rate of cell proliferation or cell death (data not shown). These results indicate that the EphB6 receptor exerts dual functions that lead to either cell adhesion/spreading or repulsion/rounding, depending on the ligand concentrations.

Biphasic Effects of Ephrin-B2 on Migration of HEK-EphB6 Cells—Because many signaling molecules regulating cell adhesion are also involved in cell migration, we next examined if ephrin-B2 affects migration of HEK-EphB6 cells. To test this, we set up a transfilter assay system, in which a membrane with defined uniform pore size separates upper and lower chambers. The lower side of porous membrane was coated with different concentrations of ephrin-B2-Fc or Fc. HEK-EphB6 cells were then plated in the upper chamber and allowed to migrate to the lower side of the filter overnight.

When the porous membrane was coated with different concentrations of ephrin-B2-Fc, HEK-EphB6 showed a biphasic behavior in cell migration (Fig. 3, A and B). In response to low concentrations (0.04–1.0 μg/ml) of ephrin-B2-Fc, migration of HEK-EphB6 cells was stimulated in a dose-dependent manner. In contrast, when the cells were tested with high concentrations (5.0–12.5 μg/ml) of ephrin-B2-Fc, cell migration was significantly reduced. Remarkably, at 12.5 μg/ml ephrin-B2-Fc, fewer HEK-EphB6 cells migrated to the lower side of membrane compared with the control, indicating that ephrin-B2 inhibits HEK-EphB6 cell migration at this concentration. The Fc-coated membranes exerted no obvious effects on HEK-EphB6 cell migration, and migration of control HEK293T cells transfected with the pcDNA3.1 vector was not affected by ephrin-B2-Fc or Fc (Fig. 3B). Similar results were obtained in experiments in which the membranes were coated with pre-clustered ephrin-B2-Fc (data not shown).

To investigate whether the effects of ephrin-B2-Fc on cell migration are dependent on cell types, we tested CHO cell lines that stably express EphB6 (CHO-HEP cells) (35) in this cell migration assay. As observed in HEK-EphB6 cells, migration of CHO-HEP cells was stimulated by lower concentrations of ephrin-B2-Fc, and inhibited by higher concentrations of ephrin-B2-Fc (Fig. 3C). These results indicate that the biphasic effect in cell migration represents a global function of EphB6 and is not restricted to a small subset of cell types.

The biphasic response of HEK-EphB6 cells and CHO-HEP cells in the cell migration assay was consistent with the results of cell adhesion assay described above. Moreover, the ephrin-B2 concentrations at which the transition of effects (promotion to inhibition) was observed were comparable between the two assays (i.e., between 1 and 5 μg/ml).
A Truncated EphB6 Receptor Exerts Monophasic Stimulatory Effects on Cell Adhesion and Migration—The results of the cell adhesion and migration assays suggest that EphB6 mediates two types of signaling (adhesion/attraction and de-adhesion/repulsion) in response to different concentrations of ephrin-B2. Because the EphB6 receptor is devoid of tyrosine kinase activity, we were next interested in whether the cytoplasmic domain of EphB6 is essential for mediating signals for the biphasic responses in cell adhesion/migration. To test this, we constructed a truncated form of EphB6 that lacks most of the

Fig. 2. Dual functions of EphB6 in cell adhesion to ephrin-B2-containing substratum. HEK-EphB6 cells and vector-transfected HEK293T cells were plated onto substratum coated with different concentrations of ephrin-B2-Fc or Fc. A, cell adhesion after 30-min incubation. Adhesion efficiencies after subtracting the control adhesion (to BSA) are presented. Values represent means ± S.E. B, cell morphology of HEK-EphB6 cells and vector-transfected HEK293T cells after 48 h incubation on substratum coated with BSA or different concentrations of ephrin-B2-Fc. HEK-EphB6 cells showed well spread morphology on low concentrations of ephrin-B2-Fc, whereas high concentrations of ephrin-B2-Fc induced cell rounding and detachment from the substratum.

Fig. 3. Biphasic effects of EphB6 on transfilter cell migration. HEK-EphB6 cells (A and B) or CHO-HEP cells (C) were placed in the upper chamber of a Transwell apparatus, in which the lower side of filter was coated with different concentrations of ephrin-B2-Fc or Fc, and the cells were allowed to migrate to the lower side at 37 °C overnight. Vector-transfected cells were used as controls. The cells that had migrated to the lower side of filter were stained, photographed (A), and quantified (B and C). Control migration to BSA-coated filters is shown as 0. Migration of HEK-EphB6 cells and CHO-HEP cells was stimulated by low concentrations of ephrin-B2-Fc, but inhibited by higher concentrations of ephrin-B2-Fc.
Functional Transition of EphB6 in Cell Adhesion/Migration

Fig. 4. Expression and ligand binding activity of a truncated EphB6 receptor in HEK-EphB6ΔC cells. A, expression of a truncated EphB6 receptor (EphB6ΔC) in HEK-EphB6ΔC cells detected by immunoblot analysis. B, ligand binding activity of EphB6ΔC (dark staining) detected by affinity probe in situ using ephrin-B2-AP.

Cytoplasmic domain (EphB6ΔC). The cytoplasmic region of EphB6ΔC has only four amino acids and lacks all the known signaling motifs conserved among the Eph receptors, including the two juxtamembrane tyrosine residues that are the major autophosphorylation sites in kinase-active Eph receptors (42–44). HEK293T cells were stably transfected with an EphB6ΔC expression construct, and several independent clones were established (HEK-EphB6ΔC cells). As in HEK-EphB6 clones, expression and ligand binding activity of the truncated receptor were confirmed by immunoblotting and affinity probe in situ using ephrin-B2-AP.

HEK-EphB6ΔC cells were then tested for adhesion activity to ephrin-B2-coated substratum (Fig. 5A). At all concentrations of ephrin-B2 used in the assay, HEK-EphB6ΔC cells always showed a dose-dependent enhancement in cell adhesion. In striking contrast to HEK-EphB6 cells, cell adhesion of HEK-EphB6ΔC was not inhibited by high concentrations of ephrin-B2. After 48-h incubation, HEK-EphB6ΔC cells showed a spreading morphology not only at low concentrations, but also at high concentrations of ephrin-B2-Fc (Fig. 5B). Consistent with these results, in the transfilter assay, HEK-EphB6ΔC cells showed a monophasic and dose-dependent promotion of migration in response to ephrin-B2-Fc (Fig. 5, C and D), and inhibition of cell migration at high concentrations of ephrin-B2-Fc was not observed. The degree of cell migration induced by low concentrations of ephrin-B2-Fc was slightly lower than that observed for HEK-EphB6 cells, suggesting that the cytoplasmic domain may also be involved in signaling for stimulation of cell migration. These results indicate that the extracellular domain of EphB6 is capable of promoting cell adhesion and migration in response to low concentrations of ephrin-B2, but the cytoplasmic domain is essential for cell repulsion and inhibition of migration induced by high concentrations of ephrin-B2.

EphB6 Is Tyrosine-phosphorylated by High Concentrations of Ephrin-B2—Previous studies have shown that EphB6 can be phosphorylated on its tyrosine residues by ligand stimulation (30). Because EphB6 can exert both positive and negative effects on cell adhesion and migration, we next investigated how tyrosine phosphorylation of EphB6 related to the biphasic cellular behavior. To test this, HEK-EphB6 cells were plated onto substratum coated with different concentrations of ephrin-B2-Fc and incubated for 60 min at 37 °C. The EphB6 receptor was then immunoprecipitated with anti-EphB6 antibody and immunoblotted with anti-phosphotyrosine antibody (Fig. 6A). Tyrosine phosphorylation of EphB6 was not detectable by stimulation with low concentrations of ephrin-B2-Fc, which enhanced cell adhesion and migration of HEK-EphB6 cells. In contrast, incubation with high concentrations of ephrin-B2, which led to inhibition of cell adhesion/migration, resulted in significant tyrosine phosphorylation of the receptor. High concentrations of control Fc substratum had no effects. Tyrosine phosphorylation of EphB6 was confirmed in experiments in which immunoprecipitation with anti-phosphotyrosine antibody was followed by immunoblotting with anti-EphB6 antibody (Fig. 6B). Similar results were obtained when HEK-EphB6 cells were stimulated by soluble ephrin-B2-Fc (Fig. 6C).

An Src Family Kinase Constitutively Associates with and Tyrosine Phosphorylates EphB6—Because the kinase domain of EphB6 is catalytically inactive, the tyrosine phosphorylation of EphB6 observed above must be mediated by a separate tyrosine kinase. Interestingly, when HEK-EphB6 cells were treated with high concentrations of ephrin-B2-Fc, tyrosine phosphorylation of a 60-kDa protein that was co-immunoprecipitated with EphB6 was also induced (Fig. 6A). Because members of the Src family tyrosine kinases have similar molecular weights (42, 44, 45), we tested whether this protein is an Src family member. We treated HEK-EphB6 cells with different concentrations of ephrin-B2-Fc, and performed immunoprecipitation with anti-EphB6 antibody followed by immunoblotting with anti-pan-Src antibody or antibodies against specific Src family members. As shown in Fig. 6A, the 60-kDa protein could be detected with anti-pan-Src antibody. In addition, anti-Fyn antibody specifically recognized the 60-kDa protein. Intriguingly, although its tyrosine phosphorylation was significantly induced by stimulation with high concentrations of ephrin-B2, amounts of Fyn co-immunoprecipitated with EphB6 did not change by ligand treatments, indicating that Fyn associates with EphB6 both in the absence and presence (low and high concentrations) of ligand.

To confirm that an Src family kinase is tyrosine phosphorylated in response to high concentrations of ephrin-B2, HEK-EphB6 cells were treated with ephrin-B2-Fc, and cell lysates were immunoprecipitated with anti-pan-Src antibody and immunoblotted with anti-phosphotyrosine antibody (Fig. 6B). As expected, robust tyrosine phosphorylation was detected by high concentrations of ephrin-B2. In contrast, when lysates of HEK-EphB6ΔC cells were tested, tyrosine phosphorylation was not detectable (Fig. 7B), indicating that the cytoplasmic domain of EphB6 is essential for the ephrin-B2-induced tyrosine phosphorylation of Src family kinases.

Finally, we examined whether EphB6 phosphorylation is mediated by an Src family kinase. HEK-EphB6 cells were pretreated with the Src inhibitor, PP2, and then tested for
tyrosine phosphorylation by stimulation with high concentration of ephrin-B2-Fc or Fc. A, quantification of HEK-EphB6ΔC cell adhesion after 30-min incubation. Ephrin-B2-Fc promoted adhesion of HEK-EphB6ΔC cells in a dose-dependent manner. B, morphology of HEK-EphB6ΔC cells after 48-h incubation on substratum coated with BSA or different concentrations of ephrin-B2-Fc. HEK-EphB6ΔC cells showed well spread morphology both on low and high concentrations of ephrin-B2-Fc. C and D, cell migration assay. HEK-EphB6ΔC cells were tested in the transfilter migration assay as in Fig. 3. Migration of HEK-EphB6ΔC cells was stimulated by ephrin-B2-Fc. Note that in both cell adhesion and migration assays, inhibitory effects by high concentrations of ephrin-B2-Fc were not observed.

DISCUSSION

Biphasic Functions of the Kinase-defective EphB6 Receptor and Functional Transition from Promotion to Inhibition—Although "kinase-active" Eph receptors have been shown to play important roles in cell adhesion and migration both in vitro (25, 27, 38–41, 46–50) and in vivo (51–54), little is known about the functions and signaling mechanisms of the kinase-defective EphB6 receptor in cellular behavior. In the present study, we have demonstrated that the EphB6-ephrin-B2 interaction directly regulates cell adhesion and migration and that, despite its catalytically defective kinase domain, EphB6 retains the ability to mediate signaling that controls cellular behavior.

Our results have also shown that EphB6 exerts biphasic functions in adhesion and migration in response to different concentration of ligand, with a functional transition from promotion to inhibition. Although Eph receptors were initially described to mediate repulsive signals in growing axons, it has become clear that they have functional versatility, including...
adhesive and attractive functions. For example, it has been previously reported that EphB ligands act as bifunctional guidance cues for migrating trunk neural crest cells in vivo; they repel early neural crest cells that migrate through the ventrolateral pathway, and later stimulate the migration of melanoblasts into the dorsolateral pathway (54). In addition, in the developing visual system, ephrin-Bs have been shown to act as both attractants and repellents to retinal axons (55–57). Whereas in vivo studies have suggested that a transition from attraction to repulsion mediated by the EphB-ephrin-B interaction is involved in the establishment of topographic retinal axon mapping (57), such functional transition has not been directly shown for EphB receptors. Our results therefore represent a first demonstration that an EphB receptor can exert a promotion-inhibition transition in regulation of cellular behavior. Interestingly, it has recently been demonstrated in an in vitro axon outgrowth assay that in response to ephrin-A5, retinal axons show a graded, concentration-dependent transition from growth promotion to inhibition (58). Together with the results in the present study, these findings suggest that bifasic action may be a common feature of Eph receptor functions, shared by both EphA and EphB receptors, and by both kinase-active and kinase-defective receptors. Although its in vivo functions in cell adhesion and migration have not been identified, EphB6 is expressed in various tissues both during development and in the adult (28, 29, 35). It is particularly intriguing that EphB6 is expressed in the ganglionic eminence, a source of tangentially migrating neurons and glial cells into the cerebral cortex, raising the possibility that EphB6 may act as a guidance receptor in neuronal migration.

Whereas the molecular mechanism that regulates the transition from promotion to inhibition has not been elucidated, multiple factors could be involved in determining the final output of cell behavior, including the status of receptor multimerization, profiles of phosphorylation in the cytoplasmic domain, and combinations of signaling molecules recruited to the receptor. Although not directly addressed in the present study, our results suggest that in the case of EphB6, tyrosine phosphorylation of the receptor acts as a switch of the transition in cell behavior mediated by the receptor-ligand interaction. This concept is consistent with the previous studies demonstrating that kinase-active Eph receptors can promote cell adhesion in kinase-independent manners. For example, although interaction between cells expressing EphA7 and ephrin-A5 resulted in cell repulsion, co-expression of a truncated form of EphA7 that lacks the tyrosine kinase domain suppresses tyrosine phosphorylation of the full-length receptor and shifts the cellular response from repulsion to adhesion (27). Another study has shown that EphA8 promotes integrin-mediated cell attachment in a tyrosine kinase activity-independent fashion (47). These findings suggest that adhesive/attractive functions of Eph receptors can be exerted by tyrosine kinase-independent signaling, whereas repulsive functions require tyrosine kinase activity and receptor phosphorylation. It should be emphasized, however, that adhesion/attraction does not always equal kinase-independent signaling, because EphB1-mediated cell attachment to fibronectin is kinase-dependent (46).

**Signaling through the Kinase-defective EphB6 Receptor—**

Regarding the signaling mechanism through the EphB6 receptor, several different, but not mutually exclusive, possibilities could be considered. First, the EphB6 receptor may form a hetero-dimer or a hetero-oligomer with catalytically active Eph receptors, by analogy with the catalytically inactive ErbB3 receptor of the epidermal growth factor receptor subfamily (59). It was recently demonstrated that EphB6 can associate with and be transphosphorylated by EphB1, if the both receptors are overexpressed in the same cells (30). Although it remains to be determined whether such hetero-receptor complexes are formed and function in vivo, these findings raise the interesting possibility that EphB6 may modulate the signaling and function of catalytically active Eph receptors. In our system, however, it does not seem likely that this mechanism plays the
main role in the regulation of cell adhesion and migration, because ephrin-B2-AP did not show detectable binding to the parental HEK293T cells used in our experiments (Fig. 1B).

Second, the cytoplasmic domain of EphB6 may recruit a specific set of signaling molecules and mediate intracellular signaling in an intrinsic kinase activity-independent manner. Although we showed that a truncated EphB6 receptor that lacks the cytoplasmic domain can promote cell adhesion and migration, our results do not rule out the possibility that the cytoplasmic domain of EphB6 can transduce signals for stimulation of cell adhesion and migration. Interestingly, the degree of cell migration induced by low concentrations of ephrin-B2 is slightly higher in HEK-EphB6 cells than in HEK-EphB6ΔC cells (Figs. 3A and B) and 5(C and D)). Because the full-length and truncated EphB6 receptors are expressed at comparable levels in these cell lines, this may suggest that the cytoplasmic domain of the receptor transduces signals in stimulation of cell migration. Remarkably, the cytoplasmic domain of EphB6 retains all the known signaling motifs conserved among the Eph receptors, including the two tyrosine residues in the juxtamembrane region that are the major phosphorylation sites in kinase-active Eph receptors, the sterile α motif (SAM) domain, and the carboxyl-terminal PDZ domain-binding motif. Indeed, it has been shown that the PDZ domain of the Ras-binding protein AF6 can interact with the carboxyl-terminal of EphB6 (60, 61). In addition, a protooncogene product c-Cbl can constitutively associate with the EphB6 receptor (30, 32), and cross-linking of EphB6 in Jurkat cells results in Cbl dephosphorylation and dissociation from Src homology 2 domain-containing tyrosine phosphatase-1 (32).

Our results that an Src family kinase associates with and tyrosine phosphorylates EphB6 have added further evidence that the cytoplasmic domain of EphB6 can mediate signaling. Although Src family members have been reported to associate with kinase-active Eph receptors (42, 44, 45, 50, 62–64), their association is preceded by receptor autophosphorylation and is largely dependent on intrinsic kinase activity of the receptor. When the receptor kinase activity is impaired, the interactions between Eph receptors and Src family kinases are significantly reduced (62, 63). Our results are therefore in striking contrast to the previous results on kinase-active Eph receptors, because the association of the EphB6 receptor with an Src family kinase is independent of phosphorylation or kinase activity of the receptor, and ligand stimulation induces phosphorylation of an Src family kinase, which in turn phosphorylates EphB6. Further studies will be needed to determine the mode of their molecular interaction and downstream signaling mechanisms of EphB6.

Third, the extracellular domain of EphB6 may interact with other transmembrane molecules, which mediate intracellular signaling that regulates cell adhesion and migration. Our observations that the EphB6ΔC receptor can still promote cell migration could be explained by this mechanism. Although molecules that interact with the EphB6 extracellular domain have not been identified, it is of particular interest that the extracellular domain of Eph receptors has been shown to interact with the N-methyl-n-aspartate receptor (65) and Ryk (16, 66). More investigations, including identification of molecules that directly associate with EphB6 and downstream signaling pathways, will be required to determine molecular mechanisms by which this receptor exerts multiple cellular functions.

Catalytically Active and Inactive Receptor Tyrosine Kinases—To date, a relatively small number of receptor tyrosine kinases have been found to have a catalytically inactive kinase domain. However, inactive receptor tyrosine kinases have been present throughout most of metazoan existence. This raises the intriguing question of why the kinase-defective receptors, which are likely to have been generated by inactivating mutations following gene duplication (67), have been positively selected in metazoan evolution. A plausible explanation would be that by forming a complex with kinase-dead receptors, receptor tyrosine kinases could modify their ligand affinity/specificity and signaling pathway, and thus obtain more functional diversity. Another possibility would be that, by separating the kinase activity from the receptor, tyrosine phosphorylation of receptors could be more precisely controlled. Usually, receptor phosphorylation is triggered by receptor dimerization/oligomerization, and its regulation is largely dependent on ligand-receptor interaction (e.g. ligand concentration, receptor distribution on the cell surface, and ligand-receptor affinity). If receptor phosphorylation is mediated by an independent kinase (e.g. Src family kinases), it would become possible to control the phosphorylation after the receptor-ligand interaction occurs. These functional flexibilities are likely to have been advantageous for metazoan organisms to properly respond to environmental changes.

At present, kinase-defective Eph receptors have been identified only in mammals (human and mouse). As more metazoan genomes are sequenced, it will be intriguing to see if orthologs of EphB6 or other kinase-defective Eph receptors exist in lower vertebrates and/or invertebrates. It will also be of great interest to determine what additional signaling pathways and functions the EphB6 receptor has obtained by inactivating its intrinsic kinase. Clearly, the Eph family will act as a good model to study the function of catalytically-defective receptor tyrosine kinases in relation to their catalytically active counterparts.

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Functional Transition of EphB6 in Cell Adhesion/Migration

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