Mechanisms of Signal Transduction:

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Ephrin-B1 Reverse Signaling Activates JNK through a Novel Mechanism That Is Independent of Tyrosine Phosphorylation*

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Eph receptors and their cognate ligand ephrins play important roles in various biological processes such as cell migration, axon guidance, and synaptic plasticity. One characteristic feature of the Eph-ephrin signal transduction is that, upon interaction with the receptor, the transmembrane B-class ephrins become tyrosine-phosphorylated and transduce intracellular signals that lead to reorganization of the cytoskeleton. Although *in vitro* and genetic studies have demonstrated unequivocally the significance of this reverse signaling, the underlying mechanism remains unclear. We report here that transfection of ephrin-B1 into 293 cells resulted in robust increase in JNK activity, whereas expression of truncated ephrin-B1 lacking the cytoplasmic domain had a negligible effect, indicating that the induction of JNK activity was attributed mainly to the reverse signaling. The ephrin-B1-mediated JNK activation was reduced significantly by dominant-negative TAK1, MKK4, or MKK7. Ephrin-B1 over-expressing 293 cells became rounded in morphology. Surprisingly, ephrin-B1 that lacked all six intracellular tyrosine residues still triggered JNK activation and rounding morphology of the transfected cells. Consistent with these observations, activation of JNK and the resulting morphological changes mediated by ephrin-B1 could be abolished by the JNK inhibitor SP600125 but not the Src inhibitor PP2. Taken together, our findings have identified a novel reverse signaling pathway transduced by ephrin-B1, which is independent of tyrosine phosphorylation but involves the activation of JNK through TAK1 and MKK4/MKK7 and leads to changes in cell morphology.

Eph receptors, the largest family of receptor tyrosine kinases comprising 14 members in mammals, play critical roles in diverse biological processes during development as well as in the mature animal (1, 2). They are activated by membrane-bound ligands called ephrins, which are classified into two subclasses based on their modes of membrane anchorage. The ephrin-A ligands (ephrin-A1–A5) are glycosylphosphatidylinositol-linked and prefer to bind to EphA receptors (EphA1–A8). The ephrin-B ligands (ephrin-B1–B3), which possess a transmembrane moiety and a short cytoplasmic domain, bind to EphB receptors (EphB1–B6). The interactions between ephrins and Eph receptors are generally promiscuous within each subclass. There is little cross-reactivity between members from different subclasses, with the notable exception of EphA4 (3).

Based on the prominent expression of ephrins and Eph receptors in the brain, early studies focused on their significance in the nervous system. Elegant *in vitro* and genetic studies have demonstrated that ephrins act as a repulsive axon guidance cue, which are essential in the formation of topographic mapping in various regions of the central nervous system. Moreover, interactions between Eph receptors and ephrins also govern neural crest cell migration and segmental patterning in the brain. Besides the fact that Eph receptors and ephrins perform important functions during development, more recent studies have unraveled their significance in the mature nervous system, especially in the aspect of synapse formation and functioning. Eph receptors and ephrins are localized at the neuronal synapses as well as the neuromuscular junction (4, 5), and ephrin-B ligands are involved in the formation of neuronal synapses and long-term potentiation in the hippocampus (6–8). Recent studies have also demonstrated the regulation of cell adhesion by Eph receptors, which is central to their participation in vascular development and tumorigenesis outside of the nervous system (9, 10).

One unusual feature of Eph-ephrin interaction is that signals can be transduced bidirectionally in both the ligand- and receptor-expressing cells (11). The glycosylphosphatidylinositol-linked A-class ephrins lack a cytoplasmic domain and intrinsic kinase activity, requiring the recruitment of other signaling molecules to trigger the reverse signaling. One possible candidate is the non-receptor tyrosine kinase Fyn, which was found necessary for the reverse signaling mediated by ephrin-A5 (12). The reverse signaling of ephrin-A5 leads to modulation of integrin functions and in turn to the regulation of cell adhesion (13, 14). Interactions between the glycosylphosphatidylinositol-linked ephrins and VAB-1, the *Caenorhabditis elegans* homolog of EphA receptors, regulate the organization of epidermal cells independently of the kinase activity of VAB-1, thereby providing genetic evidence for the significance of ephrin-A reverse signaling.

Upon interaction with EphB receptors, the transmembrane B-class ephrins also transduce intracellular signal. Ample evidence from different *in vivo* studies supports the biological significance of ephrin-B reverse signaling in axon guidance, hind brain segmentation, and neuronal plasticity. Axons of the anterior commissure, which express the ephrin-B ligands rather than EphB receptors, migrate aberrantly in EphB2 null mice but not in transgenic mice, in which the kinase domain of EphB2 is deleted (15). Similarly, axon pathfinding of retinal

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ganglion cells within the retina depends on EphB2 in a kinase-independent manner (16). During segmentation of the hind brain, EphB-ephrin-B interactions at the boundary of adjacent rhombomeres are crucial to prevent the intermingling of distinct cell populations, and bidirectional signaling of both EphB receptors and ephrin-B ligands is required (17, 18). Mice with targeted deletion of EphB2 display defects in neuronal plasticity, which can be rescued by EphB2 truncated at the carboxyl terminus (7).

The signaling pathways following ephrin-B activation have begun to be elucidated; they may be classified into the phosphotyrosine-dependent pathway and PDZ[1]-dependent pathway (2). In the former, ephrin-B is tyrosine-phosphorylated upon interaction with EphB receptors by the Src family kinases (SFK) (19–21). Sprouting angiogenesis of endothelial cells induced by EphB4-Fc is abolished by the Src inhibitor PP2, suggesting that tyrosine phosphorylation by SFK is essential for ephrin-B reverse signaling (21). On the other hand, a protein tyrosine phosphatase (PTP-BL) is recruited to the activated ephrin-B1 in a delayed kinetics via interaction between the PDZ domain of PTP-BL and the carboxyl tail of ephrin-B1, after which ephrin-B1 is dephosphorylated and the phosphotyrosine-dependent reverse signaling is switched off (21). The tyrosine phosphorylation of ephrin-B1 results in the binding of the adaptor protein Grb4 via interaction with its SH2 domain. The latter in turn recruits various signaling molecules such as focal adhesion kinase (FAK) and Cbl-associated protein (CAP) to the ephrin-B signaling complexes via its multiple SH3 domains, eventually leading to disassembly of F-actin and rounding cell morphology (22).

In addition to the phosphorylation-dependent pathway, another pathway in the reverse signaling that requires the carboxyl-terminal PDZ-binding motif of ephrin-B1 has been elucidated. Besides PTP-BL, a number of PDZ domain-containing proteins such as GRIP-1, GRIP-2, and syntenin have been identified as interacting with the carboxyl tail of ephrin-B1 (4, 23, 24); however, their significance with respect to the propagation of reverse signaling is not clear. Recently a novel protein, called PDZ-RGS3, was found to interact with ephrin-B1 via its PDZ domain. Interestingly, PDZ-RGS3 contains a regulator of heterotrimeric G protein signaling (RGS) domain, which accelerates the G protein catalytic cycle and thus inhibits the signaling transduced by G protein-coupled receptor. It was found that interaction between ephrin-B1 and PDZ-RGS3 was essential in the ephrin-B1-mediated cell dissociation in *Xenopus* embryo (25). More importantly, the activation of ephrin-B1 reverse signaling inhibits chemoattraction of cerebellar granule cells induced by the chemokine SDF-1, and the inhibition depends on cross-talk between the ephrin-B1 reverse signaling and the G protein-coupled receptor signaling via PDZ-RGS3. Although it is not clear how activation of ephrin-B1 regulated the activity of PDZ-RGS3, it is apparent that the association between ephrin-B1 and PDZ-RGS3 is constitutive and does not depend on activation by EphB2-Fc (25).

Despite tremendous advance in recent years, details of the reverse signaling of the B ephrins remain largely unclear. One candidate protein that has been identified to interact with the SH3 domain of Grb4 is axin (22). Axin is a member of the RGS protein family and acts as a negative regulator of Wnt signaling (26). In addition, axin was recently demonstrated to differentially activate the MAP kinase c-Jun NH_2-terminal kinase (JNK) (27). In view of this finding and the reported regulation of MAP kinase activity by the forward signaling of Eph receptors (28–31), the role of JNK in mediating ephrin-B1 reverse signaling has been investigated. Here we report a novel reverse signaling pathway mediated by ephrin-B1 in 293 cells, which involves activation of JNK via TAK1 and MKK4/MKK7 and leads to a morphological change in the transfected cells. Most surprisingly, this JNK-dependent pathway does not require tyrosine phosphorylation of ephrin-B1.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The full-length cDNA encoding mouse ephrin-B1 was derived from two expressed sequence tag clones (GenBank™ accession number AI131834 and AA003279). The full-length cDNA for mouse Grb4 was from a mouse expressed sequence tag clone (image.3648783) and was Myc epitope-tagged. Both ephrin-B1 and Grb4 were cloned into the mammalian expression vector pCMV5. Point mutations of ephrin-B1 and Grb4 were generated by *in vitro* site-directed mutagenesis using the QuikChange™ site-directed mutagenesis kit (Stratagene). Grb4 dominant-negative constructs were designed as described (32). Deletion mutants were achieved by PCR-based mutagenesis. In general, to avoid unwanted mutations resulting from PCR reactions, we fused only small fragments encompassing the mutated area to wild-type DNA fragments. All PCR amplified DNA products were verified by sequencing. Expression vectors for HA-MEKK1-C-K/M, HA-ASK-K709R, HA-TAK1-K63W, MKK4-DN, MKK7-DN, and FLAG-tagged JNK were as described as previously (27). Myc-PAK H82,86L was a gift from Dr. J. Han (the Scripps Research Institute).

Transient Transfection and Immunokinase Assays—Human embryonic kidney 293T cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU of penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Transfections were performed in 60-mm dishes using Dosper liposomal transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. The total amount of transfected DNA was adjusted to 4 μg with the empty vector pCMV5 where necessary. Cells were harvested at 40 h after transfection and lysed as described previously (27). FLAG-tagged JNK1 was immunoprecipitated using mouse monoclonal anti-FLAG M2 beads (Sigma), and the JNK activity was determined as described previously using 1 μg of GST-c-Jun- (1–79) (Stratagene) as substrate followed by Western blotting using Phospho-c-Jun antibody (Cell Signaling Inc.) to examine the phosphorylation of c-Jun (27). Fold activation of the kinase was determined by an imaging analyzer (Amersham Biosciences model 425E) and normalized to their expression levels. Data are expressed as fold kinase activation compared with that in vector-transfected cells, with the values representing the mean ± S.D. from at least three separate experiments. Transfected 293T cells were stimulated for 2 h by EphB2-Fc (7 μg/ml, R&D Systems, Inc.) pre-clustered by anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.) as described previously (22).

Immunoprecipitation and Western Blot Analysis—Transiently transfected 293T cells in 60-mm dishes were lysed in lysis buffer, sonicated three times for 5 s each, and centrifuged at 13,000 rpm for 30 min at 4 °C. Ephrin-B1 proteins were immunoprecipitated from the cell lysate with anti-ephrin-B1 antibody (sc-910, Santa Cruz Biotechnology., Inc.) and protein A/G Plus-agarose beads and eluted with SDS sample buffer. The boiled samples were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). After blocking with 5% skim milk in phosphate-buffered saline (PBS) with 0.1% Tween 20 for 1 h, the membranes were probed with anti-phosphotyrosine antibody (clone 4G10, Upstate Biotech.). The membranes were then stripped and reprobed with anti-ephrin-B1 antibody. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Biosciences) using horseradish peroxidase-conjugated antibodies.

Staining of 293 Cells by Ephrin- and Eph-Fc Chimeric Proteins—293 cells were stained by ephrin-B1-Fc, EphB2-Fc (R&D Systems, Inc.) or Fc fragment of Human IgG (Jackson ImmunoResearch Laboratories, Inc.) as described previously (33). Briefly, the cells were incubated with 1 μg/ml chimeric proteins in PBS plus 10% calf serum for 30 min. After washing three times by PBS plus 10% calf serum, the cells were fixed by methanol for 2 min followed by rehydration with PBS. After heating at 70 °C for 30 min, the cells were incubated with anti-human antibody conjugated with alkaline phosphatase (Promega) for 1 h. The cells were then washed three times by PBS and incubated with the phosphatase

[1] The abbreviations used are: PDZ, PSD95/Dlg/ZO-1; SFK, Src family kinases; MAP, mitogen-activated protein; MAP3K, MAP kinase kinase kinase; MKK, MAP kinase kinase; PAK, p21-activated kinase; JNK, c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; GFP, green fluorescence protein; PTP, protein tyrosine phosphatase; SH2 and SH3, Src homology 2 and 3; RGS, regulator of heterotrimeric G protein signaling; HA, hemagglutinin; GST, glutathione *S*-transferase.

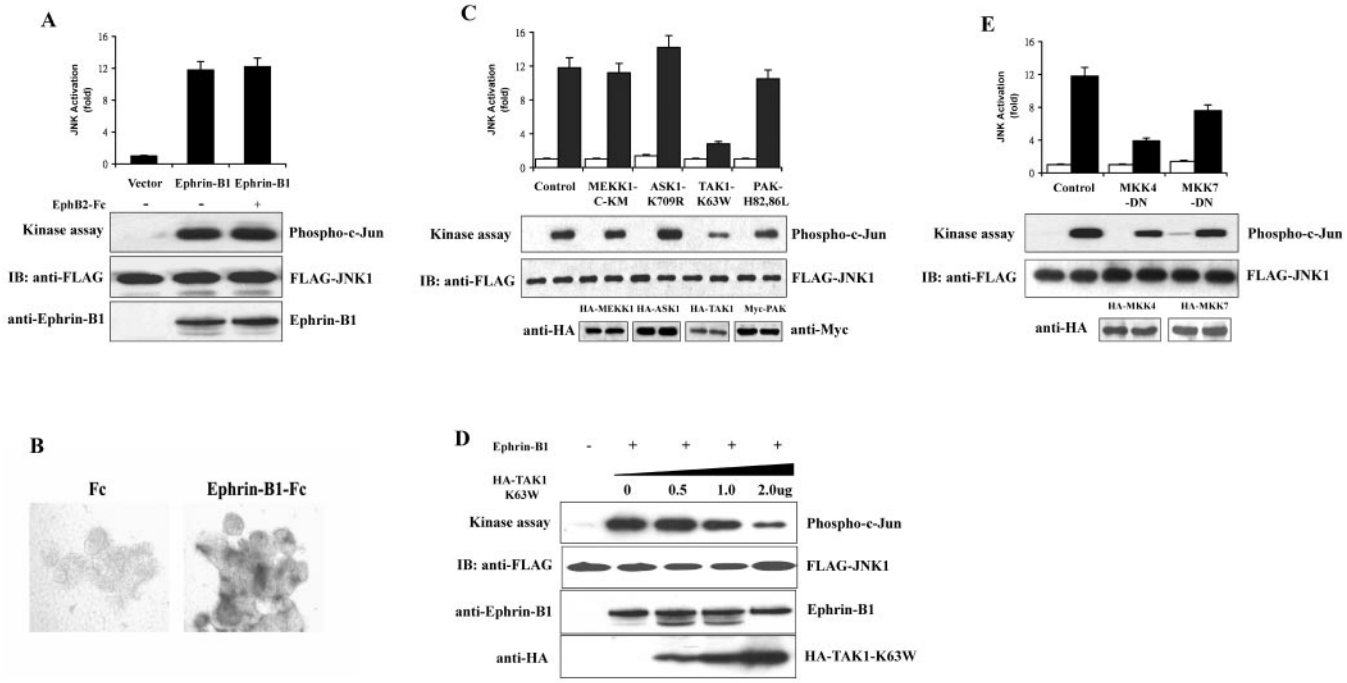

FIG. 1. **Activation of JNK by exogenous expression of ephrin-B1 in 293T cells.** *A*, 293T cells were transfected with FLAG-JNK1 together with empty vector or ephrin-B1. The cells were then incubated with (+) or without (−) EphB2-Fc. To measure JNK activity, GST-c-Jun was added in the *in vitro* kinase assay and blotted with anti-phosphoserine c-Jun antibody. The intensity of the bands was quantified (mean ± S.D., $n = 3$). Expression of the constructs was confirmed by Western blotting with anti-FLAG and anti-ephrin-B1 antibodies. *IB*, immunoblot. *B*, endogenous EphB receptors were expressed in 293 cells, as revealed by staining with ephrin-B1-Fc followed by anti-Fc-alkaline phosphatase. Staining by Fc was employed as negative control. *C*, TAK1 was essential for ephrin-B1-mediated JNK activation. 293T cells were transfected with FLAG-JNK1 and dominant-negative constructs of MEKK1, ASK1, TAK1, or PAK1 with (*solid bars*) or without (*open bars*) ephrin-B1. JNK activity was measured as the serine phosphorylation of GST-c-Jun. Expression of the various dominant-negative constructs was confirmed by Western blotting with anti-HA or anti-Myc antibodies. Equal loading was indicated by blotting with anti-FLAG antibody. *D*, TAK1 dominant-negative inhibited the ephrin-B1-mediated JNK activity in a dose-dependent manner. Increasing amounts of TAK1 dominant-negative (0.5–2 μg DNA) increasingly inhibited the JNK activation induced by over-expressed ephrin-B1. *E*, ephrin-B1-mediated JNK activation depended on MKK4 and MKK7. 293T cells were transfected with FLAG-JNK1 and dominant-negative constructs of MKK4 or MKK7 or empty vector for control, together with (*solid bars*) or without (*open bars*) ephrin-B1. JNK activity was measured as the serine phosphorylation of GST-c-Jun. Expression of the dominant-negative constructs was confirmed by Western blotting with anti-HA antibody, whereas Western blotting with anti-FLAG antibody indicated similar loading.

inhibitor levamisole (1 mM, Zymed Laboratories Inc.) in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 5 min before the addition of the AP substrates nitro blue tetrazolium (4.4 μl/ml) and 5-bromo-4-chloro-3-indolyl phosphate (3.3 μl/ml, Invitrogen).

Morphological Assay of Transfected 293 Cells—293 cells in 35-mm dishes were co-transfected with 1 μg of GFP and 3 μg of pCMV5, FLAG-JNK1, or various ephrin-B1 constructs by calcium phosphate precipitation. The cells were washed three times in PBS on the next day, and the cell morphology was examined 5 h later under fluorescence microscope. To examine the effects of inhibitors, the cells were washed three times in PBS on the next day after transfection and then incubated with the JNK inhibitor SP600125 (30 μM, Calbiochem), the Src inhibitor PP2 (1, 10, or 25 μM, Calbiochem), or Me_2SO for 2–5 h before examination of the cell morphology. To quantify the results, the number of flattened and rounded cells that were GFP-positive was counted in eight different fields randomly chosen from each dish. The results from three independent experiments were analyzed (mean ± S.E., $n = 3$).

RESULTS

Activation of JNK by Ephrin-B1 Requires TAK1, MKK4, and MKK7—To examine whether ephrin-B1 reverse signaling could activate JNK, 293T cells were co-transfected with ephrin-B1 and FLAG-JNK1. The kinase activity of JNK was assessed by phosphorylation of GST-c-Jun following immunoprecipitation. Although low levels of FLAG-JNK activity were observed in 293T cells co-transfected with vector alone, exogenous expression of ephrin-B1 resulted in a robust increase in JNK activity (Fig. 1*A*). JNK activity was not further elevated in the presence of exogenous EphB2-Fc chimeric protein. One possible explanation for the lack of a requirement for exogenous EphB2-Fc is that 293T cells express endogenous EphB receptors, which interact with the exogenous ephrin-B1, leading to the activation of JNK. Indeed, 293 cells could be positively stained by ephrin-B1-Fc, indicating endogenous expression of EphB receptors (Fig. 1*B*). Alternatively, over-expressed ephrin-B1 may spontaneously form dimers and transduce reverse signaling.

Several MAP kinase kinase kinases (MAP3K) have been shown to activate the JNK pathway (34–36). To investigate whether and if so which of the upstream MAP3Ks was responsible for ephrin-B1-mediated JNK activation, dominant-negative constructs of different MAP3K, as well as the p21-activated kinase (PAK), were co-transfected with ephrin-B1 in 293T cells. Over-expression of the dominant-negative constructs alone did not affect the JNK activity (Fig. 1*C, open bars*), and over-expression of dominant-negative MEKK1, ASK1, or PAK also did not affect ephrin-B1-induced JNK activation (Fig. 1*C, solid bars*). However, co-expression of dominant-negative TAK1 significantly reduced ephrin-B1-induced JNK activity in a dose-dependent manner (Fig. 1, *C* and *D*).

JNK is phosphorylated and becomes activated by MKK4 and MKK7, two MAP kinase kinases downstream of MAP3K (34–36). To examine their possible role in mediating ephrin-B1-induced JNK activation, dominant-negative MKK4 and MKK7 were co-transfected separately with ephrin-B1 in 293T cells. Both dominant-negative MKK4 and MKK7 could partially inhibit the ephrin-B1-induced JNK activation (Fig. 1*E*). These

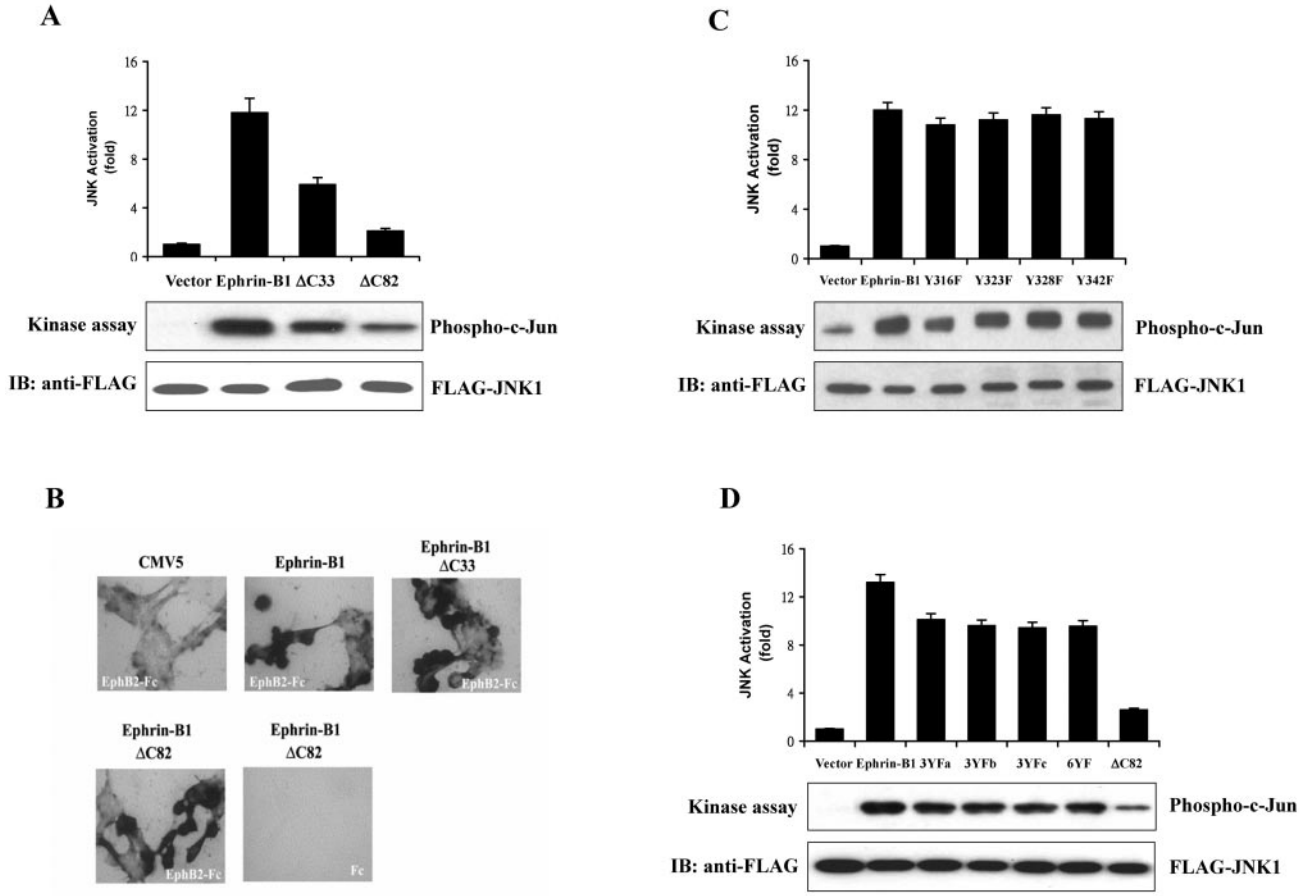

FIG. 2. **Activation of JNK by ephrin-B1 was dependent on reverse signaling but independent of intracellular tyrosine phosphorylation.** *A*, 293T cells were transfected with empty vector, ephrin-B1, or truncated ephrin-B1 that lacked the terminal 33 amino acids (Δ*C33*) or the entire cytoplasmic domain (Δ*C82*). Serine phosphorylation of c-Jun was used as a measure of JNK activity. *IB*, immunoblot. *B*, confirmation of the exogenous expression of ephrin-B1/ΔC33 and ΔC82 by immunostaining. 293 cells transfected with the different constructs or empty vector (*CMV5*) were stained by EphB2-Fc. Staining by Fc was employed as a negative control. *C*, JNK activation was triggered by exogenous expression of ephrin-B1 in which an individual tyrosine residue was substituted. Tyrosine residue 316, 323, 328, or 342 of ephrin-B1 was substituted by phenylalanine. *D*, activation of JNK by exogenous expression of ephrin-B1 that lacked multiple tyrosine residues. Three individual tyrosine residues (tyrosine residues 323, 342, and 343 for *3YFa*; tyrosine residues 328, 342, and 343 for *3YFb*; tyrosine residues 323, 328, and 342 for *3YFc*) or all six of the tyrosine residues (*ephrin-B1/6YF*) of ephrin-B1 were substituted by phenylalanine. In all cases, the different constructs were individually co-transfected into 293T cells with FLAG-JNK1. GST-c-Jun was added in the *in vitro* kinase assay and blotted with anti-phosphoserine antibody. The intensity of the phosphoserine bands was quantified (mean ± S.D., $n = 3$). Similar loading was indicated by Western blotting with anti-FLAG antibody.

data demonstrate that exogenous expression of ephrin-B1 in 293T cells leads to activation of JNK via TAK1 and MKK4/MKK7.

Activation of JNK by Ephrin-B1 Was Dependent on Reverse Signaling but Independent of Ephrin-B1 Tyrosine Phosphorylation—Because of the endogenous expression of EphB receptors in 293T cells, the increase in JNK activity upon over-expression of ephrin-B1 could be attributed to both forward and reverse signaling. To verify their relative significance, the JNK activity was compared between over-expression of wild-type ephrin-B1 and a truncated ephrin-B1 in which the transmembrane domain was retained but the entire cytoplasmic domain of 82 amino acids was deleted (ephrin-B1/ΔC82). The expression of ephrin-B1/ΔC82 could not be verified by Western blotting with anti-ephrin-B1 antibody, because the antibody recognized an epitope near the carboxyl terminus of ephrin-B1. However, staining with EphB2-Fc chimera showed that both ephrin-B1 and ephrin-B1/ΔC82 were expressed and targeted to the membrane (Fig. 2*B*). The reverse signaling-defective ephrin-B1 drastically lost the ability to activate JNK (Fig. 2*A*), indicating that JNK activation was attributable for the most part to the reverse signaling.

The carboxyl-terminal 33 amino acids are virtually identical among the three ephrin-B ligands, and these include the five conserved tyrosine residues and the PDZ-binding motif (19). To investigate the significance of the carboxyl-terminal 33 amino acids, a truncated ephrin-B1 that lacked the carboxyl terminus (ephrin-B1/ΔC33) was transfected into 293T cells. Over-expression of ephrin-B1/ΔC33 induced significant JNK activity compared with empty vector or ephrin-B1/ΔC82, although the activation was lower than that induced by wild-type ephrin-B1 (Fig. 2*A*). Staining with EphB2-Fc chimera showed that ephrin-B1/ΔC33 was expressed and targeted to the membrane, similar to the full-length ephrin-B1 (Fig. 2*B*). This surprising finding suggests that truncated ephrin-B1 that lacks the intracellular tyrosine residues and the PDZ-binding motif can still transduce reverse signaling to activate JNK.

Tyrosine residues 312, 317, and 331 of chicken ephrin-B1 were identified as the major phosphorylation sites (37). To further address the role of ephrin-B1 tyrosine phosphorylation in JNK activation, the six intracellular tyrosine residues of ephrin-B1 were singly or multiply substituted by phenylalanine (Tyr to Phe substitutions). Transfection of these mutated constructs into 293T cells induced robust JNK activation (Fig.

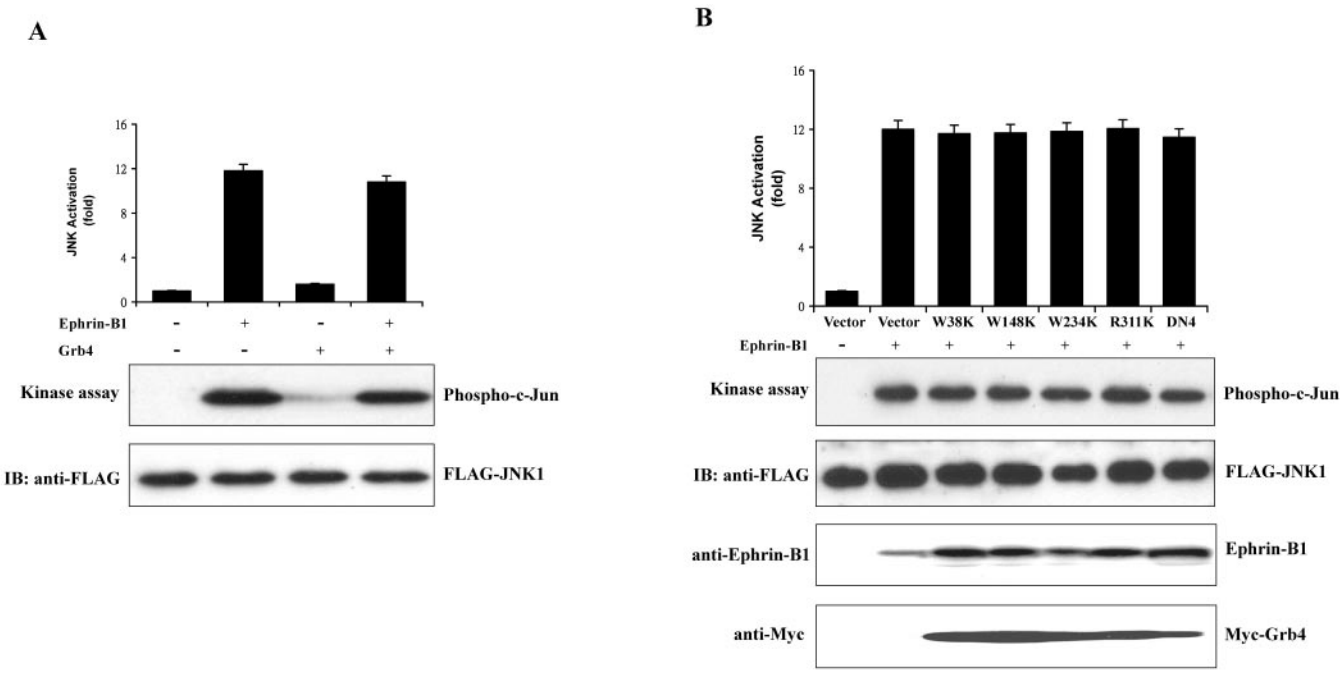

FIG. 3. **Activation of JNK by exogenous expression of ephrin-B1 was independent of Grb4.** *A*, 293T cells were transfected with empty vector, ephrin-B1, Grb4, or ephrin-B1 plus Grb4, together with FLAG-JNK1. Serine phosphorylation of c-Jun was used as a measure of JNK activity. *IB*, immunoblot. *B*, over-expression of various dominant-negative constructs of Grb4 did not inhibit the ephrin-B1-mediated JNK activation. Tryptophan residues 38, 148, 234, or arginine residue 311 of Grb4 were either individually or concomitantly (*DN4*) substituted by lysine. The different Grb4 mutants or wild-type Grb4 were co-transfected with ephrin-B1 and FLAG-JNK1, and the JNK activity was measured as the serine phosphorylation of c-Jun. Expression of ephrin-B1 and the different Grb4 constructs was verified by Western blotting with anti-ephrin-B1 and anti-Myc antibodies, respectively. The intensity of the phosphoserine bands was quantified (mean ± S.D., $n = 3$). Similar loading was indicated by Western blotting with anti-FLAG antibody.

2, *C* and *D*). In particular, ephrin-B1 in which all six tyrosine residues were substituted (ephrin-B1/6YF) could activate JNK to an extent similar to that of wild-type ephrin-B1.

Grb4 is an SH2 domain adaptor molecule that has been identified as interacting with the activated ephrin-B1 that harbors phosphotyrosine residues, leading to downstream cytoskeletal reorganization (22). The role of Grb4 in mediating ephrin-B1-induced JNK activation in 293T cells was therefore investigated. Over-expression of Grb4 alone did not increase the JNK activity, and the ephrin-B1-induced JNK activity was not affected by co-expression of Grb4 (Fig. 3*A*). Similarly, over-expression of different dominant-negative Grb4, which contained single or multiple substitutions of tryptophan or arginine by lysine, could not inhibit the ephrin-B1 induced JNK activity (Fig. 3*B*). These observations suggest that the recruitment of Grb4 is not involved in the ephrin-B1-induced JNK activation, which is consistent with the findings that over-expression of ephrin-B1/6YF can effectively activate JNK (Fig. 2*D*).

Ephrin-B1 Was Tryrosine-phosphorylated by Src Kinases upon Over-expression in 293T Cells—The results thus far indicated that exogenous expression of ephrin-B1 in 293T cells could turn on the reverse signaling and induce JNK activity independently of tyrosine phosphorylation. These observations apparently disagreed with the phosphorylation-dependent pathway in which ephrin-B1 was tyrosine-phosphorylated by SFK in order to switch on the reverse signaling (21, 22). The reason for the discrepancy is unclear. However, it is possible that the mechanism of ephrin-B1 reverse signaling is cell type-specific, such that the activation of ephrin-B1 in 293T cells does not involve tyrosine phosphorylation by SFK as described in other cell systems. Alternatively, tyrosine phosphorylation of ephrin-B1 by SFK upon activation occurs in 293T cells but is not involved in JNK activation. To distinguish between the two possibilities, tyrosine phosphorylation of ephrin-B1 was examined in transfected 293T cells. Wild-type ephrin-B1, but not mutants in which multiple tyrosine residues were substituted, became tyrosine-phosphorylated when exogenously expressed in 293T cells without the addition of EphB2-Fc (Fig. 4*A*). Moreover, tyrosine phosphorylation of ephrin-B1 was abolished when the transfected cells were treated with the Src kinase inhibitor PP1 (Fig. 4*B*). These results therefore indicate that ephrin-B1, when over-expressed in 293T cells, becomes tyrosine-phosphorylated by SFK but that this phosphorylation pathway is not essential for the observed JNK activation.

Reverse Signaling of Ephrin-B1 Led to Rounding Morphology of 293 Cells That Was Dependent on JNK Activity but Not Ephrin-B1 Tyrosine Phosphorylation—The biological significance of the JNK pathway in mediating ephrin-B1-induced response was then examined. When 293 cells were co-transfected with ephrin-B1 and GFP, the GFP-positive cells became rounded and shrunk (Fig. 5*A*), whereas the untransfected cells (GFP-negative) remained spread out and flattened (data not shown). In contrast, when 293 cells were co-transfected with GFP and empty vector or Flag-JNK, both the GFP-positive and GFP-negative cells were spread out and flattened. The induction of cell rounding by over-expressed ephrin-B1 was consistent with the previous study reporting the cellular changes of baby hamster kidney cells in response to EphB2-Fc (22). The fact that GFP-positive but not GFP-negative cells became rounded after co-transfection with ephrin-B1 indicated that the cell rounding resulted mainly from the reverse signaling of ephrin-B1 rather than the activation of endogenous EphB receptors in 293 cells. This was further supported by the observation that ephrin-B1/ΔC82 was much less active than wild-type ephrin-B1 in mediating the rounding morphology of the transfected cells (Fig. 5). On the other hand, 293 cells that over-expressed ephrin-B1/6YF or ephrin-B1/ΔC33 possessed a

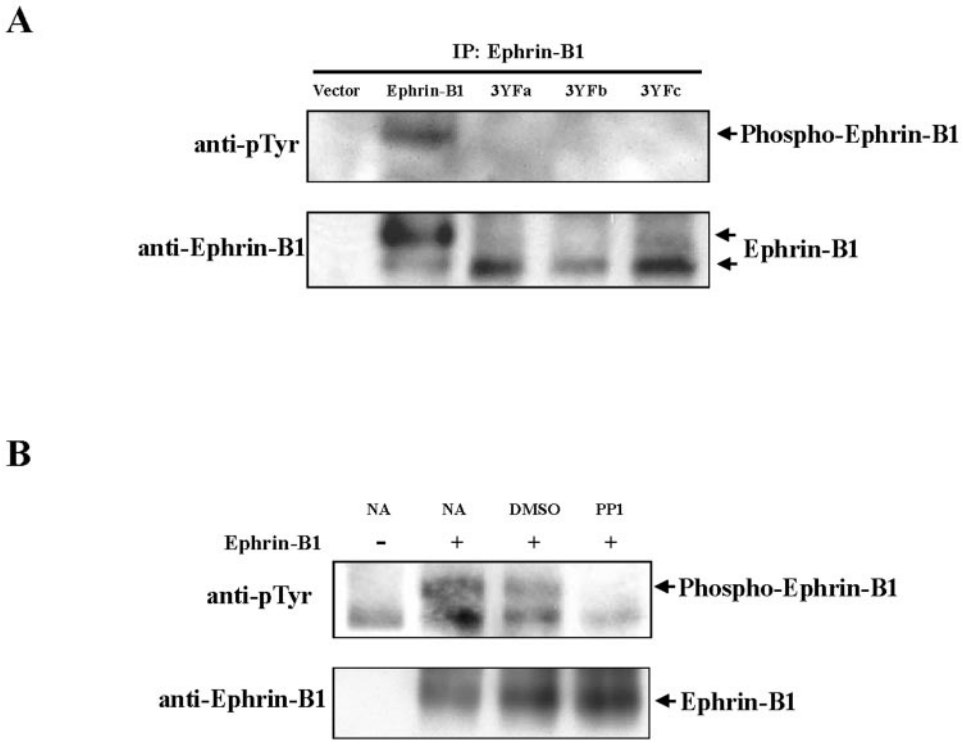

FIG. 4. **Ephrin-B1 was tyrosine-phosphorylated by Src kinases upon exogenous expression in 293T cells.** *A*, wild-type ephrin-B1 or ephrin-B1 mutants that contained multiple tyrosine substitutions were individually transfected into 293T cells. After immunoprecipitation (*IP*) by ephrin-B1 antibody, the product was immunoblotted by anti-phosphotyrosine (*anti-pTyr*) antibody. Phospho-ephrin-B1 is indicated by an *arrow* (*upper panel*). The membrane was stripped and reprobed with anti-ephrin-B1 antibody to verify expression of the different ephrin-B1 constructs (*arrows* in *lower panel*). The difference in migration between wild-type ephrin-B1 and the 3YF mutants in the anti-ephrin-B1 blot is most likely attributable to the amino acid substitutions and difference in tyrosine phosphorylation. *B*, tyrosine phosphorylation of ephrin-B1 was dependent on Src family kinases. 293T cells transfected with empty vector (−) or wild-type ephrin-B1 were untreated (*NA*) or treated with dimethyl sulfoxide (*DMSO*) or the Src kinase inhibitor PP1. Ephrin-B1 was immunoprecipitated, and the product was immunoblotted by anti-phosphotyrosine antibody. Phospho-ephrin-B1 is indicated by an *arrow* (*upper panel*). The membrane was stripped and reprobed with anti-ephrin-B1 antibody to verify similar expression of ephrin-B1 with or without PP1 (*arrow* in *lower panel*). The migration of phospho-ephrin-B1 is different from the nonphosphorylated form.

rounded morphology, indicating that activation of ephrin-B1 could induce cell rounding independently of tyrosine phosphorylation and PDZ domain interaction. This was consistent with the finding that over-expression of either mutant could induce significant JNK activation (Fig. 2, *A* and *D*).

The morphological assay for different ephrin-B1 mutants revealed a general correlation between the induction of JNK activation and the induction of cell rounding. To further verify the role of JNK in mediating the ephrin-B1-induced cell rounding, 293 cells transfected with ephrin-B1 or ephrin-B1/ΔC82 were treated with the JNK inhibitor SP600125, which selectively inhibits JNK but not other MAP kinases. The IC_{50} of SP600125 for ERK2 or p38 is more than 100-fold higher than that for JNK in the kinase assay. Moreover, adding SP600125 (25 μM) to Jurkat T cells does not affect the phosphorylation of ERK and p38 or the degradation of IκBα, an indication of NFκB signaling (38). Treatment of transfected 293 cells with SP600125 (30 μM) completely abolished the ephrin-B1-induced phosphorylation of c-Jun, without affecting the exogenous expression level of ephrin-B1 (Fig. 6, *A* and *B*). More importantly, the presence of SP600125 significantly inhibited the ephrin-B1-induced cell rounding of transfected 293 cells (Fig. 6, *C* and *D*), thereby indicating the important role of JNK in mediating ephrin-B1 reverse signaling. Because induction of JNK activity and rounding morphology was observed when over-expressing ephrin-B1/ΔC33 or ephrin-B1/6YF in 293 cells, it could be anticipated that the ephrin-B1-mediated cell rounding was independent of tyrosine phosphorylation. Indeed, treating transfected 293 cells with the Src inhibitor PP2 (1 μM) could not inhibit the JNK activation nor cell rounding induced by over-expressing ephrin-B1 (Fig. 6, *B–D*). PP2 at a low concentration (1 μM) was demonstrated to be effective in inhibiting the Src-mediated ephrin-B1 phosphorylation (21), and a higher concentration of PP2 (25 μM) led to the detachment of both transfected and untransfected cells (data not shown). The selective inhibition of the ephrin-B1-mediated morphological changes by the JNK inhibitor but not the Src inhibitor therefore provides additional evidence for the phosphotyrosine-independent JNK pathway in ephrin-B1 reverse signaling.

DISCUSSION

Our current findings have identified the MAP kinase JNK as an important mediator of ephrin-B1 reverse signaling. Over-expression of ephrin-B1 in 293T cells dramatically increased the activity of JNK. We further demonstrated that TAK1 was the main upstream activator of ephrin-B1-mediated JNK activation via MKK4 and MKK7. Moreover, we showed that the reverse signaling of ephrin-B1 led to rounding morphology of the transfected 293 cells, which required the activity of JNK. Finally, we provided evidence that this ephrin-B1-induced JNK activation and the subsequent morphological change were independent of tyrosine phosphorylation.

Our study identified the kinase cascade responsible for the JNK activity induced by ephrin-B1 reverse signaling. Co-expression of dominant-negative TAK1, MKK4, or MKK7 could each individually inhibit the ephrin-B1-induced JNK activity, suggesting that ephrin-B1 reverse signaling results in the activation of TAK1, which then activates MKK4 and MKK7, leading to JNK activation. Because axin selectively regulates JNK activity via MEKK1 but not TAK1 (27), the fact that

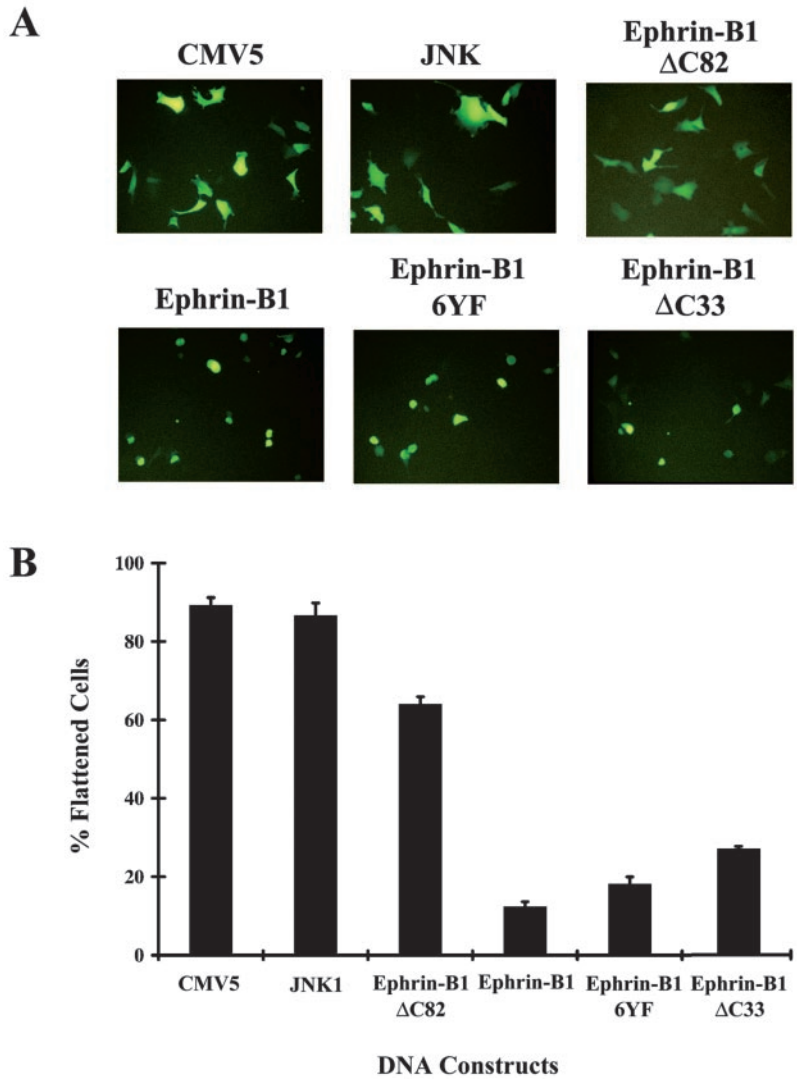

FIG. 5. **Reverse signaling of ephrin-B1 led to a morphological change in transfected 293 cells independent of ephrin-B1 tyrosine phosphorylation.** *A*, 293 cells were co-transfected with GFP and various ephrin-B1 constructs. One day after transfection, morphology of the GFP-positive cells was examined; representative fields are shown in the fluorescent micrographs. Cells that over-expressed wild-type ephrin-B1 or ephrin-B1/6YF were rounded and shrunken, whereas most of the cells transfected with ephrin-B1/ΔC82 remained flattened, similar to those transfected with empty vector (*CMV5*), indicating that the ephrin-B1-mediated change in 293 morphology was attributable largely to the reverse signaling. *B*, quantifying the morphological changes of transfected 293 cells. The number of GFP-positive cells that were flattened or rounded, respectively, was counted in three independent experiments, and the percentage of GFP-positive cells that were flattened was calculated (mean ± S.E., $n = 3$).

dominant-negative MEKK1 could not inhibit the ephrin-B1-mediated JNK activation was consistent with our observation that the JNK activation was not affected by dominant-negative axin (data not shown). Indeed, our data also showed that Grb4, which could interact with axin via its SH3 domain (22), was not required in the ephrin-B1-induced JNK activity.

Over-expressing ephrin-B1 in 293 cells, which expressed endogenous EphB receptors, could activate both forward signaling from the EphB receptors and reverse signaling from the exogenous ephrin-B1. In particular, a previous study reported the phosphorylation of JNK during forward signaling of EphB1 (28). It is therefore important to distinguish which pathway was responsible for the observed JNK activation and induction of cell rounding. Our data strongly favored an important contribution by the reverse signaling. First, the activation of JNK upon over-expressing ephrin-B1 was much higher than that of ephrin-B1/ΔC82. Second, full activation of JNK by over-expressing ephrin-B1 was observed in the presence of EphB2-Fc, which could interact with the exogenous ephrin-B1 and therefore act as the antagonist for forward signaling. Finally, over-expression of ephrin-B1 only resulted in rounding morphology of the transfected (GFP-positive) cells, whereas the untransfected cells, which expressed endogenous EphB receptors and were in contact with the transfected cells, remained spread out and flattened. We therefore conclude that it is the reverse signaling that activates JNK and causes the morphological change. Nonetheless, a low level of forward signaling may be switched on when ephrin-B1 is over-expressed in 293 cells, which accounts for the low level of JNK activation and subtle morphological change in 293 cells transfected with ephrin-B1/ΔC82.

Forward signaling of Eph receptors exerts opposite effects in cell morphology and adhesion, depending on the specific cell types (28, 39–43). With regard to the reverse signaling, differential effects in cell adhesion were also reported (13–14, 22, 44, 45). Our observations favor the loss of cell adhesion mediated by ephrin-B reverse signaling. In addition, our study represents the first demonstration that JNK activity is crucial for ephrin-B-mediated morphological change. Earlier studies have shown that JNK is phosphorylated when the forward and reverse signaling pathways are switched on (28, 45). These studies, however, did not address the significance of JNK in mediating the cellular response as a result of ephrin-B or EphB signaling. By constructing various ephrin-B1 mutants, we have observed a general correlation between the activation of JNK and the induction of cell rounding. Moreover, the role of JNK was verified by the selective inhibition of cell rounding in the presence of the JNK inhibitor SP600125 but not the Src kinase inhibitor PP2.

Unexpectedly, ephrin-B1 activates JNK and causes subsequent cell rounding independently of tyrosine phosphorylation. First, over-expressed ephrin-B1/6YF or ephrin-B1/ΔC33, neither of which contained any intracellular tyrosine residue, did induce JNK activation and cell rounding to a similar extent as

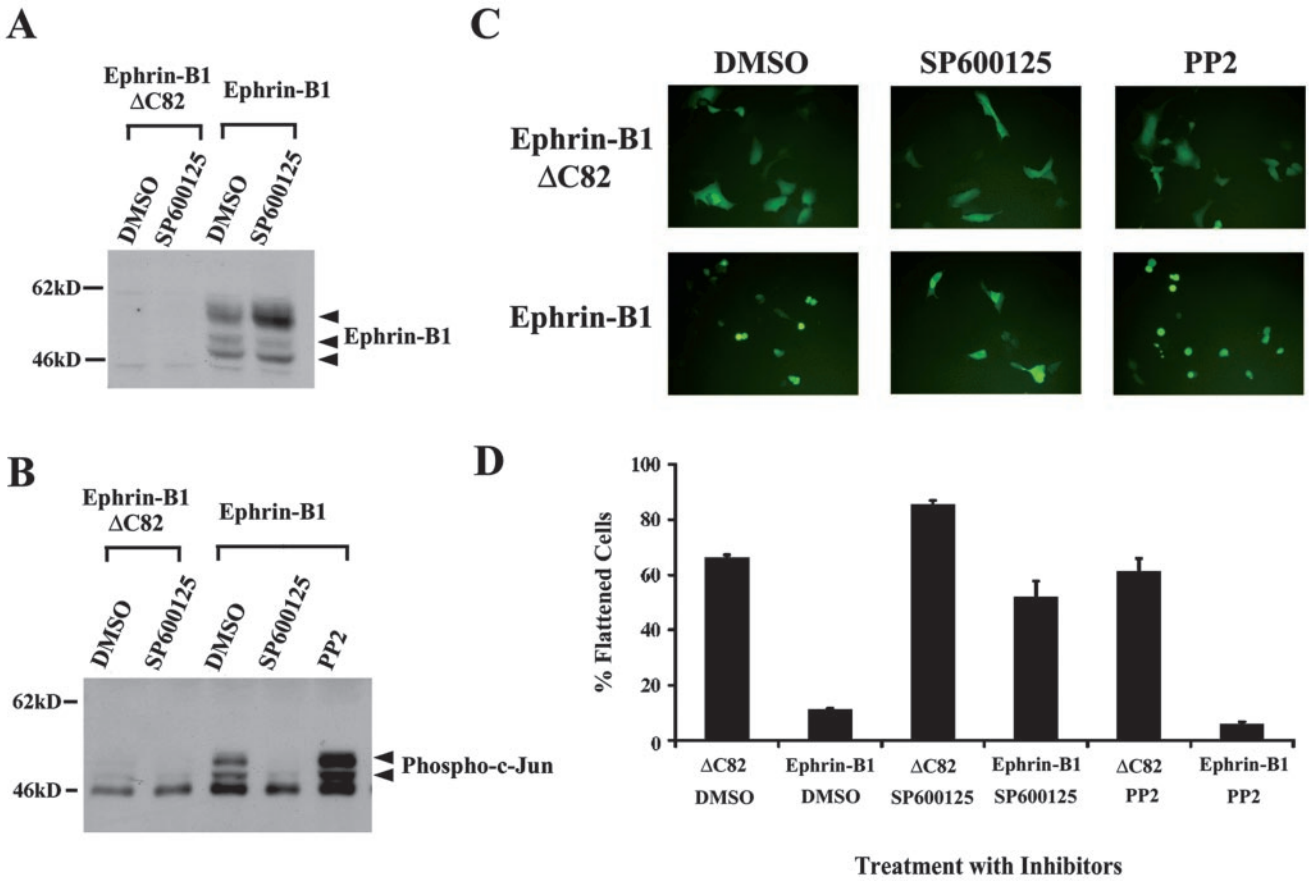

FIG. 6. **Ephrin-B1-mediated cell rounding was reversed by JNK inhibitor but not Src kinase inhibitor.** *A*, JNK inhibitor SP600125 did not affect the exogenous expression of ephrin-B1. Cells transfected with ephrin-B1 or ephrin-B1/ΔC82 were incubated with dimethyl sulfoxide (*DMSO*) or the JNK inhibitor SP600125 (30 μM) for 2 days. Cell lysate was immunoblotted with anti-ephrin-B1 antibody. *B*, JNK activation by exogenous expression of ephrin-B1 in 293 cells was abolished by SP600125 but not by the Src kinase inhibitor PP2. 293 cells were transfected with ephrin-B1 or ephrin-B1/ΔC82 and treated with dimethyl sulfoxide, SP600125 (30 μM), or PP2 (25 μM) for 2 days. Cell lysates were immunoblotted with anti-phosphoserine c-Jun antibody. *C*, 293 cells were co-transfected with GFP and ephrin-B1 or ephrin-B1/ΔC82. One day after transfection, cells were treated with Me_2SO, SP600125 (30 μM), or PP2 (1 μM) for 5 h, and the morphology of the GFP-positive cells was examined. Representative fields are shown in the fluorescent micrographs. Cells transfected with ephrin-B1/ΔC82 were flattened with or without the inhibitors, whereas ephrin-B1 over-expressing cells were rounded and shrunk when incubated with Me_2SO or PP2. However, a large proportion of the 293 cells that over-expressed ephrin-B1 remained flattened in the presence of SP600125. *D*, quantification of the morphological changes of transfected 293 cells. The number of GFP-positive cells that were flattened or rounded, respectively, was counted in three independent experiments, and the percentage of GFP-positive cells that were flattened was calculated (mean ± S.E., $n = 3$).

the wild-type ephrin-B1. Second, Grb4 was shown to be the critical adaptor that mediated the phosphotyrosine-dependent reverse signaling (22), and yet ephrin-B1-induced JNK activation was not affected by various Grb4 dominant-negative constructs. Finally, the presence of Src inhibitor abolished the tyrosine phosphorylation of ephrin-B1 but not the activation of JNK and subsequent cell rounding. Observations obtained from over-expressing ephrin-B1/ΔC33 further suggested that reverse signaling could be triggered without interaction with PDZ domain proteins. Our results therefore differ from the previous findings showing the importance of tyrosine phosphorylation or PDZ-binding motif in ephrin-B1 reverse signaling (21, 22, 25, 45). However, it is noteworthy that, in those studies, ephrin-B1 was activated by the addition of EphB-Fc to cells that endogenously expressed ephrin-B ligands. When ephrin-B1 was over-expressed in 293 cells, the ligands became activated without the addition of exogenous EphB-Fc, reminiscent of the situation in which Eph receptor was over-expressed and triggered forward signaling in the absence of exogenous ephrin-Fc (40, 46–48). Because 293 cells expressed endogenous EphB receptors, the activation of ephrin-B1 might require interaction with EphB receptor, although we did not rule out the possibility that ephrin-B1 became self-activated upon over-expression, similar to the situation when the receptor tyrosine kinase was over-expressed (46). Activation of ephrin-B1 in this way, which might be more prolonged compared with activation by EphB-Fc addition, could bypass the requirement of tyrosine phosphorylation and PDZ-protein interaction and trigger reverse signaling. With regard to the possibility of phosphotyrosine-independent reverse signaling, it was noteworthy that ephrin-B2 molecules, which contained only the ectodomains, were associated as dimers in crystal (49). This was consistent with our observation that ephrin-B1 becomes dimerized when over-expressed in 293 cells, which is independent of tyrosine phosphorylation (data not shown). Moreover, ephrin-B1 was recently shown to interact with the protein Dishevelled independently of tyrosine phosphorylation or interaction with the PDZ domain protein (50). Finally, the injection of a mutant ephrin-B1 that lacked three of the five conserved tyrosine residues plus the PDZ-binding motif into a *Xenopus* embryo induced a rounding morphology of the cells lining the blastocoel roof, although it failed to cause the cell dissociation observed when wild-type ephrin-B1 was injected (44).

Taken together, the present study has revealed a novel signaling pathway transduced by ephrin-B1 that is independent of tyrosine phosphorylation and PDZ-protein interaction. Moreover, identification of JNK as a critical mediator of ephrin-B1-induced morphological change has added to the enlarging pic-

ture that JNK activity is involved in developmental processes. Previous studies have unraveled the significance of JNK in determining cell polarity and morphogenesis (51, 52). Our findings have raised the intriguing possibility that JNK may also play a role in various developmental processes that involve the participation of ephrins, such as axon guidance, segmentation, and synaptogenesis.

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