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EphrinB1 Interacts with CNK1 and Promotes Cell Migration through c-Jun N-terminal Kinase (JNK) Activation*

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Background: EphrinB1 affects cell adhesion and migration *in vitro* and *in vivo*.
Results: CNK1 interacts with ephrinB1 in cancer cell lines, and the presence of CNK1 is required for RhoA-mediated JNK activation and cell migration.
Conclusion: CNK1 mediates ephrinB1 signaling that promotes cell migration through RhoA and JNK activity.
Significance: CNK1 scaffold links ephrinB1 signaling to JNK activation.

The Eph receptors and their membrane-bound ligands, ephrins, play important roles in various biological processes such as cell adhesion and movement. The transmembrane ephrinBs transduce reverse signaling in a tyrosine phosphorylation-dependent or -independent, as well as PDZ-dependent manner. Here, we show that ephrinB1 interacts with Connector Enhancer of KSR1 (CNK1) in an EphB receptor-independent manner. In cultured cells, cotransfection of ephrinB1 with CNK1 increases JNK phosphorylation. EphrinB1/CNK1-mediated JNK activation is reduced by overexpression of dominant-negative RhoA. Overexpression of CNK1 alone is sufficient for activation of RhoA; however, both ephrinB1 and CNK1 are required for JNK phosphorylation. Co-immunoprecipitation data showed that ephrinB1 and CNK1 act as scaffold proteins that connect RhoA and JNK signaling components, such as p115RhoGEF and MKK4. Furthermore, adhesion to fibronectin or active Src overexpression increases ephrinB1/CNK1 binding, whereas blocking Src activity by a pharmacological inhibitor decreases not only ephrinB1/CNK1 binding, but also JNK activation. EphrinB1 overexpression increases cell motility, however, CNK1 depletion by siRNA abrogates ephrinB1-mediated cell migration and JNK activation. Moreover, Rho kinase inhibitor or JNK inhibitor treatment suppresses ephrinB1-mediated cell migration. Taken together, our findings suggest that CNK1 is required for ephrinB1-induced JNK activation and cell migration.

Members of the Eph family have been implicated in regulating numerous morphogenetic processes such as axon outgrowth, neural crest, and retinal progenitor cell migration, hindbrain segmentation, skeletal patterning, mesoderm/ectoderm boundary formation, and angiogenesis (1, 2). Interactions between the Eph receptor tyrosine kinases residing on one cell with their membrane-bound ligands on another cell results in bi-directional signaling. Although evidence is emerging that both Eph receptors and ligands ultimately affect Rho family signal transduction, various signaling molecules and pathways intersect with Eph receptor or ligand signaling (3), and further studies are needed to define the Eph/ephrin signal transduction systems and how they are regulated. Eph/ephrin signaling emanating from cell-cell contact events during development leads to cell sorting and boundary formation between receptor and ligand bearing cells. Motile ligand or receptor bearing cells respond to contact with cells bearing the cognate receptor or ligand by adhesion or repulsion. Alternative growth factors and signaling pathways can mediate or regulate Eph/ephrin signaling to assist the cognate receptor or ligand bearing cells to regulate the movement and positioning of cells (2). These ligands and receptors play a role in several morphogenetic events during development, but when de-regulated can lead to cancer invasion and metastasis. The de-regulation of this signaling system is linked to the promotion of more aggressive and metastatic tumor phenotypes in a large variety of human cancers, including breast, lung, prostate, colon, and melanoma (4, 5). Recent data show that members of the Eph/ephrin family mediate cell-cell interactions both in tumor cells and in the tumor microenvironment (*i.e.* stroma and vasculature) (6–8). Thus, gaining an understanding of the mechanism and pathways that promote Eph receptor and ephrin signaling and how they are regulated is likely to have biomedical importance.

Eph and ephrin signaling has become an area of intense interest because of the wide ranging influence on control of cell survival (9), endocytosis (10) cell adhesion (11), cell movement (3), and metastasis (12). Reverse signaling through the intracellular domain of transmembrane ephrins is now a widely accepted concept. The B-type transmembrane ephrin ligands do not possess any intrinsic catalytic activity for signaling, but rely upon a scaffolding activity that recruits signaling molecules to transmit functional effects within the cell. It has been shown that ephrin-Bs utilize both phosphorylation-dependent and -independent signaling pathways, which may be viewed as three possible modes of reverse signaling: 1) one mode where tyrosine phosphorylation of the intracellular domain of ephrinB leads to recruitment of signaling molecules that exert a functional effect. Phosphorylation can be initiated through the binding and clustering of Eph receptors that lead to activation

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of a Src family kinase, which phosphorylates the intracellular domain of B-type ephrins (13, 14). Alternatively, a growth factor receptor (*i.e.* FGFR, PDGFR, TIE-2) or cell surface molecule (Claudin) induces this phosphorylation event (13–17); 2) the second mode of signaling is through an unphosphorylated ephrinB that associates with a protein complex to transduce a signal, but upon tyrosine phosphorylation, the interaction of ephrinB with the signaling complex is disrupted or modulated (18); 3) a possible third mode may exist where tyrosine phosphorylation occurs, but is not required for specific signaling events that may use non-canonical SH2/PDZ-independent forms of reverse signaling (19).

There are a number of proteins that have been shown to interact with ephrinBs and promote a functional effect, including PDZ-RGS3 (GTP exchange factor) (20), ZHX2 (a zinc finger homeodomain protein) (21), Connexin 43 (gap junction communication protein) (22), Dishevelled (a scaffold for Wnt/PCP signaling) (23, 24), and Par-6 (a central scaffold in the Par polarity complex) (25). Although these molecules associate with ephrinB in a phosphorylation-independent manner, Grb4, an adaptor protein, has been shown to associate with ephrinB1 in a phosphorylation-dependent manner and mediate functional effects on cell morphology (26, 27). STAT3 is also among the group of phosphorylation-dependent ephrinB-associated signaling molecules (28).

It was previously shown that overexpression of ephrinB1 in HEK 293 cells resulted in JNK activation and cell rounding, but did not require the C-terminal 33 amino acids of ephrinB1 nor tyrosine phosphorylation (29). In another study, activation of ephrinB1 by EphB1/Fc induced phosphorylation of JNK but mutants of ephrinB1 bearing cytoplasmic deletions fail to activate JNK (30). Although JNK activation is a downstream event in ephrinB reverse signaling (29), its precise role in cell-cell and cell-substrate modulation is not yet clear.

In the current study, we examine how an ephrinB1-interacting protein, Connector Enhancer of KSR1 (CNK1),[2] contributes to ephrinB1 signaling. CNK1 is a scaffold protein that possesses multiple protein interaction domains, including a sterile α motif (SAM), a conserved region in CNK (CRIC) domain, and a PSD-95/DLG-1/ZO-1 (PDZ) domain, and a pleckstrin homology (PH) domain. (31). This scaffold links Rho and Ras signal transduction pathways (32), and is critical in the activation of the PI3K/AKT cascade in the insulin signaling pathway (33), as well as the FoxO signaling network (34). Here, using HEK 293T, HT-29, and HeLa cell lines, we show that CNK1 links ephrinB1 to RhoA-dependent JNK activation and cell migration.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Full-length human ephrinB1 cDNA clone was obtained from Open Biosystems (GenBank^{TM} accession: NM_004429). HA-tagged ephrinB1 was generated by PCR and subcloned into pCDNA3.1 or pCDH-CMV-MCS-EF1. Pyo-tagged CNK1 and siRNA-resistant CNK1 have been described previously (33). Full-length human p115 RhoGEF cDNA clone was obtained from Open Biosystems (GenBank^{TM} accession: BC067262). Flag-tagged p115 RhoGEF was generated by PCR and inserted into pCDNA3.1. Flag-tagged MKK4 was obtained from Addgene. Various HA-tagged deletion mutants of ephrinB1 (Δ4, Δ20, Δ30, Δ34, Δ38, Δ60, and TmCyt) were generated by PCR. Other deletion or substitution mutants of ephrinB1 (Y6F) and CNK1 (ΔCRIC, ΔPDZ, ΔPH, Y456F, Y519F, and Y665F) were generated using QuickChange Site-directed mutagenesis according to the manufacturer's instruction (Agilent). Rho kinase inhibitor (Cat. number: 555550) and JNK inhibitor (Cat. Number: 420119) were purchased from Calbiochem.

Cell Culture and Generation of Stable Cell Line—HEK 293T, HeLa, and HT-29 cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. Stable HeLa cell lines expressing ephrinB1 proteins were generated by lentiviral infection. Briefly, pCDH-CMV-MCS-EF1 expressing Mock or HA-tagged ephrinB1 was transfected into lentiviral packaging cell lines HEK 293T cells using the MISSION lentiviral packaging mix (Sigma). The culture supernatant containing virus particles was harvested 48 h after transfection. Cells were grown in 6-well plates to 60–70% confluency, and 1 ml of viral supernatant containing 8 μg of polybrene was added. After 48 h, 2 μg/ml puromycin (Clontech) was added to the cultures for selection. After 14 days, puromycin-resistant cell pools were established.

Immunoprecipitation and Western Blot Analysis—Cells were lysed in cold lysis buffer (20 mmol/liter Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Roche), 1 mM sodium vanadate) for 30 min at 4 °C, and lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C. Equivalent amounts of protein lysate were incubated with the anti-HA, anti-Flag (Applied Biological Materials), anti-ephrinB1 (R&D Systems), anti-CNK1 (BD Bioscience), or anti-Pyo antibodies at 4 °C for 2 -16 h, and then incubated with protein-A/G-agarose (Santa Cruz Biotechnology) at 4 °C for 1 h. The antibody recognizing the Pyo epitope has been described previously (33). Immunoprecipitates were washed with either RIPA or lysis buffer three times. Immunoprecipitated proteins were eluted with SDS-sample buffer and analyzed by SDS-PAGE and Western blotting. Immunoblotting was conducted using anti-Flag-HRP (Sigma), anti-HA-HRP (Roche), anti-ephrinB1 (R&D Systems, or Santa Cruz Biotechnology C18), anti-CNK1 (BD Bioscience), anti-pSrc, Src, pJNK, pMKK4, MKK4, pMKK7, and MKK7 (Cell Signaling), anti-RhoA and JNK (Santa Cruz Biotechnology), anti-phosphotyrosine (Millipore) and α-tubulin (Sigma).

In Vitro Kinase Assay—250 ng of purified GST-CNK1 (Novus) or extracellular domain of ephrinB1 (Novus) was incubated with or without 100 ng of recombinant active Src (Signalchem) in kinase buffer (Cell Signaling) containing 50 μM ATP for 20 min at 30 °C. Samples were immunoblotted with anti-phosphotyrosine antibody.

RNA Interference Experiments—For siRNA-mediated depletion, the silencer select negative control #1 siRNA (Ambion), and silencer select pre-designed siRNA (siCNK1-1, Ambion)

[2] The abbreviations used are: CNK, Connector Enhancer of KSR; SAM, sterile α motif; PH, pleckstrin homology; Tm-cyto, transmembrane and cytoplasmic domain; FN, fibronectin; RhoA, Rhotekin-agarose.

and stealth siRNA (siCNK1-2, Invitrogen) for targeting CNK1 genes were transfected into cells using siPORT™ NeoFX™ transfection reagent (Ambion) following the instructions of the manufacturers.

Rho GTPases Activity Assay—The activity of Rho GTPases was measured by affinity precipitation using the Rho Assay Reagent (Millipore). Briefly, cells at 80% confluency were lysed in 1 ml of Mg^{2+} Lysis/Wash buffer (Millipore) containing 1 mM sodium vanadate and protease inhibitors. Lysates were clarified by centrifugation, and equalized for total volume and protein concentration. Rhotekin-agarose was immediately added to equal amounts of cell lysates and incubated for 1 h at 4 °C. The agarose beads were centrifuged at 12,000 rpm for 30 s, and the bead pellet was washed three times with Mg^{2+} Lysis/Wash buffer. The bead pellet was finally suspended in Laemmli sample buffer and then analyzed by SDS-PAGE and Western blotting.

Fibronectin Plating—Petri dishes were coated with 20 μg/ml of fibronectin in PBS at 4 °C for overnight. The plates were washed with PBS and blocked by incubating with DMEM containing 0.5% BSA (replating medium) for 1 h at 37 °C. Cells were starved in the media containing 0.2% FBS for 16 h, and then harvested and resuspended in replating medium to 2×10^5 cells/ml and then held in suspension for 2 h. 5 ml or 10 ml cell suspension was replated onto 10-cm or 15-cm plate pre-coated with fibronectin and processed for Western blotting or immunoprecipitation, respectively.

Cell Motility Assay—For wound healing assays, 2×10^5 cells/well in 70 μl medium with 0.2% FBS were seeded into Culture-inserts (Ibidi) on 10 μg/ml of fibronectin-coated plates. After the cells were confluent, the cells were pretreated with 0.5 μg/ml Mitomycin C (Sigma) for 1 h. Culture inserts were removed, and cells were incubated with fresh medium containing 0.2% FBS for 18 h. The photographs of wound closure were taken using a phase-contrast microscope with a digital camera. The gap area was quantified using Image J software. The %wound closure is determined as the (gap area at 0 h-gap area at 18 h)/gap area at 0 h × 100. For transwell migration assays, 24-well cell culture plate inserts with 8-μm pore size polycarbonate membrane (Corning) were pre-coated with 100 μl of fibronectin solution (10 μg/ml) and incubated overnight at 4 °C. All the cells were pre-incubated in serum-free media for 24 h. 5×10^4 cells in 100 μl of serum-free medium were placed in the upper chamber of a transwell plate and cultured for 16 h. After incubation, each well was washed with PBS and fixed for with 100% methanol for 15 min and then stained with GIEMSA (Sigma) for 20 min. Non-migrating cells were removed with a cotton swab. The number of migratory cells in 4 random fields was counted, and three independent inserts were analyzed.

RESULTS

EphrinB1 Interacts with CNK1—We recently reported that in HEK 293T cells overexpressing CNK1, ephrinB1 was found in CNK1 immune-complexes by mass spectrometric analysis (33). We first examined the expression of these two proteins in human cell lines. Western analysis indicates that both ephrinB1 and CNK1 are amply expressed in HT-29 cells, while little ephrinB1 and almost no CNK1 is detected in HEK 293T cells.

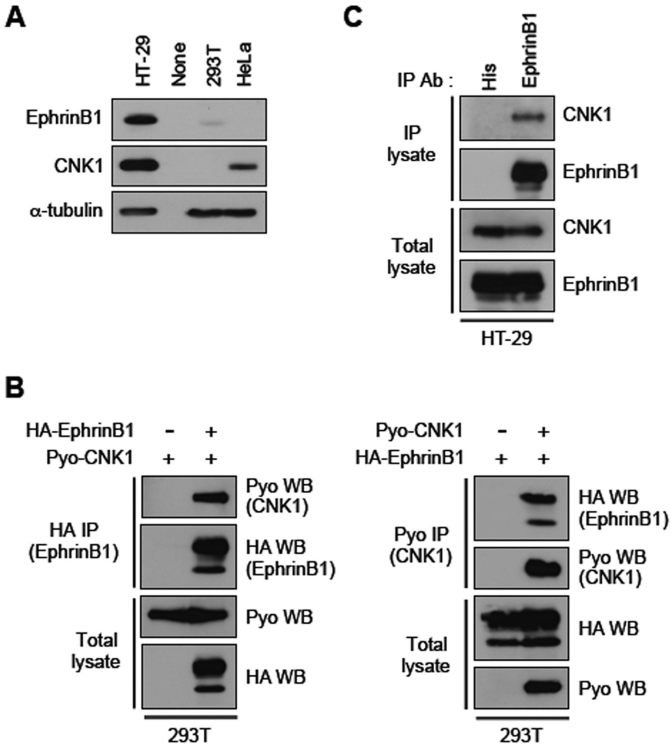

FIGURE 1. **EphrinB1 is associated with CNK1.** *A*, Western blot analysis of ephrinB1 and CNK1 in human cell lines. *B*, HEK 293T cells were transfected with HA-tagged ephrinB1 and Pyo-tagged CNK1 as indicated. Cell lysates were immunoprecipitated with HA (*left panel*) or Pyo (*right panel*) antibodies. Immunoprecipitates and total lysates were immunoblotted with HA or Pyo antibodies. *C*, HT-29 cell lysates were immunoprecipitated with ephrinB1 or His (as control) antibodies. Immunoprecipitates and total lysates were immunoblotted with ephrinB1 or CNK1 antibodies.

HeLa cells have abundant CNK1, but no detectable ephrinB1 (Fig. 1*A*). Thus, we exogenously expressed HA-tagged ephrinB1 along with Pyo-tagged CNK1 in HEK 293T cells and performed coimmunoprecipitation (Co-IP) analyses. CNK1 was detected in ephrinB1 immune-complexes (Fig. 1*B*), and ephrinB1 was detected in CNK1 immune-complexes (Fig. 1*B*). To confirm the interaction between these two proteins exists among endogenous proteins, HT-29 colon carcinoma cells were examined. As expected, CNK1 was associated with ephrinB1 immune-complexes from these cells, indicating that both proteins can interact *in vivo* (Fig. 1*C*).

PDZ Binding Motif of EphrinB1 and the CRIC Domain of CNK1 Is Important for the Association—To assess which amino acids in ephrinB1 are critical for an interaction with CNK1, progressive deletions were generated in the C-terminal region of the intracellular domain of ephrinB1 (Fig. 2*A*). These HA-tagged ephrinB1 deletion mutants were co-expressed with CNK1 in HEK 293T cells, and Co-IPs and Western analyses were performed. Deletion of the C-terminal four amino acids, which also represents a PDZ-binding motif, dramatically reduced the interaction between the two proteins. In contrast, the controls of wild type ephrinB1 (WT) and a mutant consisting of only the transmembrane and cytoplasmic domain (Tm-cyto) showed a robust association with CNK1 (Fig. 2*B*). To determine which domains within CNK1 are critical for an interaction with ephrinB1, we generated various domain deletion

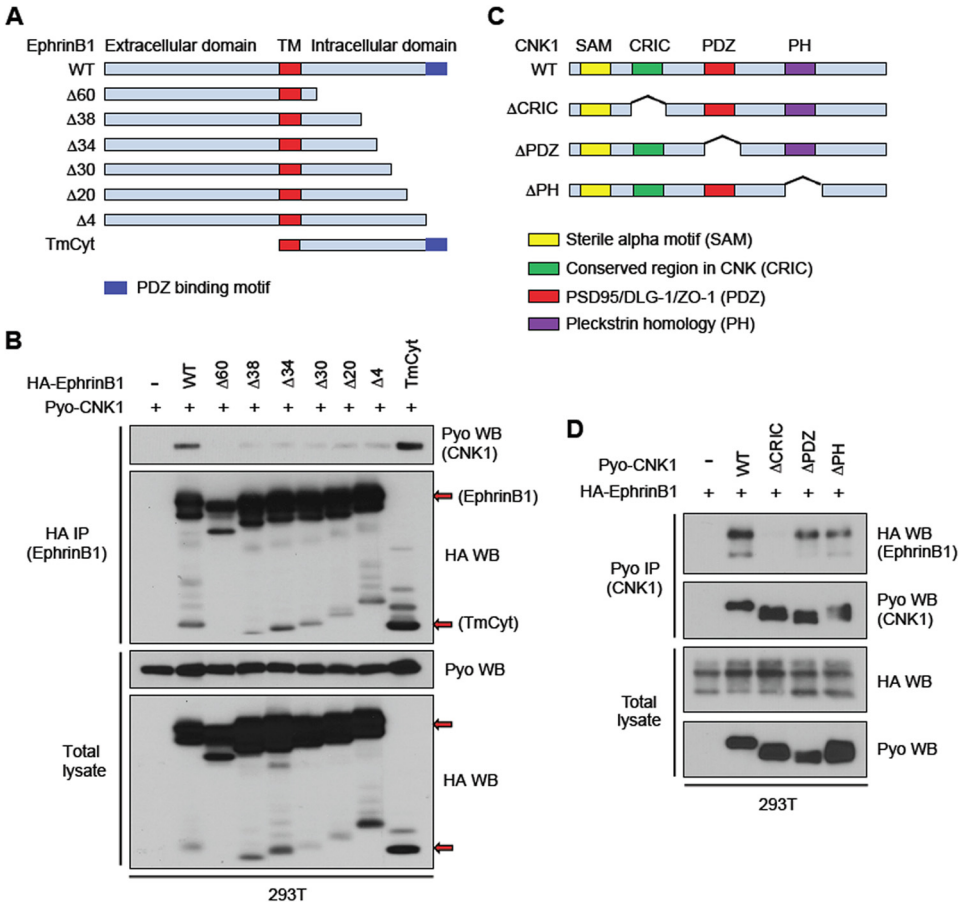

FIGURE 2. **PDZ binding motif of ephrinB1 and the CRIC domain of CNK1 are required for an interaction.** *A*, schematic representation of WT ephrinB1 or deletion mutants lacking 4, 20, 30, 34, 38, or 60 amino acids from the C terminus or the extracellular domain (TmCyt). *B*, HA-tagged WT or deletion mutants of ephrinB1 were co-transfected with Pyo-tagged CNK1 into HEK 293T cells as indicated. Proteins were immunoprecipitated with HA antibody. Immunoprecipitates and total lysates were immunoblotted with HA or Pyo antibodies. *C*, schematic representation of WT CNK1 or deletion mutants lacking the CRIC, PDZ, or PH domains. *D*, Pyo-tagged WT or deletion mutants of CNK1 were co-transfected into HEK 293T cells with HA-tagged ephrinB1 as indicated. Cell lysates were immunoprecipitated with Pyo antibody. Immunoprecipitates and total lysates were immunoblotted with HA or Pyo antibodies.

mutants in CNK1, including CRIC domain, PDZ domain, and PH domain (Fig. 2*C*).

Surprisingly, deletion of the CRIC domain causes a prominent loss of association between CNK1 and ephrinB1, while deletion of the PDZ and PH domains had no substantive effect (Fig. 2*D*).

CNK1 Promotes EphrinB1-mediated JNK Phosphorylation through RhoA Activation—We next engaged in determining whether CNK1 mediates ephrinB1 reverse signaling. In addition, we tested whether the identified motifs that are required for an interaction between ephrinB1 and CNK1 are also required for ephrinB1 signaling. To this end, we examined JNK activation, as evidenced by phospho-JNK Western analysis. HEK 293T cells were transfected with Pyo-tagged WT and ΔCRIC constructs of CNK1, along with WT ephrinB1 or Δ4 ephrinB1 (deletion of C-terminal 4 amino acids). Western analysis of the lysates shows robust phospho-JNK only with WT CNK1 and WT ephrinB1 (Fig. 3*A*), indicating that an interaction between CNK1 and ephrinB1 is critical for JNK activation.

RhoA has been shown to be important in HEK 293T cells for JNK activation (35), and in HeLa cells, serum stimulates Rho-dependent activation of the JNK cascade through CNK1 (36). Therefore, we examined whether ephrinB1, which is known to activate RhoA in embryonic development (23, 37–39), required CNK1 for RhoA activation. HEK 293T cells were transfected with WT HA-tagged ephrinB1 and/or Pyo-tagged CNK1 and activated RhoA was pulled down using the Rhotekin-binding domain protein. RhoA was detected using RhoA specific antibodies, and phospho-JNK was also detected using phosphospecific antibodies (Fig. 3*B*). Activated RhoA levels are increased in the presence of CNK1 alone. JNK activation, as measured by an increase in phospho-JNK detection, occurs only in the case where both CNK1 and ephrinB1 are both present (Fig. 3*B*). To confirm that ephrinB1-mediated JNK activation is RhoA-dependent, HEK 293T cells co-expressing both ephrinB1 and CNK1 were also transfected with similar amounts of HA-tagged dominant negative constructs for the GTPases RhoA, Rac, and Cdc42 (Fig. 3*C*). Western analysis shows that only dominant-negative RhoA prevented an increase in phospho-JNK detection (Fig. 3*C*), indicating that ephrinB1-mediated JNK activation is through a RhoA-dependent mechanism. Together, these data show that CNK1 promotes ephrinB1-mediated JNK phosphorylation through RhoA activation.

EphrinB1/CNK1 Binding Links RhoA with JNK Signaling Components—Since CNK1 has been shown to bind the GTP exchange factor, p115RhoGEF, we wanted to examine whether

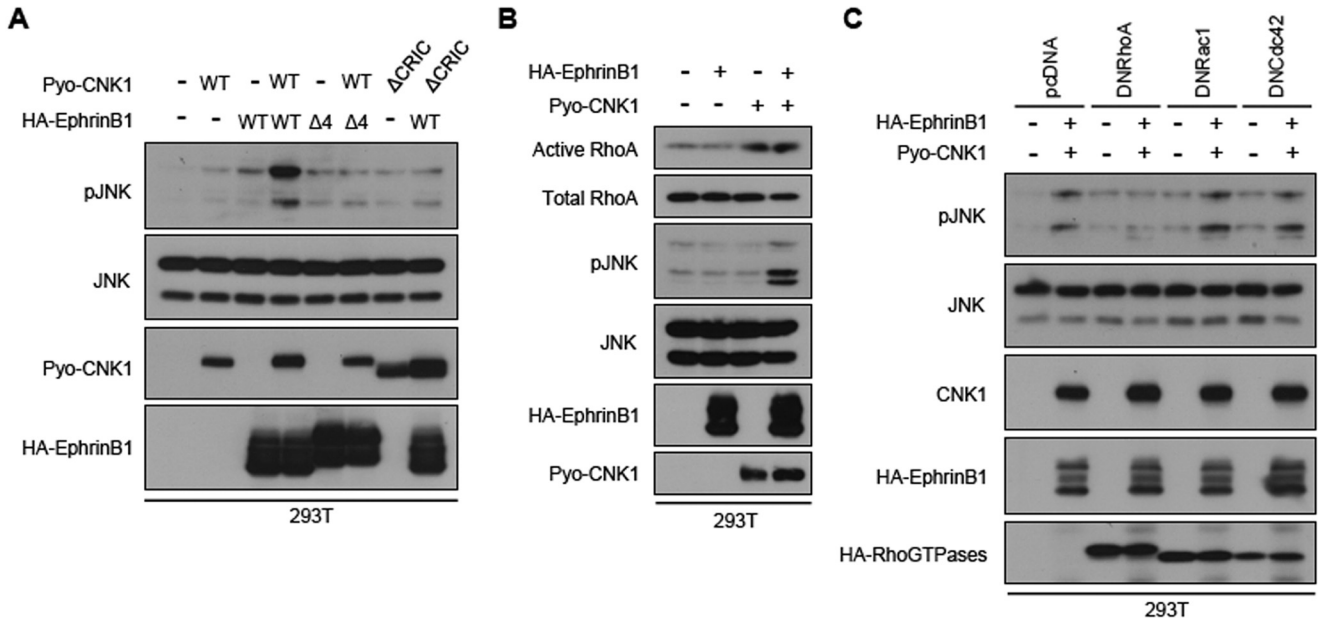

FIGURE 3. **CNK1 cooperates with ephrinB1 for JNK activation through RhoA.** *A*, HA-tagged WT or Δ4 mutant of ephrinB1 was co-transfected into HEK 293T cells with Pyo-tagged WT or a ΔCRIC mutant of CNK1. Western analysis was performed with the indicated antibodies. *B*, HEK 293T cells were transfected with HA-tagged ephrinB1 and/or Pyo-tagged CNK1. A pull-down assay using Rhotekin-agarose (RhoA) was performed. Samples and total lysates were immunoblotted with RhoA, phospho-JNK, JNK, HA, or Pyo antibodies. *C*, HA-tagged ephrinB1 and Pyo-tagged CNK1 were co-transfected into HEK 293T cells with a vector control, DN-RhoA, DN-Rac1, or DN-Cdc42. Western analysis was performed with the indicated antibodies. (*A*, *B*, and *C*) 30 h after transfection, cells were serum-starved for 18 h.

CNK1 links the RhoA GEF to ephrinB1. We co-expressed various combinations of HA-tagged ephrinB1, Pyo-tagged CNK1, and Flag-tagged p115RhoGEF into HEK 293T cells and performed Flag IPs to capture p115RhoGEF immune-complexes. Western analysis of these complexes shows that robust levels of ephrinB1 are only found in the p115RhoGEF immune-complexes when CNK1 is also expressed (Fig. 4*A*). In ephrinB1 immune-complexes, p115 RhoGEF is found prominently only when CNK1 is also present (Fig. 4*A*). Finally, in CNK1 immune-complexes p115 RhoGEF is found regardless of whether ephrinB1 is present (Fig. 4*A*). These data indicate that a trimolecular complex is formed where ephrinB1 interacts with CNK1, which interacts with p115RhoGEF. It has been reported that the activation of JNK by ephrinB1 requires MKK4, a JNK activating kinase (29). To assess how and whether MKK4 associates with the ephrinB1/CNK1 complex, various combinations of HA-tagged ephrinB1, Pyo-tagged CNK1, and Flag-tagged MKK4 were expressed in HEK 293T cells. Western analysis of Flag (MMK4), Pyo (CNK1), and HA (ephrinB1) IPs shows that ephrinB1 can interact with MKK4 in the absence of CNK1, but CNK1 requires the presence of ephrinB1 to complex with MKK4 (Fig. 4*B*). Collectively, these data indicate that ephrinB1 binds to MKK4 and CNK1, and that CNK1 links ephrinB1 to p115RhoGEF. Since activation of the Rho/Rho kinase pathway may lead to MKK4 activation (35), a possible conduit between Rho and JNK activation may be revealed by the ephrinB1/CNK1 interaction.

Src Inhibition Reduces EphrinB1/CNK1 Binding, and JNK Activation—Because Src is known to be active in HEK 293T cells and to be involved in RhoA-dependent JNK activation (40), we assessed its importance to the ephrinB1/CNK1 interaction. A Western analysis of lysates from HT-29 colon carcinoma cells, HEK 293T cells, and HeLa cells shows that HT-29 and HEK 293T cells have robust basal levels of active Src, but HeLa cells have significantly less active Src (Fig. 5*A*). HEK 293T cells were transfected with constructs for Pyo-tagged CNK1 and HA-tagged ephrinB1 and treated with the Src inhibitor SKI-606 (Fig. 5*B*). Lysates were prepared and HA IPs were performed to isolate ephrinB1 immune-complexes. Western analysis shows that less CNK1 is found in the HA (ephrinB1) IPs when the Src inhibitor is present. Direct Westerns of the lysates show that JNK activation is also diminished in the presence of the Src inhibitor, as evidenced by phospho-JNK detection (Fig. 5*B*). To determine whether the interaction between the endogenous ephrinB1 and CNK1 proteins was similarly affected, HT-29 cells were examined in the absence and presence of SKI-606. EphrinB1 IPs from the HT-29 lysates were immunoblotted for CNK1 and it was found that less CNK1 was associated with the ephrinB1 immune-complexes in the presence of SKI-606 (Fig. 5*C*). Western blots of the cell lysates indicated that the basal level of phopho-JNK was also reduced by SKI-606 (Fig. 5*C*), similar to the results observed in Fig. 5*B*. These data indicate that Src inhibition reduces the ephrinB1/CNK1 association, and results in reduced JNK activation.

Tyr-665 of CNK1 Contributes to the Src-induced EphrinB1/CNK1 Binding—Because it has been shown that ephrinB1 can be phosphorylated by activated Src (41, 42), we assessed whether tyrosine phosphorylation of ephrinB1 was responsible for enhancing the interaction with CNK1. HeLa cells, which possess very low levels of endogenous active Src (Fig. 5*A*), were transfected with constitutively active Src (Y529F) and HA-tagged WT ephrinB1 or a mutant harboring phenylalanines in place of the six conserved tyrosines in the intracellular domain (Y6F). Lysates from these cells were subjected to IP of

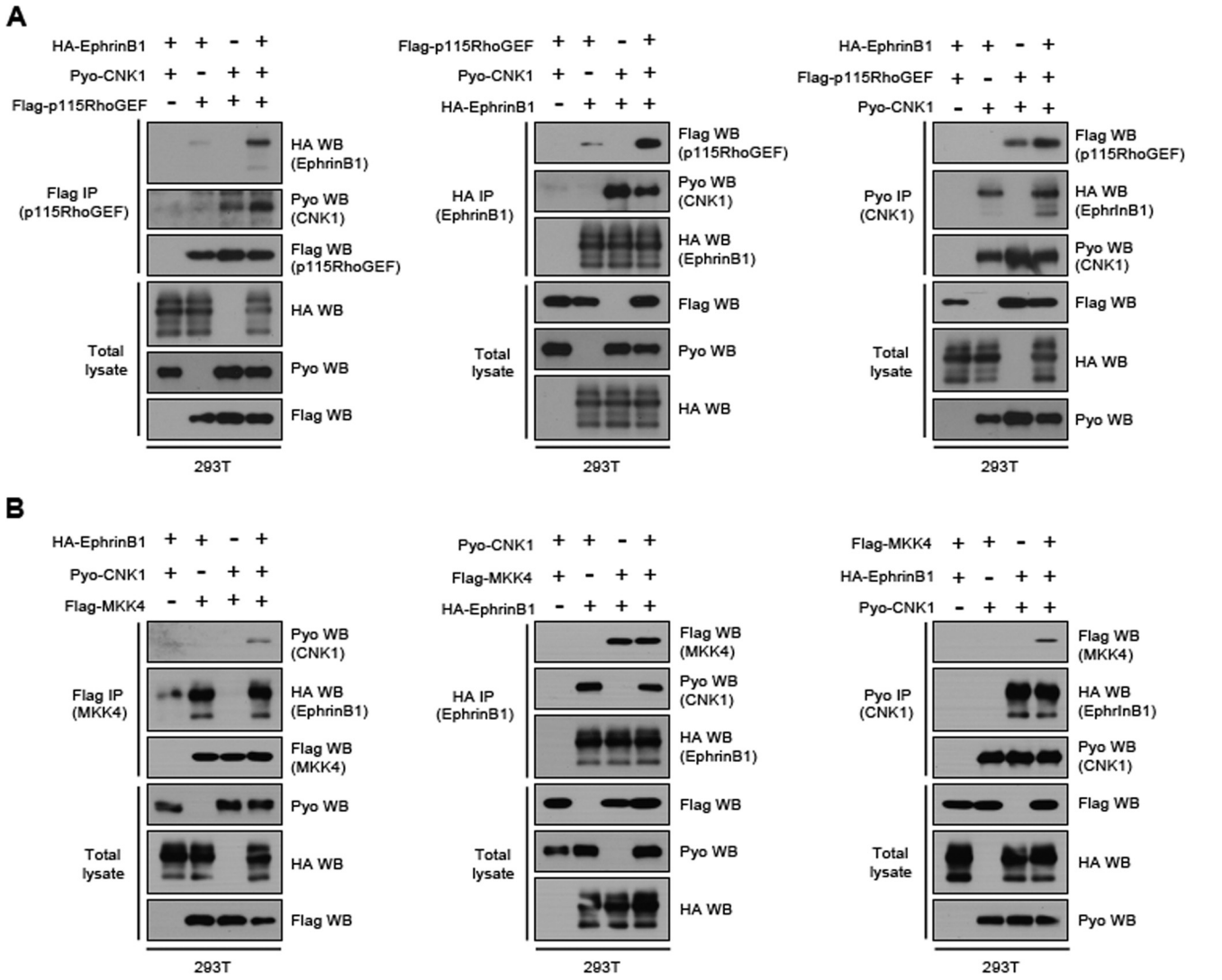

FIGURE 4. **EphrinB1 and CNK1 link components of JNK and RhoA signaling pathway.** *A*, HEK 293T cells were co-transfected with HA-ephrinB1, Pyo-CNK1 or Flag-p115RhoGEF as indicated. Cell lysates were immunoprecipitated with Flag (*left panel*), HA (*middle panel*), or Pyo (*right panel*) antibodies. Immunoprecipitates and total lysates were immunoblotted with Flag, HA, or Pyo antibodies. *B*, HEK 293T cells were co-transfected with HA-ephrinB1, Pyo-CNK1, or Flag-MKK4 constructs as indicated. Cell lysates were immunoprecipitated with Flag (*left panel*), HA (*middle panel*), or Pyo (*right panel*) antibodies. Immunoprecipitates and total lysates were immunoblotted with Flag, HA, or Pyo antibodies.

the endogenous CNK1, and Western analysis (Fig. 6*A*). In the presence of activated Src, more WT ephrinB1 was found in the CNK1 immune-complexes, and the same was true for the Y6F ephrinB1 mutant (Fig. 6*A*). These data indicate that tyrosine phosphorylation of ephrinB1 by Src does not affect its ability to interact with CNK1.

Since tyrosine phosphorylation of ephrinB1 was not responsible for modulating its interaction with CNK1, we examined whether the endogenous CNK1 is phosphorylated on tyrosine in the presence of an activated form of Src. CNK1 was immunoprecipitated and immunoblotted using anti-phosphotyrosine antibodies. CNK1 was clearly tyrosine phosphorylated in the HeLa cells expressing constitutively active Src (Fig. 6*B*). To test whether Src can directly phos-phorylate CNK1, we performed an *in vitro* kinase assay with purified Src and bacterially expressed and purified CNK1. Western analysis indicated that Src did phosphorylate CNK1 on tyrosine, but not the control of bacterially expressed and purified extracellular domain of ephrinB1 (Fig. 6*C*). Because there are putative Src phosphorylation sites within CNK1, we substituted phenylalanine for tyrosine at the three predicted Src phosphorylation sites in CNK1, which include Tyr-465, Tyr-519, and Tyr-665. Pyo-tagged versions of WT CNK1 and the three mutants (Y465F, Y519F, and Y665F) were co-expressed with HA-tagged ephrinB1 in the presence or absence of the active Y529F Src protein. CNK1 was immunoprecipitated and Western analysis was performed to detect HA-tagged ephrinB1 (Fig. 6*D*). EphrinB1 was prominently found in the immune-complexes of WT, Y465F, and Y519F versions of CNK1, but much less was detected in the Y665F mutant complexes (Fig. 6*D*). We also tested whether the Y665F mutation disrupts tyrosine phosphorylation of CNK1, and thus prevents association with ephrinB1. We co-expressed WT CNK1 or the Y665F mutant of CNK1 along with activated Src in HeLa cells. CNK1 was immunoprecipitated from lysates and immunoblotted with anti-phosphotyrosine antibodies. WT CNK1 was tyrosine phosphorylated when the constitu-

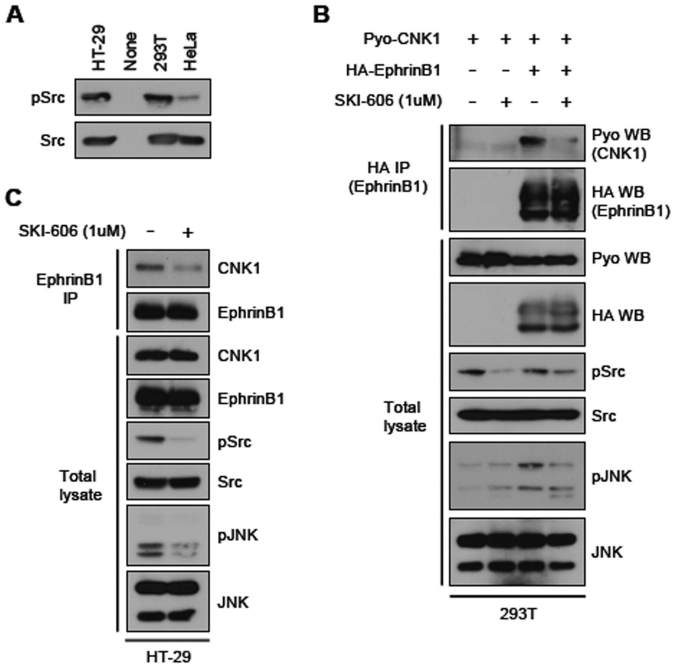

FIGURE 5. **Src regulates the interaction between ephrinB1 and CNK1.** *A*, Western blot analysis of the status of basal Src phosphorylation in human cell lines. *B*, HEK 293T cells were transfected with HA-ephrinB1 and Pyo-CNK1 as indicated. 30 h after transfection, cells were treated with 1 μM Src inhibitor (SKI-606) for 18 h. Proteins were immunoprecipitated with HA antibody and then immunoblotted with indicated antibodies. Total lysates were immunoblotted with the indicated antibodies. *C*, HT-29 cells were treated with 1 μM Src inhibitor for 18 h. Cell lysates were immunoprecipitated with ephrinB1 antibody. Immunoprecipitates and total lysates were immunoblotted with indicated antibodies.

tively active Src was present, but the Y665F mutant was not (Fig. 6*E*). These data indicate that phosphorylation of tyrosine 665 in CNK1 may be responsible for an enhanced interaction between CNK1 and ephrinB1.

One prediction from this model is that the Y665F mutant of CNK1 would be less efficient at signaling the activation of JNK downstream of the CNK1/ephrinB1 interaction. Thus, we co-expressed the phosphorylation site mutants of CNK1 with ephrinB1 in HEK 293T cells that display substantial amounts of active Src. Western analysis of lysates showed that WT, Y456F, and the Y519F mutants are able to induce the phosphorylation of JNK that is associated with its activation (Fig. 6*F*). However, the Y665F mutant failed to activate JNK (Fig. 6*F*), indicating that this phosphorylation site is key to effectively transmitting the signal downstream of the CNK1/ephrinB1 interaction.

Adhesion to Fibronectin Promotes EphrinB1/CNK1 Binding and JNK Activation—Cell adhesion to fibronectin is known to activate Src and JNK (43). Therefore, we tested whether the time-dependent adhesion to fibronectin of HeLa cells expressing HA-tagged ephrinB1 and Pyo-tagged CNK1 affected the interaction between CNK1 and ephrinB1. Lysates from HeLa cells expressing both proteins were obtained over a 90 min window and IPs and Western analysis were performed. In ephrinB1 immune-complexes, the detectable amount of CNK1 increased with time and the same was true of CNK1 immune-complexes (Fig. 7*A*). As expected, Western analysis of the lysates also shows an increase in phospho-Src detected during this time. Next, we tested whether the phosphorylation of tyrosine 665 within CNK1 is required for the increase of ephrinB1/CNK1 interaction resulting from adhesion to fibronectin. HeLa cells were co-transfected with Pyo-tagged WT CNK1 or the Y665 mutant with HA-tagged ephrinB1. Cells were replated on fibronectin-coated plates for 90 min. IPs and Western analysis showed that adhesion to fibronectin induced the phosphorylation of WT CNK1 as well as ephrinB1/WT CNK1 binding, but not the phosphorylation of the Y665F mutant, nor ephrinB1/Y665F CNK1 binding in immune-complexes (Fig. 7*B*). To further test whether a CNK1/ephrinB1 interaction is necessary for JNK activity in response to adherence to fibronectin, we used HeLa cells, which normally do not express detectable ephrinB1, but do express CNK1. We generated HeLa cell lines stably expressing an empty vector or ephrinB1, and examined whether adhesion to fibronectin induced JNK activation over a 120-min period. Western analysis was performed on lysates from the stably transformed HeLa cells using phospho-JNK as an indicator of JNK activation (Fig. 7*C*). Within 15 min of contacting fibronectin-coated plates, a robust induction of JNK activation is observed in the ephrinB1-expressing HeLa cells (Fig. 7*C*). In contrast, little JNK activation is induced in the vector-expressing cells (Fig. 7*C*). Thus, we examined whether knockdown of the endogenous CNK1 affected phospho-JNK levels in these two cell lines. The two cell lines were plated on fibronectin for 90 min, and lysates were prepared and examined by direct Western analysis. In the case of HeLa cells stably expressing ephrinB1, control siRNA-containing cells show a robust induction of phospho-JNK and phospho-MKK4 that is associated with activation. In contrast, knockdown of CNK1 results in the inhibition of the ephrinB1-mediated increase in MKK4 and JNK activation (Fig. 7*D*). As expected in the vector control cells, regardless of whether control siRNA or siRNA targeting CNK1 was present, phospho-JNK and phospho-MKK4 levels were unaffected by adherence to fibronectin. These data indicate that adhesion to fibronectin promotes ephrinB1/CNK1 interactions, which are necessary for the activation of the ephrinB1-associated MKK4, which in turn leads to JNK activation.

CNK1, RhoA, and JNK-dependent Signaling Are Required for EphrinB1-mediated Cell Migration—Because we established that adhesion of ephrinB1-expressing HeLa cells promoted JNK activation via an ephrinB1/CNK1 interaction, we tested whether the migration of these cells was also affected. HeLa cells stably transfected with empty vector or ephrinB1 were plated and a wound healing assay was performed in the presence of either control siRNA or two CNK1 siRNAs. Within 18 h, HeLa cells expressing ephrinB1 efficiently migrated into the wound created by the assay, when compared with the HeLa cells bearing the empty vector (Fig. 8, *A* and *C*). However, when the ephrinB1 expressors were also treated with CNK1 siRNAs, there was a substantial inhibition of cell migration into the wound area (Fig. 8*A*). To confirm that this inhibition of migration was specific to the knockdown of CNK1, a siRNA-resistant CNK1 construct was co-expressed with the siRNAs against CNK1 (Fig. 8*B*). The siRNA resistant construct partially, but substantially, rescues migration (Fig. 8*B*). Next, we confirmed that knockdown of CNK1 affects cell migration using the transwell migration assay. HeLa cells expressing ephrinB1 show

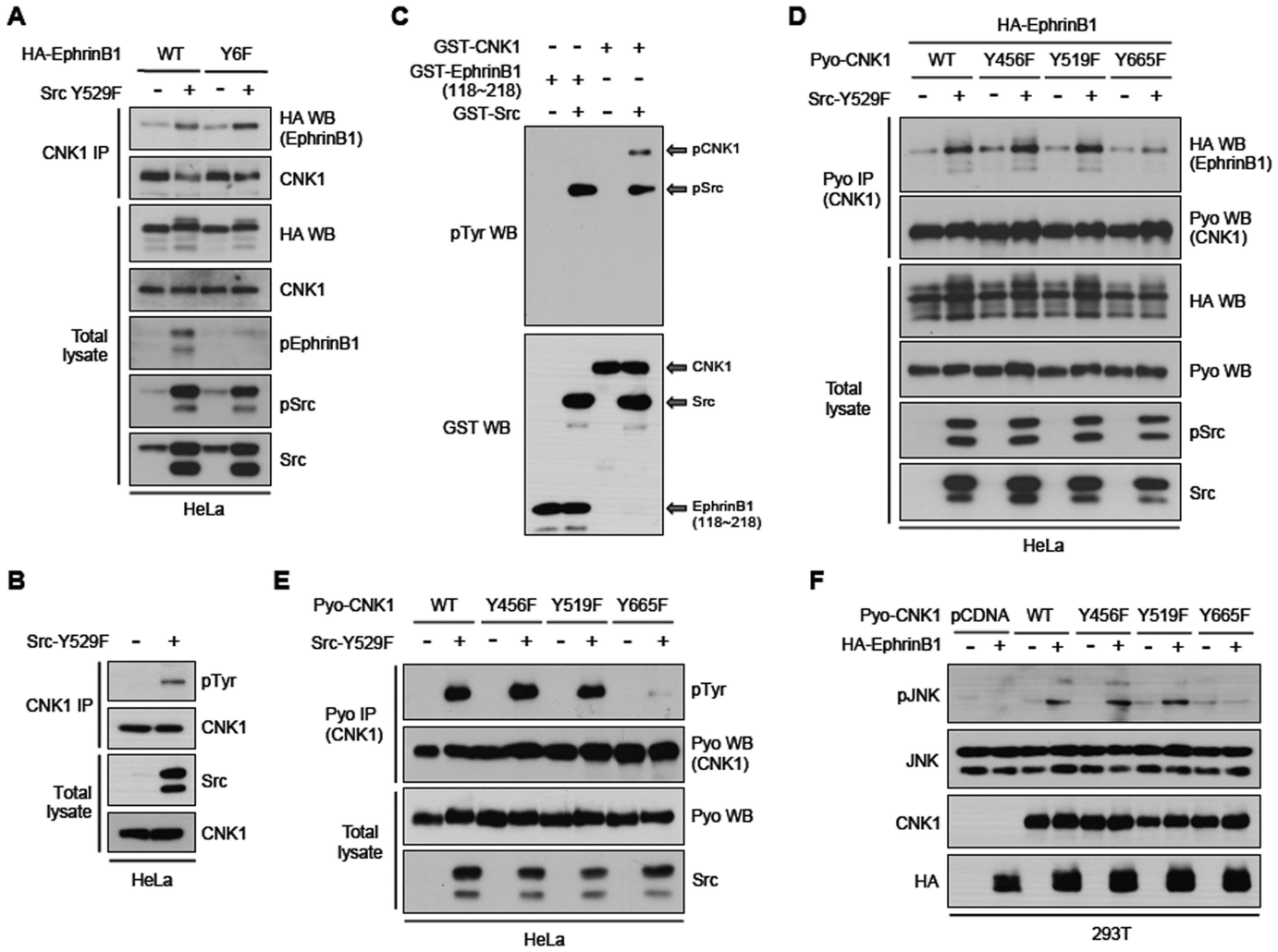

FIGURE 6. **Tyr-665 of CNK1 contributes to the Src-induced ephrinB1/CNK1 binding.** *A*, HA-tagged WT or Y6F mutant of ephrinB1 was co-transfected into HeLa cells with Src Y529F as indicated. Cell lysates were immunoprecipitated with CNK1 antibody and then immunoblotted with indicated antibodies. Total lysates were also examined by direct Western analysis. *B*, HeLa cells were transfected with Src Y529F. Cell lysates were immunoprecipitated with CNK1 antibody. Immunoprecipitates and total lysates were immunoblotted with indicated antibodies. *C*, purified GST-CNK1 or GST-EphrinB1 partial protein (negative control) were incubated with or without GST-Src in kinase buffer containing 50 μM ATP for 20 min at 30 °C. Samples are immunoblotted with phosphotyrosine or GST antibodies. *D*, pyo-tagged WT or substitution mutants (Y456F, Y519F, or Y665F) of CNK1 were co-transfected into HeLa cells with HA-tagged ephrinB1 and/or Src as indicated. Cell lysates were immunoprecipitated with Pyo antibody. Immunoprecipitates and total lysates were immunoblotted with the indicated antibodies. *E*, WT CNK1 or the substitution mutants of CNK1 was cotransfected along with activated Src in HeLa cells. CNK1 was immunoprecipitated from lysates and immunoblotted with anti-phosphotyrosine antibodies. *F*, WT CNK1 or the substitution mutants of CNK1 were co-transfected into HEK 293T cells with HA-EphrinB1. Western blot analysis was performed with the indicated antibodies.

increased migration when compared with vector control cells. However, the enhanced migration of this cell line was considerably reduced by the introduction of CNK1 siRNA (Fig. 8*C*). These results are consistent with the requirement for an ephrinB1/CNK1 interaction for RhoA-mediated JNK activation and cell migration.

To test whether JNK activation and Rho-mediated Rho kinase activation were necessary for the migration of these cells, the HeLa cells stably expressing ephrinB1 were subjected to a wound healing assay in the presence of JNK or Rho kinase inhibitors. Both the JNK and Rho kinase inhibitors markedly prevented phosphorylation of JNK (Fig. 8*D*), and cell migration into the wound area, as judged by measuring the area of the wound (gap) region after 18 h (Fig. 8*E*). Collectively, these data show that CNK1 mediates ephrinB1-induced cell migration through a RhoA and JNK-dependent mechanism (Fig. 9).

DISCUSSION

The Eph/Ephrin system plays a pivotal role in various biological processes such as cell adhesion and migration. In addition to the ability of the transmembrane ephrinBs to transduce forward signals through their cognate EphB receptors, ephrinBs also act as receptor-like molecules and can transduce reverse signals into the ephrinB-expressing cells in a tyrosine phosphorylation-dependent, PDZ-binding dependent and -independent manner. Several years ago, it was reported that ephrinB1 reverse signaling activates the JNK pathway (29, 30). Although it was shown that the intracellular domain of ephrinB1 was important for JNK activation and cell migration, the mechanism that connects ephrinB1 to JNK activation and cell migration was unclear. In the current report, we show that CNK1 mediates ephrinB1-induced JNK activation. First, we show that

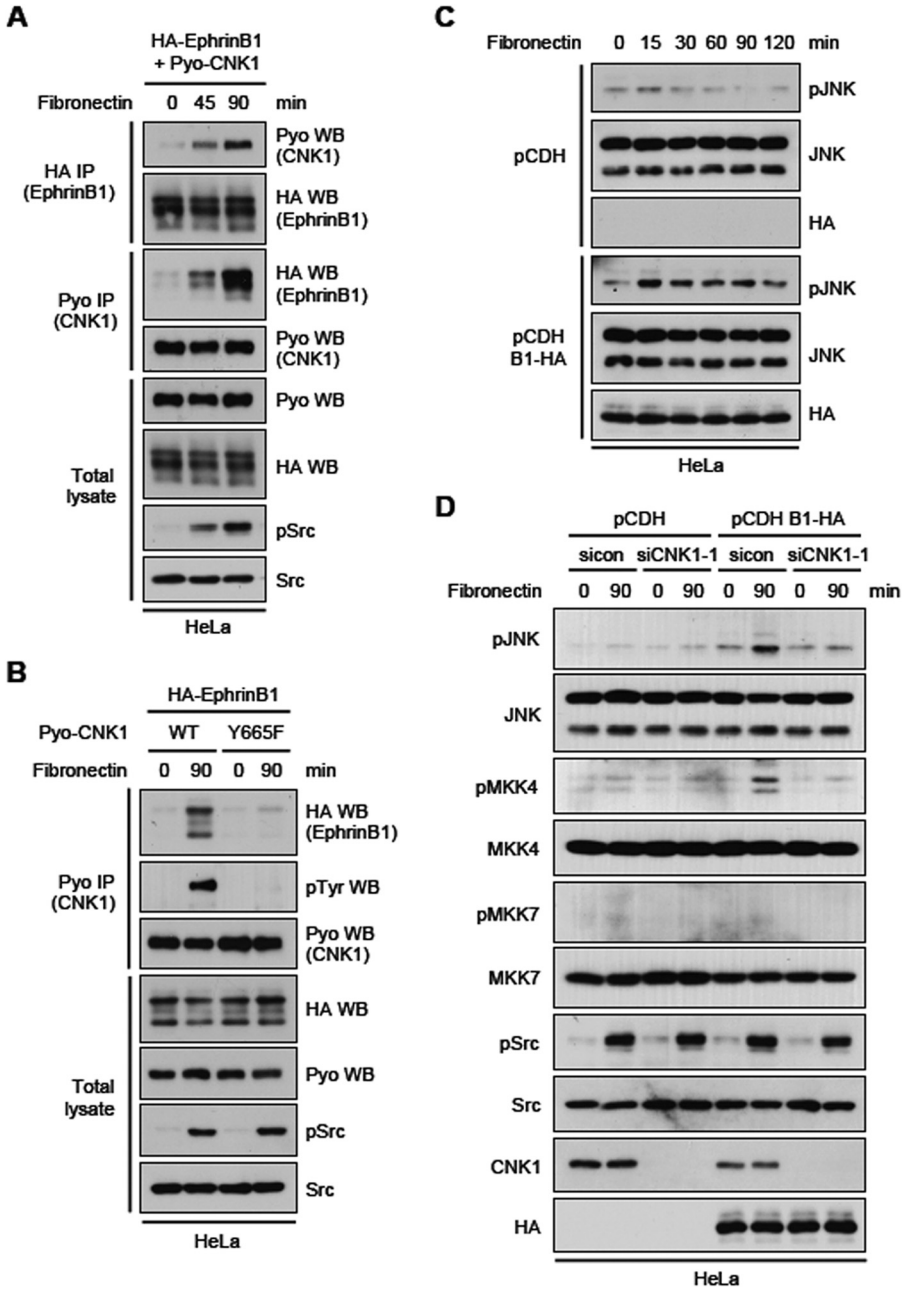

FIGURE 7. **Adhesion to fibronectin promotes ephrinB1/CNK1 binding and JNK activation.** *A*, HA-ephrinB1 was cotransfected with Pyo-CNK1 constructs in HeLa cells. *B*, construct for HA-tagged ephrinB1 was cotransfected with a Pyo-tagged WT or a Y665F mutant construct of CNK1 in HeLa cells (*A* and *B*). 30 h after transfection, cells were starved in serum-free media for 16 h. Cells were harvested, held in suspension in DMEM containing 0.5% BSA for 2 h, and plated onto fibronectin-coated dishes for the indicated times. Cell lysates were immunoprecipitated with HA or Pyo antibodies. Immunoprecipitates and total lysates were subjected to Western analysis with the indicated antibodies. *C*, HeLa-derived cell lines stably transfected with an empty (pCDH) or HA-ephrinB1-expressing vector (pCDH B1-HA) were serum-starved for 16 h and then replated onto FN-coated dishes for the indicated times. Cell lysates were analyzed by Western blot analysis using pJNK or JNK antibodies. *D*, pCDH or pCDH B1-HA cells were transfected with either control or siRNA against CNK1. 30 h after transfection, cells were serum-starved for 16 h and then replated onto FN-coated dishes for 90 min. Cell lysates were immunoblotted with indicated antibodies.

overexpression of WT ephrinB1 and WT CNK1 in HEK 293T cells increases JNK activation. However, overexpression of an ephrinB1 mutant lacking the C-terminal four amino acids of the PDZ-binding motif (Δ4 ephrinB1) in combination with WT CNK1 fails to increase JNK activation (Fig. 3*A*). Reciprocally, WT ephrinB1 expressed in combination with a CNK1 mutant harboring a deletion of the CRIC domain (ΔCRIC CNK1) also fails to increase JNK activation (Fig. 3*A*). Second, CNK1 binds to p115 RhoGEF, linking it to ephrinB1 (Fig. 4*A*; (Ref. 36). Moreover, ephrinB1 binds to an upstream activating kinase of JNK, MKK4, thus completing the connection to CNK1 and JNK activation (Fig. 4*B*). Third, a pharmacological inhibitor of Src suppresses JNK activation as well as the ephrinB1/CNK1 association (Fig. 5, *B* and *C*). Finally, adhesion to fibronectin promotes the interaction of ephrinB1 with CNK1. Depletion of endogenous CNK1 abrogates the induction of JNK activation upon fibronectin adhesion in the ephrinB1 overexpressing cells (Fig. 7*D*). Our results support a mechanism where binding of

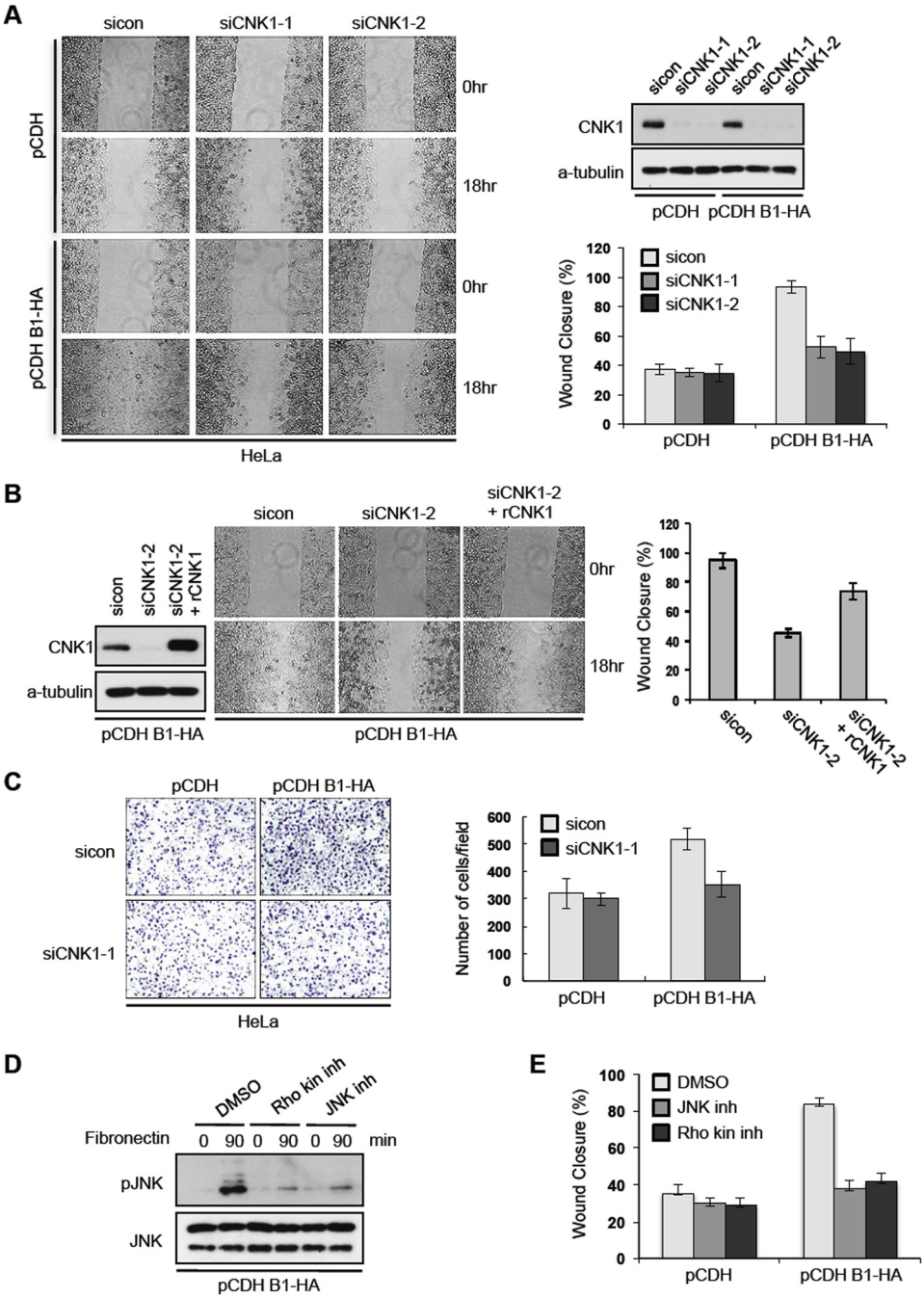

FIGURE 8. **CNK1, RhoA, and JNK-dependent signaling are required for ephrinB1-mediated cell migration.** *A*, pCDH or pCDH B1-HA cells were transfected with control or two CNK1 siRNAs and analyzed for Western analysis using the CNK1 antibody (*upper right*). 30 h after transfection, cells were plated and grown in culture inserts on FN-coated plates until confluent, at which point the culture inserts were removed. Representative images of migrating cells obtained at 0 or 18 h after wound formation (*left*). The migration was quantified by calculating the cell-covered area using Image J software as described under "Experimental Procedures" (*lower right*). *B*, control or CNK1 siRNA were transfected into pCDH B1-HA cells with a siRNA-resistant CNK1 expression vector and analyzed by Western blot using the CNK1 antibody (*left*). Wound closure assays were performed, and representative images of migrating cells obtained at 0 or 18 h after wound formation are displayed (*middle*). The migration was quantified by calculating the cell-covered area using Image J software (*right*). *C*, pCDH or pCDH B1-HA cells were transfected with control or CNK1 siRNA. 30 h after transfection, transwell migration assays were performed as described under "Experimental Procedures." The number of migrated cells in 4 random fields was counted. *D*, pCDH B1-HA cells were treated with DMSO (vehicle control), 10 μM of Rho kinase inhibitor or JNK inhibitor. Cells were replated on FN-coated dishes for indicated times. Cell lysates were immunoblotted with pJNK and JNK antibodies. *E*, pCDH or pCDH B1-HA cells were plated and grown on FN-coated plates with culture inserts. These inserts were removed, and cells were treated with DMSO, 10 μM of Rho kinase inhibitor or JNK inhibitor. Data represent the mean ± S.D. of three individual experiments.

ephrinB1 to CNK1 is important for JNK activation, with both CNK1 and ephrinB1 acting as scaffolds that connect RhoA and JNK signaling (Fig. 9).

We show that the PDZ binding motif of ephrinB1 and the CRIC domain of CNK1 are critical for these molecules to interact (Fig. 2) and lead to JNK activation (Fig. 3*A*). This is a rather unexpected result because CNK1 possesses a PDZ domain, which we expected to be important for the ephrinB1/CNK1 interaction. Interestingly, it was reported that Dishevelled directly interacts with the C-terminal region of ephrinB1,

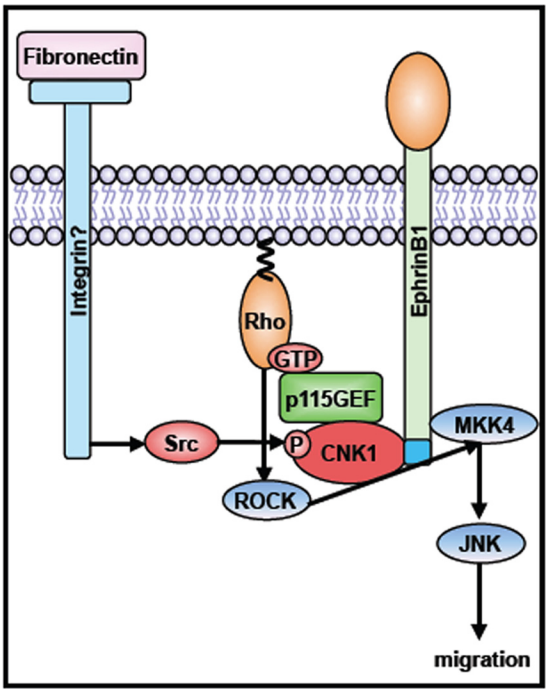

FIGURE 9. **Model for CNK1 function in ephrinB1 signaling.** Adhesion to fibronectin promotes Src activation and the phosphorylation of CNK1. The phosphorylated CNK1 interacts with ephrinB1. The binding of ephrinB1 to CNK1 connects RhoA and p115RhoGEF with ephrinB1-associated MKK4, promoting JNK activation and cell migration.

which was independent of its PDZ domain as well (23, 24). Alternatively, it is formally possible that ephrinB1 and CNK1 may indirectly bind through another, as yet unknown protein that contains a PDZ domain. Several PDZ domain-containing proteins have been reported to interact with the PDZ binding motif of ephrinBs, such as PDZ-RGS3, GRIP, PICK, or syntenin (2, 3). Moreover, we did not observe a direct association between the two proteins in a Co-IP using bacterially expressed purified CNK1 and *in vitro* translated ephrinB1 (data not shown), suggesting that the interaction may be indirect.

We also found that ephrinB1/CNK1 association is regulated by Src activity. The Src inhibitor reduces ephrinB1/CNK1 binding, while overexpression of active Src increases the binding (Figs. 5 and 6). Overexpression of active Src not only increases the association of CNK1 with WT ephrinB1, but also the Y6F mutant of ephrinB1 (Fig. 6*A*). Moreover, stimulation of ephrinB1 with EphB2-Fc increased ephrinB1 tyrosine phosphorylation but did not affect the binding of ephrinB1 to CNK1 (data not shown). Therefore, the tyrosine phosphorylation of ephrinB1 by Src is not responsible for increased interactions between ephrinB1 and CNK1. We provide strong evidence that Src-mediated phosphorylation of the tyrosine residue 665 within CNK1 is responsible for an enhanced association between ephrinB1 and CNK1. Overexpression of active Src increases the association of ephrinB1 with WT CNK1 but not a Y665F mutant of CNK1 (Fig. 6*D*). Moreover, the Y665F mutant did not promote JNK activation (Fig. 6*F*). In addition, adhesion to fibronectin promotes Src activation and the tyrosine phosphorylation of CNK1, resulting in the enhanced ephrinB1/CNK1 binding (Fig. 7, *A* and *B*). This tyrosine phosphorylation of CNK1 may create a binding site for ephrinB1, although this has still to be demonstrated. In agreement with this concept, it has been reported that CNK1 interacts with Src and is phosphorylated by Src, and thereby mediates Raf-1 activation (44).

It has been reported that CNK1 interacts with Net1 and p115RhoGEF, Rho-specific guanine nucleotide exchange factors (36). Expression of p115RhoGEF promotes MKK4/JNK activation and dominant negative RhoA expression suppresses p115RhoGEF-mediated JNK activation (45). In addition, p115RhoGEF regulates RhoA activity upon adhesion to fibronectin. Overexpression of a catalytically inactive mutant of p115RhoGEF inhibits RhoA activity and focal adhesion formation on fibronectin (46). Here, we have shown that the interaction between p115RhoGEF and ephrinB1 is markedly enhanced in the presence of CNK1, suggesting that p115RhoGEF may be involved in ephrinB1/CNK1-mediated JNK activation. Interestingly, we have also found that ephrinB1 binds to MKK4, an upstream kinase of JNK, and CNK1 interacts with MKK4 only in the presence of ephrinB1, suggesting that ephrinB1 links CNK1 to MKK4. Moreover, MKK4 is activated in ephrinB1-expressing HeLa cells upon adhesion to fibronectin, and this activation is inhibited by siRNA-mediated loss of CNK1 (Fig. 7*D*). In agreement with this, dominant-negative MKK4 has been shown to inhibit ephrinB1-induced JNK activation (29). CNK1 binds to MKK7 and mediates serum or sphingosine-1 phosphate-induced JNK activation (36), raising the possibility that MKK7 could be implicated in the ephrinB1/CNK1-mediated JNK activation. However, we have not seen any induction of MKK7 phosphorylation by adhesion to fibronectin in ephrinB1 overexpressing cells (Fig. 7*D*), which may reflect the diversity of MKK4 or MKK7 activation in response to different stimuli (35, 47).

EphrinB reverse signaling regulates cell adhesion and motility. For example, in *Xenopus* embryos, ephrinB1 promotes retinal progenitor movement by interacting with Dishevelled, and the Rho/Rock pathway has been shown to be critical for this movement (23, 24). The ephrinB1 C terminus has been demonstrated to play an important function for cell migration. The intracellular introduction of a synthetic peptide consisting of a fusion of HIV-TAT and the C-terminal 16 amino acids of ephrinB1 blocks ephrinB1-mediated signaling in scirrhous gastric cancer cell migration and dissemination (48). The stimulation of ephrinB1 by EphB1-Fc promotes attachment and migration of endothelial cells, which is dependent on the PDZ binding motif of ephrinB1 (30). However, our finding shows that ephrinB1 overexpression in HeLa cells promotes cell migration even in the absence of Eph receptor binding, and depletion of CNK1 suppresses ephrinB1-dependent cell migration. In agreement with this, it has been reported that ephrinB1 or ephrinB2 overexpression in endothelial cells increases the motility and triggers repeated cycles of actomyosin-dependent cell contractions as well as cell spreading in the absence of an Eph receptor (49). Therefore, the regulation of cell migration by ephrinBs might involve both receptor-dependent and -independent pathways.

Eph/ephrin signaling is implicated in regulating cell adhesion and cell movement during development. EphrinB1 plays a pivotal role in cell migration, invasion, angiogenesis and metastasis of cancer cells. Not surprisingly, the elevated expression of

ephrinB1 has been found in multiple cancers, such as hepatocarcinomas, gastric adenocarcinomas, and osteosarcomas (50–52). The present study has revealed CNK1 as a mediator for ephrinB1 signaling that promotes cell migration through RhoA and JNK activity. Our findings have raised the possibility that they may also play a role in various developmental processes and cancer metastasis.

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