

EphA1 interacts with integrin-linked kinase and regulates cell morphology and motility

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Summary

The Eph-ephrin receptor-ligand system is implicated in cell behavior and morphology. EphA1 is the founding member of the Eph receptors, but little is known about its function. Here, we show that activation of EphA1 kinase inhibits cell spreading and migration in a RhoA-ROCK-dependent manner. We also describe a novel interaction between EphA1 and integrin-linked kinase (ILK), a mediator of interactions between integrin and the actin cytoskeleton. The C-terminal sterile α motif (SAM) domain of EphA1 is required and the ankyrin region of ILK is sufficient for the interaction between EphA1 and ILK. The interaction is independent of EphA1 kinase activity but

dependent on stimulation of the EphA1 ligand ephrin-A1. Activation of EphA1 kinase resulted in a decrease of ILK activity. Finally, we demonstrated that expression of a kinase-active form of ILK (S343D) rescued the EphA1-mediated spreading defect, and attenuated RhoA activation. These results suggest that EphA1 regulates cell morphology and motility through the ILK-RhoA-ROCK pathway.

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Introduction

Cell-extracellular-matrix (cell-ECM) interaction is crucially involved in embryonic development and in many physiological and pathological processes, including injury repair, inflammation and metastasis. Integrins mediate cell adhesion to the ECM, and connect to the cytoskeleton and various cytoplasmic signaling proteins. Through these interactions, integrins control cytoskeletal reorganization and transmit signals into cells (Giancotti and Ruoslahti, 1999; Hynes, 1987). Focal adhesions contain integrins and multiple signaling molecules including talin, vinculin, focal adhesion kinase (FAK) and paxillin. Recently, integrin-linked kinase (ILK) has been identified as a binding partner of the β 1-integrin subunit cytoplasmic domain, and a common component of focal adhesions (Hannigan et al., 1996; Nikolopoulos and Turner, 2001). ILK plays an important role in the assembly and functions of the cell-matrix structures. ILK also acts as a regulator of cell shape and motility by connecting integrins to the actin cytoskeleton (Brakebusch and Fassler, 2003).

Eph kinases constitute the largest family of receptor tyrosine kinases, with 16 distinct members. Ligands for the EphA receptor, called ephrin-A, are anchored on the plasma membrane through a glycosylphosphatidylinositol (GPI) linkage (Manning et al., 2002), whereas EphB receptors bind ligands containing transmembrane domains (ephrin-B). Because ephrins are membrane-anchored, the Eph-ephrin interaction takes place upon contact between Eph- and ephrin-presenting cells. Previous studies have shown that, in addition to signaling by Eph kinases (forward signaling), ephrins on the opposing cells can also transmit signals (reverse signaling) to the cells interior when they are engaged with Eph receptors

(Bruckner et al., 1997; Davy et al., 1999; Holland et al., 1996). A well-characterized function of Eph receptors is to mediate cell-contact-induced repulsive guidance of axons and neural crest cells during development, although cell adhesion and attraction mediated by Eph receptors and ephrins have also been described (Fox and Kandpal, 2004; Wilkinson, 2001). In addition to the extensively studied nervous system, Eph receptors and ephrins are widely expressed in endothelial cells and epithelial cells. Much of the evidence considered points to Eph-receptor signaling leading to the regulation of the cytoskeleton and cell motility. EphA and EphB have been reported to suppress or promote integrin activity through interacting with several signaling molecules (Becker et al., 2000; Gu and Park, 2001; Huynh-Do et al., 2002; Vearing et al., 2005; Vindis et al., 2003). For example, EphB1 promotes cell adhesion to fibronectin, through an interaction with Nck or low-molecular-weight protein tyrosine phosphatase (LMW-PTP), in a kinase-dependent fashion (Huynh-Do et al., 2002). EphB2 was also shown to control integrin activity by inducing R-Ras (Zou et al., 1999). FAK may connect EphA2 receptors with integrin (Carter et al., 2002; Miao et al., 2000; Parri et al., 2007). EphA2 activity modulates cell adhesion and spreading in a FAK-dependent fashion. EphA2-mediated cell spreading on adhesive substrates depends not only on FAK, but also on Rho-family GTPases (Deroanne et al., 2003; Miao et al., 2003).

Rho-family GTPases are important regulators of the actin cytoskeleton and are likely to be involved in Eph-receptor regulation of cell morphology and cell motility. The activation of RhoA stimulates actomyosin contractility and stress-fiber formation, whereas the induction of Cdc42 and Rac1 results in the extension of filopodia and lamellipodia, respectively (Kozma et al., 1997). Recently, Eph-receptor activation has been linked to changes in the

activities of certain Rho GTPases via guanine nucleotide exchange factor (GEF) or GTPase-activating proteins (GAPs). EphA4 has been reported to bind ephexin (Shamah et al., 2001) and Vsm-RhoGEF (Ogita et al., 2003), and EphA2 to bind Tiam1 (Tanaka et al., 2004).

EphA1 was the first member of the Eph family to be identified (Hirai et al., 1987) and is expressed mainly in epithelial tissues (Coulthard et al., 2001; Maru et al., 1988). This receptor is implicated in tumorigenicity. Low expression levels of EphA1 in malignant tumors have been reported (Fox and Kandpal, 2004; Hafner et al., 2004). In breast carcinoma cells, downregulated EphA1 expression is associated with invasive behavior of the cells, which is accompanied by dysregulation of other Eph-ephrin family members (Fox and Kandpal, 2004). However, little is known about the function of EphA1 in cell motility or morphology. We report here the function of EphA1 in cell behaviors and a novel interaction between EphA1 and ILK in the regulation of cell spreading.

Results

EphA1 binds ILK

To gain insights into EphA1 functions, we performed yeast two-hybrid screening to look for EphA1 binding proteins. The bait construct consisted of the intracellular region (amino acids 570–976) of EphA1 fused to the DNA-binding domain of GAL4. We obtained 36 independent clones upon screening a human placenta cDNA library. One of the clones encoded the ankyrin repeat of ILK.

By constructing a series of mutants, we tried to identify domains within both EphA1 and ILK that mediate the interaction between the two (Fig. 1A). The cytoplasmic region of EphA1 (CP) with a kinase-inactivating mutation (CP/KD) and CP lacking the juxtamembrane domain (CP/ΔJM) bound the full-length ILK (ILK) as efficiently as the wild type (CP), whereas CP devoid of the sterile α motif (SAM) domain in the C-terminal tail (CP/ΔSAM) was incapable of interacting with ILK (Fig. 1B). However, the SAM domain alone (SAM) was not sufficient to accomplish the full binding and dramatically reduced colony formation was observed. The β-galactosidase liquid assay was performed to quantify the binding capacity. The activity of CP/ILK was 80% of that of an established positive control (p22/p47 in the Nox2 complex) (Sumimoto et al., 1996) (supplementary material Fig. S1). Deletion of the SAM domain abolished the binding down to the baseline level of a negative control. We could hardly detect statistically significant binding between the SAM domain alone and ILK.

ILK comprises four N-terminal ankyrin repeats, the C-terminal kinase domain and the central pleckstrin homology (PH)-like domain. We split the whole ILK into the ankyrin (ANK) and kinase (CAT) domains. The ANK domain was necessary and sufficient to bind EphA1CP. However, the SAM domain was not required for interaction with ANK, suggesting that ANK is capable of binding regions within CP other than the SAM domain. To further determine

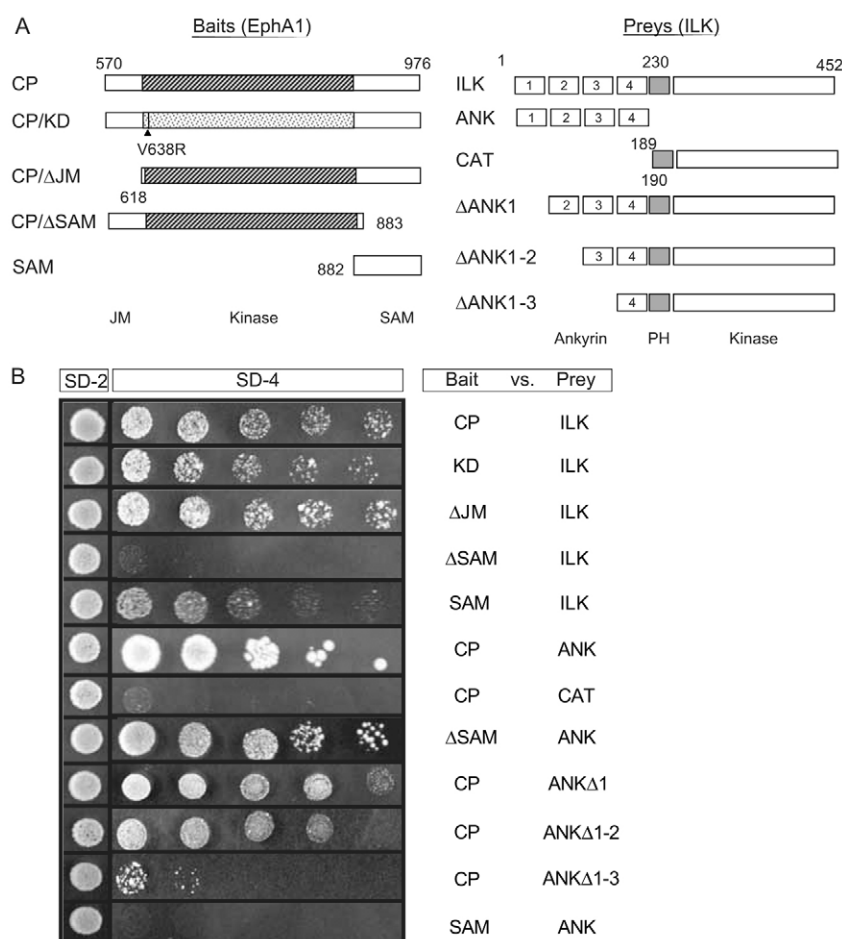


Fig. 1. Yeast two-hybrid analysis for the interaction between EphA1 and ILK. (A) Schematic representation of the EphA1 and ILK constructs used in the yeast two-hybrid analysis. Bait constructs were the GAL4 DNA-binding domain fused to: the cytoplasmic region of EphA1 (CP), kinase-dead EphA1 (CP/KD), juxtamembrane-region deletion EphA1 (CP/ΔJM), EphA1 with the C-terminal sterile α motif (SAM) deletion (CP/ΔSAM) and the SAM domain alone (SAM). Preys were the GAL4-activation domain fused to: full-length ILK (ILK), the N-terminal ankyrin-repeats (ANK), the C-terminal PH- and kinase domain (CAT), ILK with the ankyrin1 deletion (ΔANK1), ILK with the ankyrin1-2 deletion (ΔANK1-2) and ILK with the ankyrin1-3 deletion (ΔANK1-3). (B) Interactions between EphA1 and ILK in the yeast two-hybrid analysis. The left panel shows growth of transformed yeasts in synthetic dropout medium (SD-2; –Leu, –Trp). The middle panel indicates interactions between various EphA1 and ILK constructs (shown in the right panel) in SD-4 (–Leu, –Trp, –His, –Ade) in a series of dilution (dilution increases from left to right). Representative colonies from five independent experiments are shown.

which ANK domain of ILK was important for the interaction with EphA1, we generated the ILK mutants lacking each of the ANK repeats (ΔANK1, ΔANK1-2, ΔANK1-3). The interaction was drastically reduced in ΔANK1-3 (Fig. 1B) cells. SAM failed to bind ANK. Collectively, both SAM in CP and ANK3 in ILK are required for CP and ILK to bind each other. ANK is sufficient to bind CP but SAM is not sufficient to bind ILK and presumably plays an ancillary role. ANK alone acquired a higher binding ability than the whole ILK towards CP even without SAM in this particular assay.

To test the possibility that ILK and EphA1 interact in mammalian cells, we transfected HEK293 cells stably expressing GFP-tagged EphA1 (EphA1-GFP) or ΔSAM EphA1-GFP with myc-tagged ILK (ILK-myc) (WT, wild type; KD, kinase dead), and immunoprecipitated with anti-myc antibody before and after

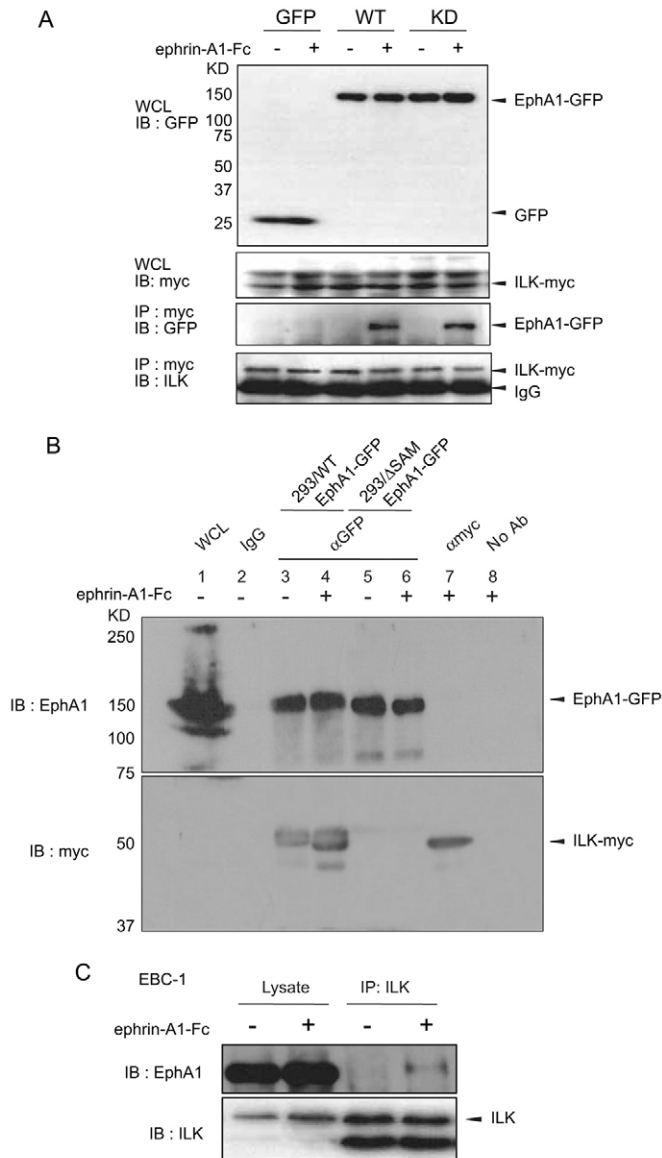


Fig. 2. Interaction between EphA1 and ILK in mammalian cells. (A) Cells expressing GFP, WT (wild-type)-EphA1-GFP or KD (kinase dead)-EphA1-GFP were transfected with ILK-myc. 24 hours after transfection, cells were serum-starved for 12 hours and stimulated with ephrin-A1-Fc (1 μ g/ml) for 10 minutes. Cell lysates were immunoprecipitated (IP) with anti-myc antibody followed by immunoblot (IB) analysis with the indicated antibodies. (B) HEK293 cells expressing WT-EphA1-GFP or Δ SAM-EphA1-GFP were transfected with ILK-myc and treated as in A. Whole cell lysate (WCL; lane 1) and immunoprecipitates by control IgG (lane 2), anti(α)-GFP (lanes 3-6), anti-myc (lane 7) and no antibody (Ab) (lane 8) were subjected to anti-EphA1 and anti-ILK immunoblotting. (C) EBC-1 cells were serum-starved overnight and stimulated with ephrin-A1-Fc (1 μ g/ml) for 10 minutes. Cell lysates were immunoprecipitated with anti-ILK antibody followed by immunoblot analysis with anti-EphA1 and anti-ILK antibody.

stimulation by a chimeric protein comprising ephrin-A1 fused to the immunoglobulin Fc region ephrin-A1-Fc. As shown in Fig. 2A, EphA1-GFP was detected in anti-myc immunoprecipitates in an ephrin-A1-Fc-dependent but EphA1-kinase-activity-independent manner. This is consistent with the yeast two-hybrid results shown in Fig. 1. To eliminate the possibility that the presence of EphA1-

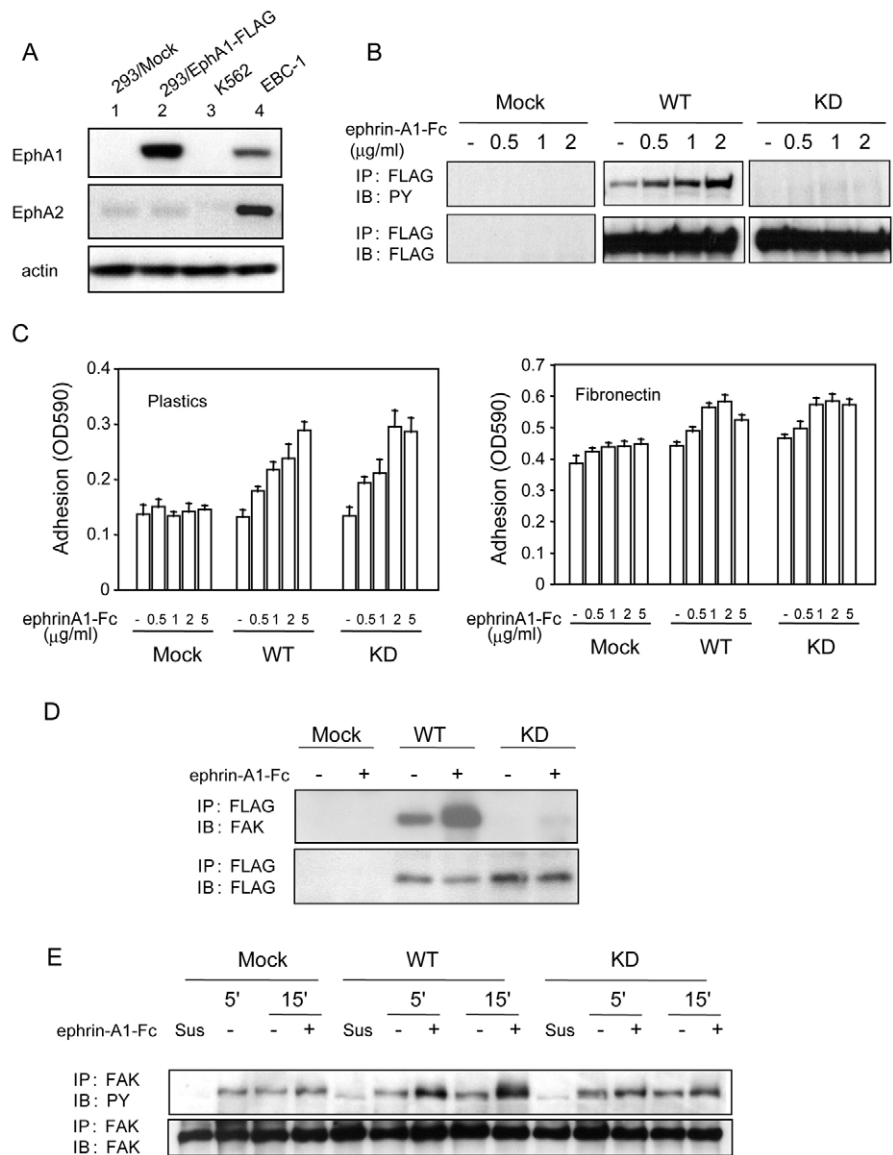
GFP in the anti-myc immunoprecipitates might be artificially mediated by protein A/G Sepharose-bound ephrin-A1-Fc in the protein lysates, the immunoprecipitation was performed with anti-GFP antibody followed by anti-ILK immunoblotting. We found that EphA1-GFP, but not Δ SAM-EphA1-GFP, was bound to ILK in an ephrin-A1-Fc-dependent manner (Fig. 2B). The requirement of the SAM domain is consistent with the yeast two-hybrid results shown in Fig. 1B. EphA1-GFP, which was contained in the anti-myc immunoprecipitates after ephrin-A1-Fc stimulation, disappeared when protein A Sepharose alone was purposely added in the absence of anti-myc antibody (Fig. 2B, lane 8). Although ephrin-A1-Fc stimulation at a high concentration of 10 μ g/ml in HEK293 EphA1-GFP cells resulted in ephrin-A1-Fc-mediated pull down of EphA1-GFP when protein lysates were incubated with protein A Sepharose alone, we could repeatedly observe no EphA1-GFP with ephrin-A1-Fc at 1 μ g/ml (supplementary material Fig. S2). Moreover, we could not detect ephrin-A1-Fc in the anti-ILK immunoprecipitates (data not shown). We also tested the interaction between ILK and EphA1, which are endogenously expressed in EBC-1 cells derived from human lung squamous cell carcinoma. As shown in Fig. 2C, binding of EphA1 and ILK was observed when cells were stimulated by ephrin-A1-Fc. These results indicate that ILK interacts with EphA1 receptor dependently on ephrin-A1-Fc but independently of EphA1 kinase activity.

EphA1 expression enhances adhesion to the ECM in a kinase-activity-independent manner

To investigate the functional roles of EphA1 in the regulation of spreading, integrin-mediated cell adhesion and integrin-mediated migration, we used HEK293 cells stably transfected with vector (pCMV-Tag4A) alone (Mock), or with FLAG-tagged WT- or KD-EphA1 receptor as a model system. Although EphA2, EphA4 and EphA6 were detectable in HEK293 cells by reverse transcriptase (RT)-mediated PCR analysis, EphA4, EphA6 and EphA1 were not detected by immunoblotting (data not shown). We compared the EphA1 and EphA2 expression levels between WT-EphA1 and EBC-1 cells, with leukemic K562 cells as a negative control (Fig. 3A). HEK293 WT-EphA1-FLAG cells expressed EphA1 about fivefold over EBC-1 cells, and both HEK293 Mock and HEK293 WT-EphA1-FLAG expressed EphA2 at a level of less than 10% of that observed in EBC-1 cells. Immunoblotting of total cell lysates showed that both WT-EphA1 and KD-EphA1 were stably expressed in HEK293 cells (Fig. 3B). This allowed us to make a direct comparison of biological activities between HEK293 WT- and KD-EphA1-FLAG cells. WT-EphA1-FLAG but not KD-EphA1-FLAG was autophosphorylated in normal culture conditions without ephrin-A1-Fc stimulation. Immunofluorescence analysis revealed cell-surface presentation of WT- and KD-EphA1-FLAG (supplementary material Fig. S3). Ephrin-A1-Fc stimulation resulted in phosphorylation of WT-EphA1-FLAG in a dose-dependent manner, whereas little tyrosine phosphorylation was observed for KD-EphA1-FLAG (Fig. 3B). Unclustered, monomeric ephrin-A1-Fc failed to stimulate EphA1 phosphorylation (data not shown).

To further confirm that WT- and KD-EphA1-FLAG were biologically functional, we performed cell adhesion to immobilized ligands. Both WT- and KD-EphA1-FLAG-transfected cells but not vector controls (Mock) could mediate adhesion to immobilized ephrin-A1-Fc (0.5-5 μ g/ml), further supporting their proper presentation on the cell surface, being capable of binding to the ligand. All three types of cells adhered

Fig. 3. Functional expression of EphA1 in HEK293 cells. (A) The expression vector alone (pCMV-Tag4, Mock) or containing cDNA encoding WT- or KD (V638R)-EphA1-FLAG was stably transfected into HEK293 (293) cells. Expression levels of EphA1 and EphA2 in HEK293 Mock cells (lane 1), HEK293 WT-EphA1-FLAG cells (lane 2), K562 cells (lane 3) and EBC-1 cells (lane 4) are shown. Lysates from the indicated cells were analyzed by anti-EphA1, anti-EphA2 and anti-actin immunoblotting. (B) Mock, WT-EphA1-FLAG (WT) and KD-EphA1-FLAG (KD) cells were serum starved for 12 hours and stimulated with the indicated concentrations of ephrin-A1-Fc for 10 minutes, then lysed. EphA1 receptors were immunoprecipitated (IP) with anti-FLAG antibody and analyzed by immunoblotting (IB) with PY-20 (PY) and M2 (FLAG) antibody. (C) Immobilized ephrin-A1-Fc mediates adhesion of WT- and KD-EphA1-FLAG cells but not Mock cells. Cells were plated onto plastic culture plates or fibronectin (FN)-coated (1 $\mu\text{g}/\text{ml}$) 96-well plates with the indicated dosage of immobilized ephrin-A1-Fc (0–5 $\mu\text{g}/\text{ml}$) for 30 minutes. Adherent cells were fixed and stained with crystal violet. Dyes were extracted and measured at A590. (D) Mock, WT-EphA-FLAG and KD-EphA-FLAG cells were treated as in B (ephrin-A1-Fc at 1 $\mu\text{g}/\text{ml}$). Anti-FLAG immunoprecipitates were subjected to anti-FAK (upper) and anti-FLAG (lower) immunoblotting. (E) Mock, WT-EphA-FLAG and KD-EphA-FLAG cells were replated on FN-coated dishes with or without immobilized ephrin-A1-Fc (1 $\mu\text{g}/\text{ml}$) for 5 or 15 minutes. Then, cells were lysed and immunoprecipitated with anti-FAK antibodies for immunoblot analysis. Phosphorylated and total FAK in suspension (Sus) and in adhered cells were detected using anti-phosphotyrosine (PY) antibody followed by anti-FAK (FAK) antibody.



to fibronectin (FN; 1 $\mu\text{g}/\text{ml}$) equally well (Fig. 3C). Experiments on vitronectin (VN)-coated plates gave similar results (data not shown). Given that there was no difference in the increased cell adhesion in an ephrin-A1-Fc-dependent fashion between plastic and FN plates, the EphA1-ephrin-A1 interaction is sufficient to cause mechanical tethering of cells. With ephrin-A1-Fc at 5 $\mu\text{g}/\text{ml}$, adhesion to FN was slightly decreased in WT-EphA1 cells but not in KD-EphA1 cells (Fig. 3C, right). Because ephrin-A1 has been reported to induce tyrosine phosphorylation of FAK and recruitment of FAK to EphA2, a closely related EphA1 homolog (Parri et al., 2007), we studied the activation of FAK upon ephrin-A1-Fc stimulation in HEK293 Mock, WT-EphA1-FLAG and KD-EphA1-FLAG cells. We found that ephrin-A1-Fc stimulation enhanced FAK phosphorylation and binding to EphA1 (Fig. 3D,E).

Ephrin-A1-stimulated EphA1 inhibits cell spreading and migration

We further studied the effect of EphA1 activation on cell spreading. HEK293 WT-EphA1-FLAG or KD-EphA1-FLAG cells were replated on FN-coated coverslips with or without immobilized ephrin-A1-Fc (1 $\mu\text{g}/\text{ml}$) and allowed to spread for 30 minutes. As shown in Fig. 4A,B, WT-EphA1-FLAG cells spread poorly, and condensed F-actin was seen at the cell periphery by ligand stimulation. EBC-1 cells that naturally express both EphA1 and EphA2 behaved in a similar fashion. By contrast, no spreading defect was observed in Mock or KD-EphA1-FLAG cells, or in cells expressing EphA1 devoid of the SAM domain (ΔSAM).

In HEK293 cells stably expressing EphA1-GFP (Fig. 2A), ephrin-A1-Fc stimulation resulted in endocytic vesicle formation of EphA1-GFP, which further supports the functional expression of this construct (Fig. 4C). An ephrin-A1-Fc-dependent spreading defect was observed also in HEK293 EphA1-GFP cells (Fig. 4D). To examine whether spreading inhibition can be observed not only in HEK293-derived cells but also in other types of cells, we expressed EphA1-GFP in Rat1 fibroblasts, breast cancer cell MCF7 of epithelial origin and endothelial NP31 cells. When all three types

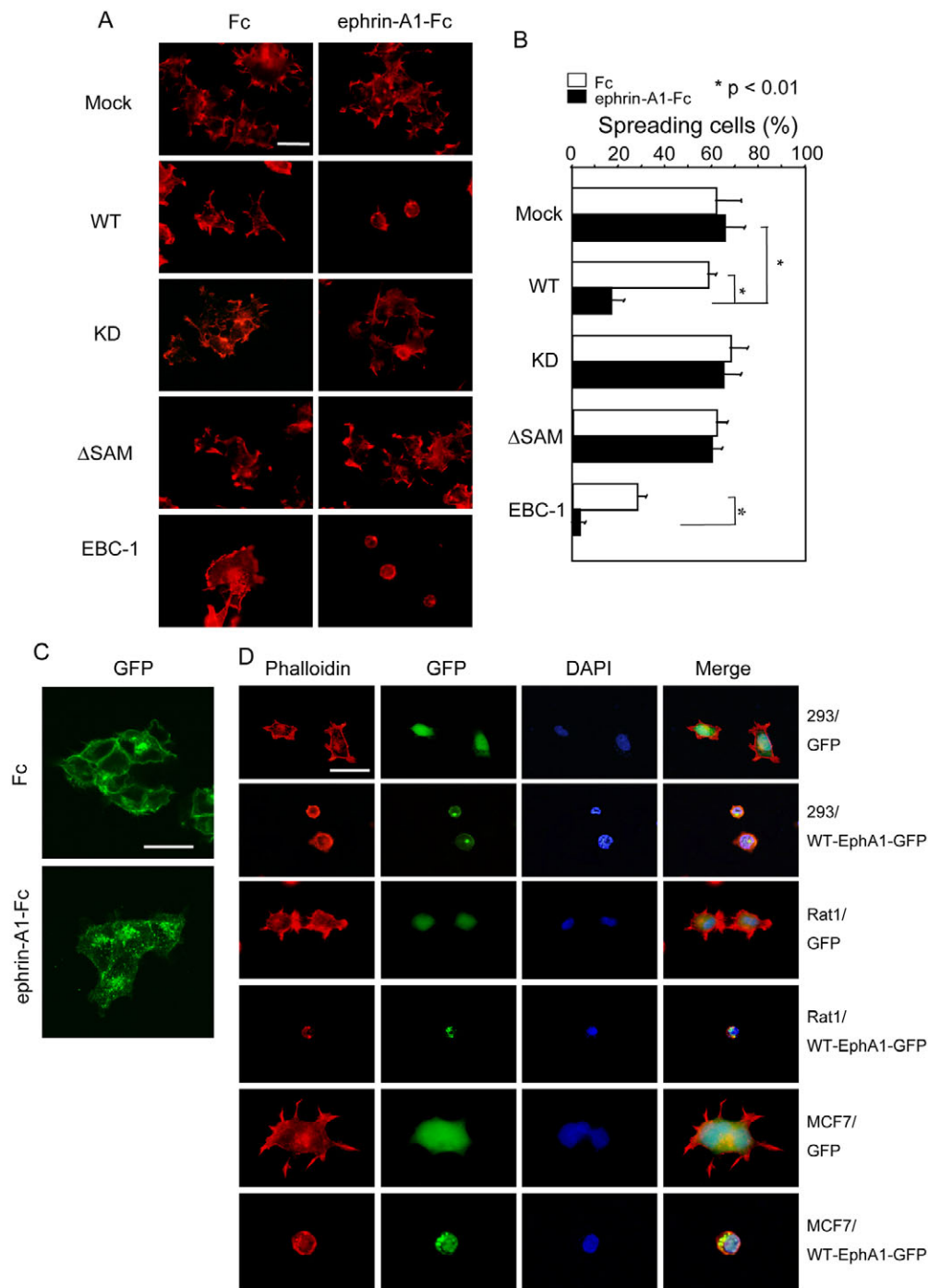


Fig. 4. EphA1 inhibits cell spreading in a kinase-activity-dependent manner. (A) Mock, WT-EphA1-FLAG (WT), KD-EphA1-FLAG (KD) and ΔSAM-EphA1-FLAG (ΔSAM) HEK293 cells (see Figs 1 and 2) and EBC-1 cells were plated onto FN-coated (1 μg/ml) coverslips with (right) or without (left) immobilized ephrin-A1-Fc (1 μg/ml) for 30 minutes and stained with rhodamine-conjugated phalloidin. (B) Quantification of spreading in the cells shown in A. (C) GFP images of HEK293 WT-EphA1-GFP cells stimulated by control Fc or ephrin-A1-Fc. (D) HEK293 (293), Rat1 and MCF7 cells expressing GFP or WT-EphA1-GFP were subjected to spreading assays as in A, and stained by phalloidin and DAPI. Only cells after ephrin-A1-Fc stimulation are shown. Scale bars: 10 μm.

of cells were subjected to spreading assays, inhibition of spreading was observed in cells expressing EphA1-GFP but not GFP alone (Fig. 4D; supplementary material Fig. S4).

To confirm that the observed spreading defect is indeed mediated by EphA1, we used NP31/ATF3-Tet cells, which we reported previously (Masuda et al., 2008). Tetracycline (Tet)-regulated expression of the transcription factor ATF3 induces EphA1 expression. We observed a spreading defect that was dependent on both Tet and ephrin-A1-Fc. We then applied anti-EphA1 small interfering RNA (siRNA) to knock down EphA1 expression. Although we repeatedly observed decreases in the

number of spreading cells after siRNA treatment, the ephrin-A1-Fc-dependent spreading defect was abrogated by anti-EphA1 siRNA (Fig. 5).

EphA1 activates RhoA-ROCK

These observations led us to test whether Rho-family GTPases were involved in EphA1-activation-induced inhibition of cell spreading. To measure Rho-family GTPase activity in spreading cells, cells were re-spread on plates coated with FN and ephrin-A1-Fc or control Fc, and were lysed 20 minutes after replating. Active Cdc42 and Rac1 were pulled down by using GST-PAK-CD, and active

RhoA was pulled down by GST-Rhotekin-RBD (Ren et al., 2000; Ridley et al., 2003). A significant elevation in RhoA activity and a reduction in Rac1 activity were observed in HEK293 WT-EphA1-FLAG cells (Fig. 6A,B). The changes were observed even before ligand stimulation, but were certainly enhanced by it. No significant changes in Cdc42 activity were detected in any of the transfected cells. As shown in Fig. 6C,D, Y-27632, an inhibitor of the Rho

effector ROCK, attenuated the negative effects of ephrin-A1-Fc on the spreading of the WT-EphA1-FLAG cells. By contrast, the ROCK inhibitor did not affect spreading of Mock and KD-EphA1-FLAG cells.

In WT-EphA1-FLAG cells, ephrin-A1-Fc stimulation led to a transient inhibition of cell spreading and elevated RhoA activity, both of which could contribute to the suppression of cell motility. Thus, we performed a cell migration assay to investigate the effect of EphA1 activation on cell migration. Migration of WT-EphA1-expressing cells but not Mock or KD-EphA1 cells was significantly inhibited by ephrin-A1-Fc compared with Fc in a dose-dependent fashion (Fig. 6E, left). To test whether inhibition of migration was attributable to the activation of the RhoA-ROCK pathway, cells were treated with Y-27632. As expected, Y-27632 rescued the inhibitory effect of EphA1 on cell migration. By contrast, Y-27632 did not affect migration of Mock or KD-EphA1-FLAG cells (Fig. 6E, right). Expression of a dominant-negative RhoA (N19) also rescued ephrin-A1-Fc-stimulated inhibition of cell migration by roughly 60% ($P < 0.02$) (data not shown).

EphA1 downregulates ILK activity

The interaction between EphA1 and ILK led us to test whether EphA1 modulates ILK kinase activity. We performed kinase assays of ILK in WT-EphA1-FLAG or KD-EphA1-FLAG cells stimulated with ephrin-A1-Fc. Mock, WT-EphA1-FLAG and KD-EphA1-FLAG cells were replated on FN-coated dishes with or without immobilized ephrin-A1-Fc for 20 minutes. Immunoprecipitated ILK was found to bind both WT-EphA1-FLAG and KD-EphA1-FLAG in an ephrin-A1-Fc-dependent manner (Fig. 7A), which is consistent with what we observed with EphA1-GFP in Fig. 2. It was then subjected to kinase assays using GST-GSK3 β as an exogenous substrate. To demonstrate the specificity of this kinase assay, we pre-treated the cells with wortmannin before they were plated on ephrin-A1-Fc to inhibit PI3-kinase, which is an activator upstream of ILK kinase and downstream of FN stimulation. As shown in Fig. 7B,C, WT-EphA1-FLAG attenuated FN-induced ILK kinase activity by roughly 45% in a ligand-dependent manner, whereas neither Mock nor KD-EphA1-FLAG cells showed significant changes, suggesting that the ILK inhibition is mediated by EphA1 activation. PI3-kinase inhibition by wortmannin at 100 nM completely blocked the ILK kinase activity (Fig. 7B). Additionally, we transfected siRNA (Troussard et al., 2003) that specifically targets ILK to evaluate the function of ILK in HEK293 cell spreading. As shown in Fig. 7D, ILK expression was markedly decreased upon anti-ILK siRNA treatment. Cells transfected with anti-ILK siRNA or control siRNA were replated on FN-coated coverslips and allowed to spread for 30 minutes. As reported previously in other cell types (Filipenko et al., 2005; Fukuda et al., 2003), anti-ILK-siRNA-transfected HEK293 cells showed impaired spreading morphology, whereas control-siRNA-transfected cells spread normally (Fig. 7D). In anti-ILK-siRNA-transfected HEK293 WT-EphA1-FLAG cells at 80 nM, cell migration was inhibited by roughly 55% without ephrin-A1-Fc treatment, as compared with that in control siRNA-transfected cells, and ephrin-A1-Fc treatments failed to enhance the inhibition (data not shown). In HEK293 cells expressing WT-EphA1-FLAG devoid of the SAM domain (the domain that facilitates the interaction with ILK), ephrin-A1-Fc-stimulated inhibition of spreading was abolished (Fig. 4A). ILK kinase activity was not significantly downregulated in vitro with Δ SAM-EphA1-FLAG. Taken together, we suppose that EphA1 activation causes an ILK-mediated spreading defect.

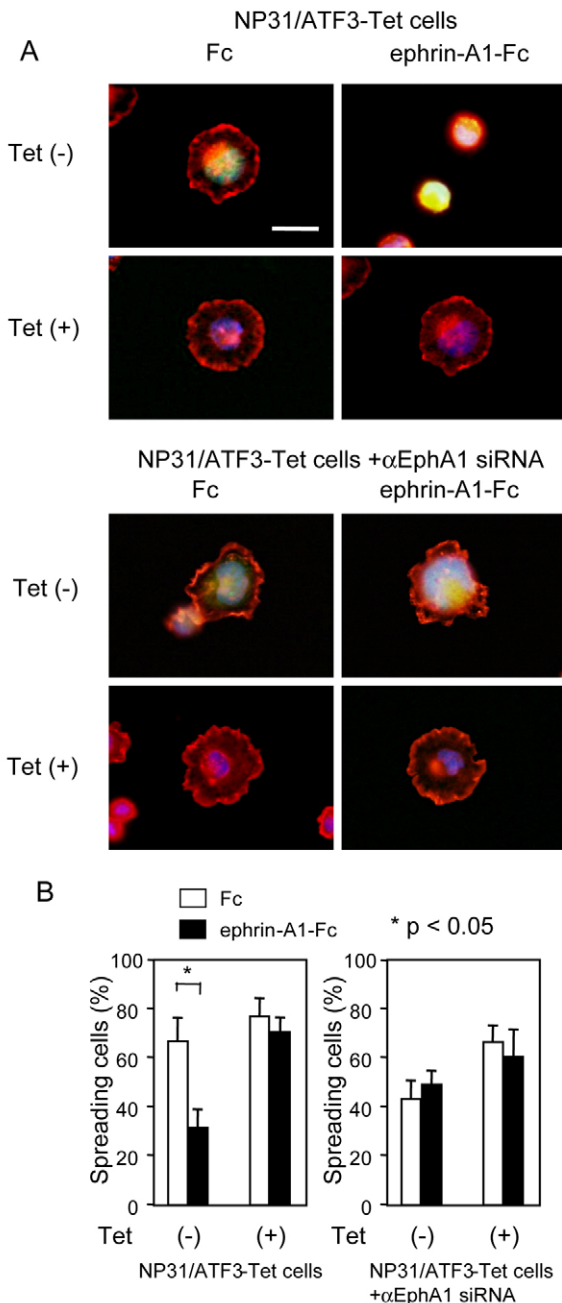


Fig. 5. EphA1 is required for the ephrin-A1-Fc-induced spreading defect in endothelial cells. (A) NP31 ATF3-Tet cells were deprived of tetracycline [Tet (-)] for 12 hours to induce EphA1 expression. Cells stimulated by ephrin-A1-Fc (right panels) with (lower four panels) or without (top four panels) anti(α)-EphA1 siRNAs at 80 nM were analyzed by spreading assays. In the Tet system, GFP is co-induced in cells deprived of tetracycline, which enables recognition of EphA1-expressing cells (green) merged with phalloidin (red) to give yellow color. DAPI is blue. Scale bar: 10 μ m. (B) Quantification of A.

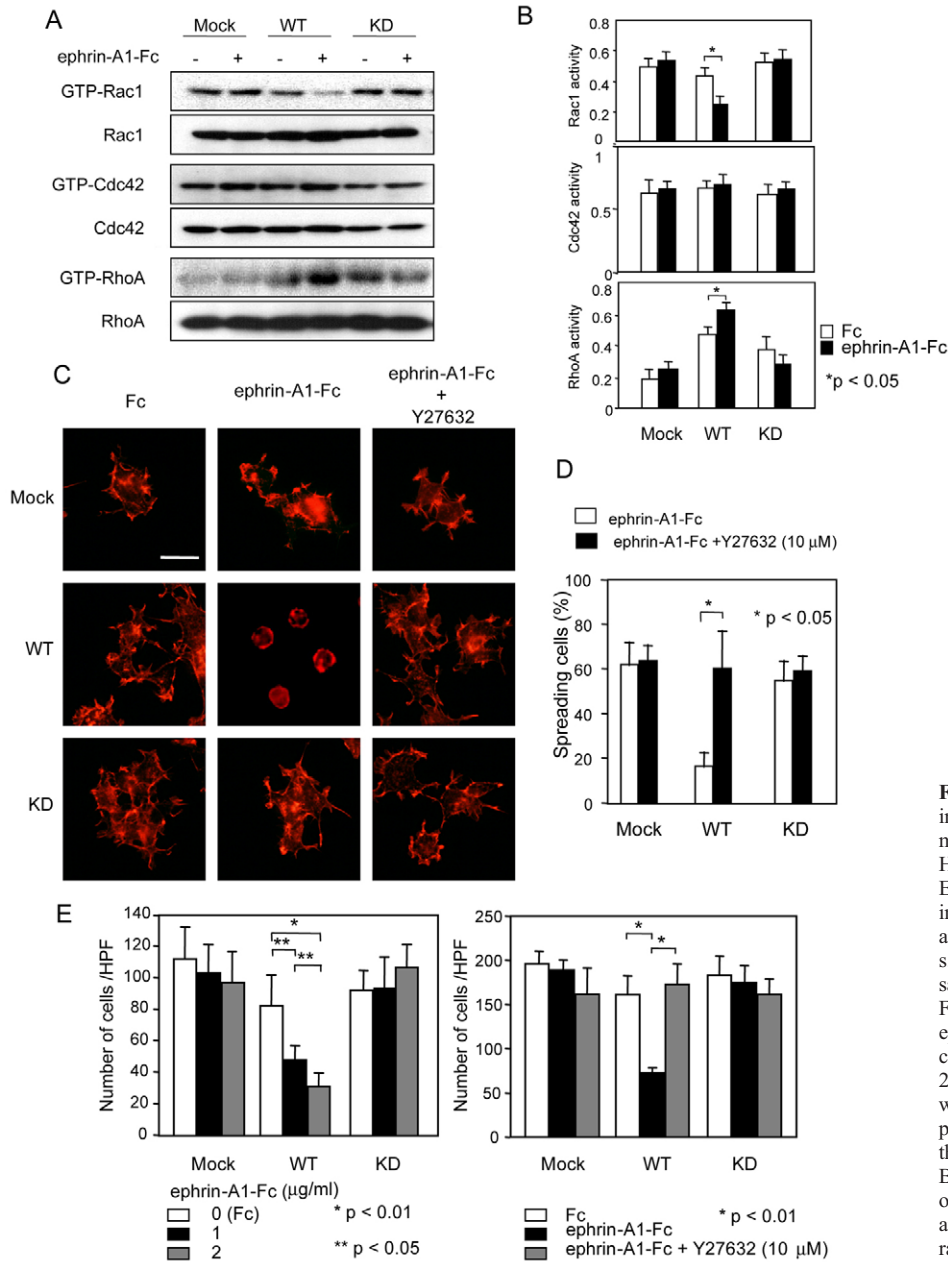


Fig. 6. ROCK inhibitor (Y-27632) attenuates the inhibitory effect of EphA1 on cell spreading and migration. (A) Activities of Rho-family GTPases in HEK293 Mock, WT-EphA1-FLAG (WT) and KD-EphA1-FLAG (KD) cells were measured by immunoblot analysis as described in the Materials and Methods. (B) Quantification of A with means \pm s.d. from three independent experiments. (C) The same set of cells as shown in A were plated onto FN-coated (1 μ g/ml) coverslips with immobilized ephrin-A1-Fc (1 μ g/ml) in serum-reduced medium containing DMSO (vehicle; middle column) or Y-27632 (10 μ M; right column) for 30 minutes. Cells were fixed and stained with rhodamine-labeled phalloidin. Scale bar: 10 μ m. (D) Quantification of the spreading of the cells shown in C. (E) Modified Boyden-chamber migration analysis of the same set of cells was performed as described in the Materials and Methods. Values show means \pm s.d. from nine randomly selected high-power fields.

A kinase-active form of ILK blocks ephrin-A1-induced suppression of cell spreading

When HEK293 WT-EphA1-GFP and Δ SAM-EphA1-GFP cells transfected with WT-ILK-myc were stimulated with ephrin-A1-Fc or control Fc, we observed retraction of processes only in ephrin-A1-Fc-treated HEK293 WT-EphA1-GFP cells (Fig. 8A). Merged images of ILK and EphA1 were observed even before ephrin-A1-Fc stimulation but were certainly enhanced by it. As shown in Fig. 7, EphA1 attenuates FN-induced ILK activity in an ephrin-A1-Fc-dependent manner. Therefore, we investigated the possible dominant active effect of a kinase-active form of ILK (S343D) (Persad et al., 2001) on the spreading of WT-EphA1-GFP cells. The same set of cells was transfected with the myc-tagged WT-ILK or ILK-S343D expression vector as shown in Fig. 8A and was subsequently subjected to spreading assays. Although we found almost no difference in spreading defect between Mock-transfected and WT-

ILK-transfected cells (data not shown), expression of ILK-S343D had an inhibitory effect (Fig. 8B). Δ SAM-EphA1-GFP cells spread well and were not influenced by ILK transfections.

In WT-EphA1-FLAG cells transfected with WT-ILK and ILK-S343D, ephrin-A1-Fc-induced attenuation of ILK activity was recovered and ephrin-A1-Fc-induced RhoA activation was markedly reduced (Fig. 8C). ILK-S343D rescued the ephrin-A1-Fc-induced suppression of cell migration as well (Fig. 8D). Thus, the kinase-active form of ILK acts as a dominant-active molecule to interfere with the interaction between EphA1 and ILK. These results further support the idea that ILK might be situated upstream of RhoA in the ephrin-A1-elicited EphA1-activation pathway.

A dominant-negative form of ILK blocks ephrin-A1-induced suppression of cell spreading

As shown in Fig. 1, ANK is necessary and sufficient to bind EphA1 in a yeast two-hybrid system. We tested it in mammalian cells. Myc-tagged ILK bound EphA1-GFP in an ephrin-A1-Fc-dependent manner (Fig. 2). Even in the absence of ephrin-A1-Fc stimulation, myc-tagged ANK but not ILK or CAT efficiently bound EphA1-FLAG (Fig. 9A), indicating that ANK alone can work as a dominant-negative form. To label transfected cells, ANK was tagged with GFP and introduced to HEK293 EphA1-FLAG cells. As shown in Fig. 9B,C, ANK rescued the ephrin-A1-Fc-induced spreading defect.

Discussion

Here, we demonstrate that ephrin-A1-stimulated EphA1 inhibits cell spreading and migration. Cellular and biochemical analysis revealed that EphA1 blocks ILK in a kinase-dependent manner, which leads to inhibition of Rac1 and stimulation of RhoA activity (Fig. 9D).

Effects of Eph-ephrin signaling on cell adhesion, spreading and migration have been reported in various cell types (Davy and Robbins, 2000; Deroanne et al., 2003; Gu and Park, 2001; Huynh-Do et al., 2002; Miao et al., 2000; Miao et al., 2003; Miao et al., 2005). For example, activation of EphA2 by ephrin-A1 inhibits the Rac1-Pak1 (p21-activated kinase 1) pathway of smooth muscle cells and represses cell spreading (Deroanne et al., 2003). EphA2 also regulates HGF-induced epithelial cell morphogenesis (Miao et al., 2003). In the case of ephrin-A5-induced growth-cone collapse, ROCK is an important mediator (Davy et al., 1999; Davy and Robbins, 2000). In this report, EphA1-ephrin-A1 interaction inhibits cell spreading on but not adhesion to FN. Activation of EphA or EphB receptors was shown to modulate integrin functions (Becker et al., 2000; Gu and Park, 2001; Miao et al., 2000; Miao et al., 2005). Similar to the previous reports (Deroanne et al., 2003; Miao et al., 2003; Miao et al., 2005), inhibition of ROCK by Y-27632 attenuated the negative effects of ephrin-A1 on the spreading of WT-EphA1 cells. It was suggested that activation of Rho and ROCK transmits a negative signal to the Cdc42-Rac pathway (Hirose et al., 1998), confirming the results of Kozma et al. (Kozma et al., 1997). Mutually antagonistic effects of Rac and Rho were also observed in focal-contact and focal-complex formation in Swiss 3T3 cells (Rottner et al., 1999). In the same cell type, it was recently shown that Rac activation antagonized Rho activity (Sander et al., 1999). The decrease in Rac1 activity and elevated RhoA activity are expected to lead to an altered balance between these GTPases, and a relative increase in RhoA activity has been previously linked to inhibition of cell motility (Etienne-Manneville and Hall, 2002; Nobes and Hall, 1999). In our experiments, we also obtained results that point to such an antagonism. Activation of EphA1 by ephrin-A1 induced activation of RhoA and downregulation of Rac1. We currently do not know whether Rac downregulation is downstream of Rho activation. Activation of the

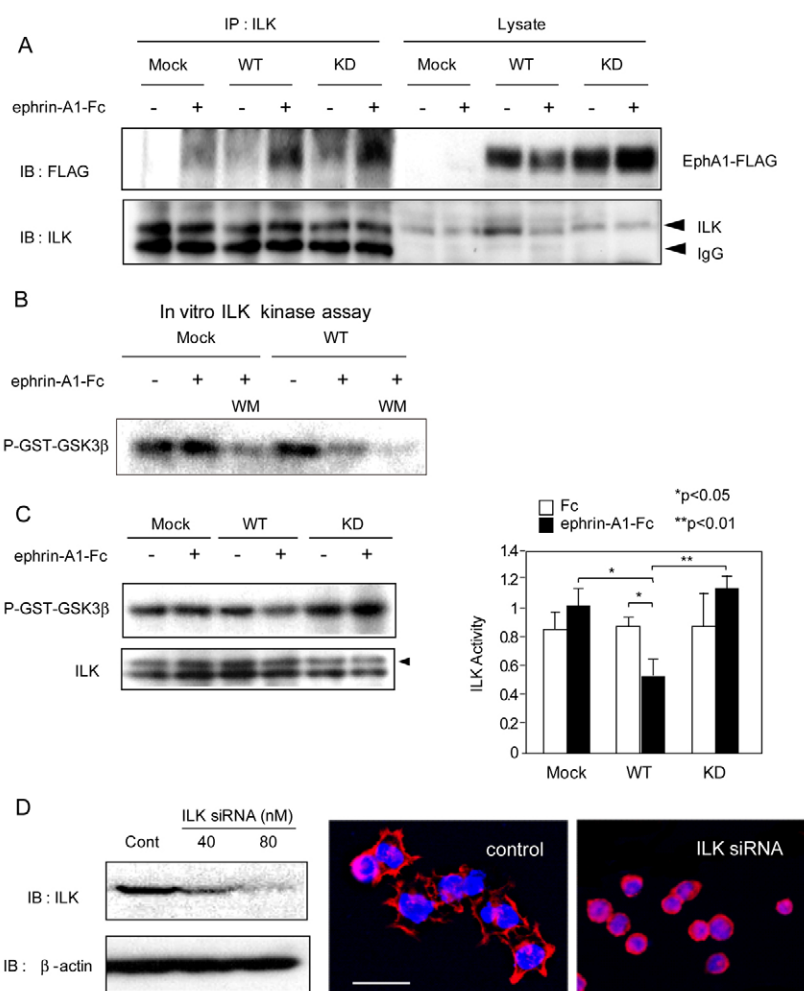
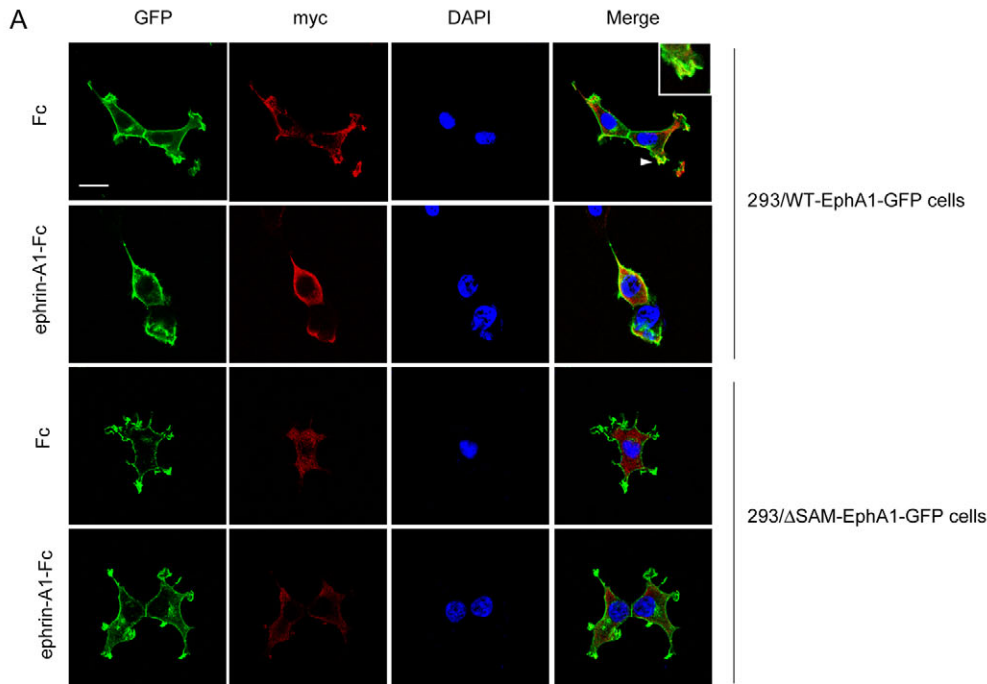


Fig. 7. EphA1 inhibits the kinase activity of ILK. (A) Anti-FLAG (upper) and anti-ILK (lower) immunoblotting of endogenous ILK immunoprecipitated with anti-ILK antibody from the same set of cells as shown in Fig. 3D. (B) Mock and WT-EphA1-FLAG (WT) cells were plated onto FN-coated (1 μ g/ml) dishes with or without immobilized ephrin-A1-Fc (1 μ g/ml) for 15 minutes. Wortmannin (WM; 100 nM) was used for 30 minutes prior to the re-spreading of cells. ILK was immunoprecipitated and subjected to ILK kinase assay as described in the Materials and Methods. (C) Normalized kinase activities against the total amount of immunoprecipitated ILK (arrowhead) were quantified. Data on the right show means \pm s.d. of three independent experiments. (D) HEK293 cells were transfected with control non-silencing RNA (Cont) or ILK siRNA (40 or 80 nM). Immunoblot analysis was performed 2 days after transfection. Cells transfected with control siRNA or ILK siRNA (80 nM) were plated on FN-coated (1 μ g/ml) coverslips for 30 minutes. Cells were then stained with rhodamine-conjugated phalloidin (red) and DAPI (blue). Scale bar: 10 μ m.

EphA1 receptor tyrosine kinase by ephrin-A1 might directly influence Rac and Rho antagonistically. Crosstalks between the Rho and Cdc42-Rac pathways have been described. LIM kinase, another serine-threonine kinase that phosphorylates and thereby inactivates the actin-depolymerizing protein cofilin (Arber et al., 1998; Maekawa et al., 1999; Yang and Mizuno, 1999), is not only activated by ROCK but also by Pak1, and the association of Pak1 with LIM kinase is increased by activated Cdc42 and Rac (Edwards and Gill, 1999). Pak is activated by GTP-bound Cdc42 and Rac (Manser et al., 1995).

Currently, not much is known as to how ligand-induced activation of EphA receptor tyrosine kinases regulates the Rho and Cdc42-Rac pathways. EphA4 directly interacts with ephexin, which has



293/WT-EphA1-GFP cells

293/ΔSAM-EphA1-GFP cells

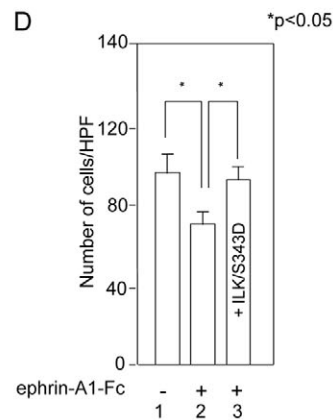
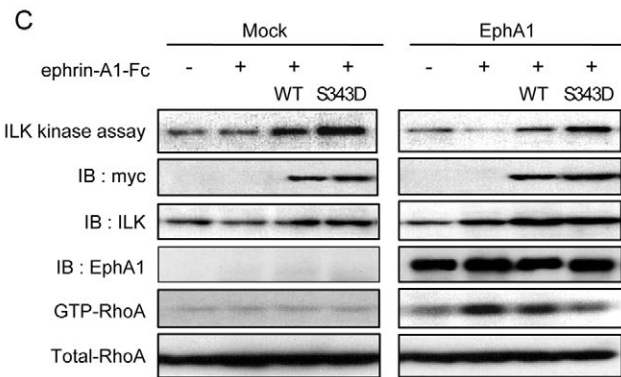
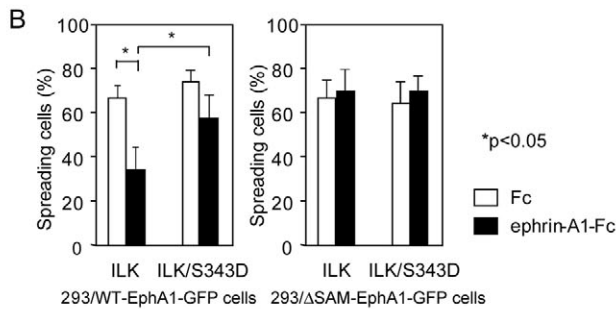


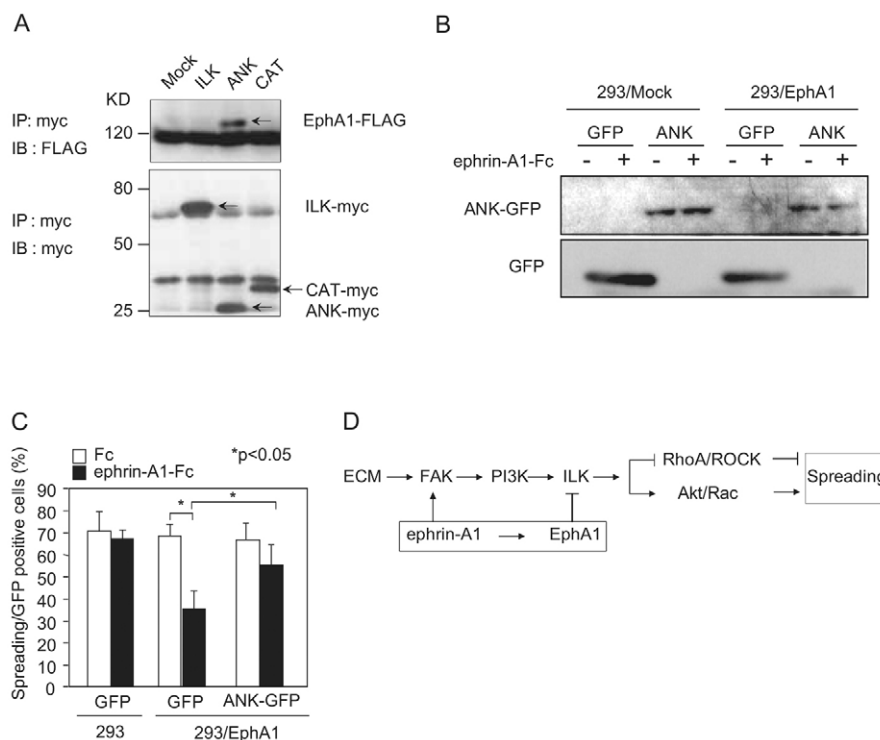
Fig. 8. An active form of ILK (S343D) blocks the inhibitory effect of EphA1. (A) HEK293 (293) WT-EphA1-GFP and ΔSAM-EphA1-GFP cells were transfected with wild-type ILK-myc (myc), starved of serum for 12 hours and then stimulated with ephrin-A1-Fc or control Fc (1 μg/ml) for 15 minutes. Cells were fixed and immunostained by anti-myc antibody (red). DAPI is blue; GFP is green. Note the colocalization of EphA1-GFP and ILK-myc in the merged images even before ephrin-A1-Fc stimulation in WT-EphA1-GFP cells (see the area with an arrowhead in the inset). Scale bar: 20 μm. (B) The same set of cells as is shown in A, with additional transfection with ILK-S343D, was subjected to spreading assays. The number of spreading cells over that of myc-positive cells was calculated. Data show means ± s.d. of three independent experiments. (C) ILK and RhoA activities in HEK293 WT-EphA1-FLAG cells transfected with wild-type ILK-myc (WT) or ILK-S343D-myc (S343D) were measured by immunoblot analysis as described in the Materials and Methods. (D) HEK293 WT-EphA1-GFP cells transfected with vector alone (lanes 1, 2) or ILK-S343D-myc (lane 3) were subjected to cell migration assays as described in Fig. 6E in the absence (lane 1) or presence (lanes 2, 3) of ephrin-A1-Fc.

GEF activity for RhoGTPases and also with Vsm-RhoGEF (Ogita et al., 2003; Shamah et al., 2001). As has been shown for EphB2 receptors (Holland et al., 1997), EphA receptors might also interact, via autophosphorylated juxtamembrane tyrosine residues, with RasGAP (Ras GTPase-activating protein), which is constitutively associated with RhoGAP. RhoGAP is a negative regulator of Rho, and the strong activation of Rho by ephrin-A1 in our experiments would require inactivation of RhoGAP activity. It remains to be shown whether additional factors (such as p62 Dok) of the RasGAP-RhoGAP complex are responsible for the inhibition (Holland et al.,

1997; Holland et al., 1996). EphA receptors have been shown recently to interact with several different downstream factors. Non-receptor tyrosine kinases such as Src, Fyn, Yes and Abl bind directly via their SH2 domain to phosphorylated tyrosine residues of several different EphA receptors (Bruckner and Klein, 1998; Kalo and Pasquale, 1999).

In this report, we identified ILK as an interacting partner of EphA1 by the yeast two-hybrid analysis, and demonstrated that the SAM domain facilitated EphA1 binding to the ANK domain of ILK in a ligand-dependent and kinase-independent manner. ILK

Fig. 9. A dominant-negative ILK rescues the EphA1-mediated spreading defect. (A) HEK293 cells were co-transfected with EphA1-FLAG and myc-tagged ILK, CAT or ANK (see Fig. 1), and anti-myc immunoprecipitates (IP) were subjected to anti-FLAG (upper) and anti-myc (lower) immunoblotting. Specific bands are indicated by arrows. (B) HEK293 (293) Mock and WT-EphA1-FLAG (EphA1) cells were transfected with GFP (control) or ANK-GFP, and their expression was confirmed by anti-GFP immunoblotting. (C) The same set of cells as is shown in B was subjected to spreading assays. (D) A model for the regulation of cell spreading by EphA1. Ephrin-A1 activates EphA1, which recruits ILK to the SAM domain of EphA1. ECM activates the FAK-PI3K-ILK cascade. EphA1 could activate FAK but also inhibit ILK downstream of FAK. ILK suppresses Rho-ROCK but its de-inhibition by EphA1 through ILK inhibition results in the spreading defect. The kinase activity of ILK is negatively regulated by EphA1 kinase (through a direct or indirect mechanism). The suppression of Rac1 and activation of the RhoA-ROCK pathway might result in the increase of cell contractility, and inhibition of cell spreading and migration.



serves as a scaffold that brings together other proteins that are associated with the actin cytoskeleton. Specifically, the N-terminus of ILK mediates interactions with PINCH and ILK-associated phosphatase (ILKAP; also known as PP2C δ), whereas the C-terminal half binds to integrins β 1 and β 3, paxillin, actopaxin, and affixin (Fukuda et al., 2003; Hannigan et al., 1996; Nikolopoulos and Turner, 2000; Nikolopoulos and Turner, 2001; Nikolopoulos and Turner, 2002). ILK not only serves as a molecular scaffold at the cell-ECM adhesion sites but also participates in signal-transduction pathways. Downstream targets of ILK signaling include PKB (Akt), GSK3 β , β -catenin and the myosin light chain (Ren et al., 1999; Ren et al., 2000). Cell-ECM interactions regulate ILK activity, and these events are probably mediated by PI3K (Fig. 9D).

Currently, we do not know the mechanism of how activated EphA1 negatively regulates the kinase activity of ILK. ILK did not appear to be phosphorylated by EphA1 (data not shown). ILKAP is a serine-threonine phosphatase that has been shown to negatively regulate ILK kinase activity (Leung-Hagesteijn et al., 2001). In addition to ILKAP, other inhibitors of ILK have been identified. PTEN is a 3'-inositol lipid phosphatase and its activity is required for tumor-suppressor function. ILK can potentially be activated by a PH-like-domain-mediated interaction with 3'-phosphorylated inositol lipids. Several studies have shown that *PTEN*-null prostate carcinoma cells have constitutively increased levels of ILK activity, and transfection of *PTEN* into *PTEN*-null cells downregulated ILK activity (Obara et al., 2004). Dab2 (also known as DOC2) is a candidate tumor suppressor and downregulates ILK activity by unknown mechanisms (Wang et al., 2001). Dab2 contains the phosphotyrosine-binding (PTB) domain in its N-terminus.

The LIM-domain-only adapter protein PINCH links ILK to activated growth-factor receptors such as EGFR via another adapter, Nck (Persad and Dedhar, 2003). ILK and PINCH are concentrated in peripheral ruffles of cells spreading on FN (Tu et al., 1999).

EphA1 might not require those adapters for interaction with ILK and binding is likely to be more direct, via non-SAM regions within the cytoplasmic domain. Because the SAM domain participates in homo- and hetero-interactions with diverse partners, including protein, RNA, Zn²⁺ and lipid (Qiao and Bowie, 2005), it might bring another molecule that is required for EphA1 to efficiently bind ILK. As in the case of EphA4, EphA1 still retains its kinase activity on stimulation by ephrin-A1-Fc even in the absence of the SAM domain (data not shown) (Park et al., 2004). Because the SAM domain alone failed to bind ILK, it plays a certain ancillary role for the EphA1-ILK interaction.

The kinase-active form of ILK (S343D) served in a dominant-negative manner, and its expression resulted in the restoration of reduced ILK activity and attenuation of the ephrin-A1-Fc-induced spreading defect in WT-EphA1 cells, and also in a reduction of RhoA activity. This implies that kinase activity of ILK is important for EphA1-mediated cellular responses. The ANK domain of ILK worked in a dominant-negative manner, and attenuated ephrin-A1-induced RhoA activation and the spreading defect (Fig. 9C). These data suggest that ILK is involved in EphA1-initiated RhoA activation. ILK has been implicated in integrin-mediated signaling to the actin cytoskeleton (Sakai et al., 2003; Yamaji et al., 2001). The mechanisms whereby ILK exerts these effects are largely unknown, although they potentially involve a combination of ILK-associated kinase activity, focal-adhesion localization and scaffold activity. Of interest, ILK has been linked to early cell-spreading events (Chun et al., 2003; Filipenko et al., 2005; Fukuda et al., 2003; Tu et al., 2001; Wu and Dedhar, 2001; Yamaji et al., 2001; Zhang et al., 2002). It is well established that Rho family GTPases can control cell spreading and cell motility, and that ILK regulates cytoskeletal reorganization by virtue of its ability to activate the small GTPases Rac1 and Cdc42 via the guanine nucleotide exchange activity of alpha-PIX (Filipenko et al., 2005). Depletion of ILK by siRNA resulted in a spreading defect (Fig. 7D) (Filipenko et al.,

2005; Fukuda et al., 2003) and a reduction of Rac1 activity in epithelial cells (Filipenko et al., 2005). Given that RhoA and ILK kinase activity do not fully correlate (Fig. 8D), ILK does not necessarily inhibit RhoA directly, and EphA1 might stimulate RhoA-ROCK and inhibit ILK in different signaling pathways. We demonstrated that activation of EphA1 receptor resulted in downregulation of Rac1 activity and of ILK kinase activity. These data suggest that attenuation of ILK kinase activity by EphA1 could lead to inhibition of alpha-PIX activity for Rac1, and result in a shift of balance among Rho-family GTPases to enhance RhoA activity.

Materials and Methods

Reagents

Ephrin-A1-Fc was purchased from R&D Systems. The Fc fragment of human IgG and goat anti-human Fc, FN, poly-L-lysine and anti-FLAG antibody were from Sigma. Mouse monoclonal anti-phosphotyrosine PY20 was from ICN. Mouse monoclonal anti-RhoA, rabbit polyclonal anti-Rac1, anti-Cdc42 and mouse monoclonal anti-myc antibody were from Santa Cruz Biotechnology. Mouse monoclonal anti-GFP and anti-FAK antibodies were from BD Biosciences. Mouse monoclonal anti-ILK antibody was from Upstate. Anti-phospho-GSK3 β antibody was from Cell Signaling. G418 and Y-27632 were from Calbiochem.

Plasmid construction

The full-length DNA fragment encoding WT-EphA1 was prepared by polymerase chain reaction (PCR) with eph cDNA (Maru et al., 1988) as a template. PCR was performed by KOD plus polymerase (Takara) with primers 5'-GAATTCACC-ATGGAGCGCGCTG-3' (forward) and 5'-GTCGACGTCCTTGAATCCCTG-AATACT-3' (reverse), and cloned in frame into the *EcoRI* and *SaI* site of pCMV-Tag4A (Stratagene) to generate FLAG-tagged WT-EphA1, and also into pEGFP-N3 (Clontech) to generate GFP-tagged EphA1. The kinase dead (KD) form of EphA1, in which valine 638 in the ATP-binding region was changed to arginine (V638R) (Shamah et al., 2001; Shu et al., 1994), was prepared with primers 5'-GACACTGTCATAGGAGAAGGAGAGTTTGGGGAACGGTAT-3' (forward) and 5'-GTCGACGTCCTTGAATCCCTGAATACT-3' (reverse). The PCR product was digested with *Tth*1111 and *SaI*, and replaced by the corresponding sequence of WT-EphA1. Δ SAM-EphA1 was constructed by PCR with primers 5'-GAATTCACCATGGAGCGCGCTG-3' (forward) and 5'-TTGCTCCAGATGTGCCTGAAG-3' (reverse).

Cell culture and transfection

HEK293, NP31, MCF7 and Rat1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin and L-glutamine. The mammalian expression vectors for FLAG-tagged WT- and KD-EphA1 with a single mutation in the kinase domain (V638R) were transfected into HEK293 cells using SuperFect transfection reagent (Qiagen). Clonal G418-resistant cells were isolated and expanded. Expression of WT- or KD-EphA1 was analyzed by immunoblotting with anti-FLAG antibody. All the experiments were performed with a mixture of more than ten G418-resistant populations of clonal cells. NP31/ATF3-Tet cells were described previously (Masuda et al., 2008). siRNA targeting human ILK (Troussard et al., 2003) or rat EphA1 (Masuda et al., 2008) and a control non-silencing sequence were synthesized by Japan Bioservice. Cells were transfected with siRNA using TransIT-TKO reagent (Mirus). ILK expression was evaluated by immunoblotting 2 days after transfection.

Immunofluorescent staining

HEK293 cells expressing FLAG- or GFP-tagged WT-, KD- or Δ SAM-EphA1 with or without transfection with ILK-myc were plated on coverslips, unstimulated or stimulated by ephrin-A1-Fc after serum starvation and fixed with 4% paraformaldehyde for 1 hour at room temperature. Then cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and washed with PBS. Cells were incubated with anti-FLAG or anti-myc antibodies for 1 hour at room temperature and washed with PBS. Next, cells were incubated with Cy3- or TRITC-conjugated anti-mouse IgG (Jackson ImmunoResearch) for 1 hour at room temperature. Cells were co-stained with phalloidin and/or DAPI when indicated. Samples were photographed using an OLYMPUS BX-51 fluorescence microscope equipped with DP70 digital camera system and analyzed.

Cell-adhesion assay

Standard cell-adhesion assays were carried out as described previously (Miao et al., 2000). Briefly, 96-well plates were coated with ECM proteins such as VN, FN and poly-L-lysine at 5 μ g/ml at 4°C overnight. Then, ephrin-A1-Fc or Fc was clustered with goat anti-human Fc at a ratio of 1:5 on ice for 30 minutes before adding to ECM-coated wells. Non-specific binding sites were blocked with 1% BSA/phosphate-

buffered saline (PBS) at room temperature for 1 hour. Cells were serum starved overnight, and were detached with 2 mM EDTA and washed with adhesion medium (serum-free DMEM containing 0.1% BSA). Approximately 1×10^4 cells were plated in each well in adhesion medium and allowed to adhere at 37°C for 30 minutes. Adherent cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. A590 was measured with extracted dye using an enzyme-linked immunosorbent assay reader (Molecular Devices).

Cell spreading

The cells were plated on FN-coated (5 μ g/ml) coverslips with or without immobilized ephrin-A1-Fc (1 μ g/ml) in adhesion medium and allowed to spread for 30 minutes at 37°C. The cell morphology was observed under an Olympus IX70 fluorescent microscope equipped with digital camera and recorded. Non-spreading cells were defined as round cells, whereas spreading cells were cells with extended processes as described (Fukuda et al., 2003; Zhang et al., 2002). The percentage of cells with spreading morphology was quantified by analyzing at least 300 cells from four randomly selected fields.

Cell-migration assay

Cell-migration assays using modified Boyden chambers were performed essentially as described (Miao et al., 2000). To test the migration of transfected HEK293 cells, the underside of filter inserts (8- μ m pore size, COSTER) was coated with 50 ng of FN in 10 μ l of PBS. Non-specific binding sites were blocked with 1% BSA/PBS. Approximately 1×10^4 cells in serum-free DMEM containing 0.1% BSA were plated on the top of the insert and allowed to migrate through the filter at 37°C overnight. Ephrin-A1-Fc or Fc at 1 μ g/ml was added to the lower chamber. Cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Cells that went through the filter and stayed on the underside of inserts were counted.

Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 25 mM NaF, 10% glycerol, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, 2 μ g/ml leupeptin and 2 μ g/ml aprotinin for 30 minutes at 4°C. Lysates were clarified at 13,000 g for 5 minutes. Immunoprecipitation was carried out using antibodies at 1 μ g/mg of total protein at 4°C for 1 hour. Immune complexes were collected using protein-A-Sepharose beads (Amersham Biosciences) for 1 hour at 4°C. The beads were then washed with immunoprecipitation washing buffer containing 20 mM Tris-HCl (pH 7.4), 10% glycerol, 50 mM NaCl, 0.2% NP-40, 0.5 mM PMSF and 0.5 mM Na₃VO₄. The samples were boiled in reducing SDS sample buffer and separated on polyacrylamide gel. Proteins were transferred to PVDF membranes (Bio-Rad) and probed with the indicated antibodies.

Rho-GTPases activity assay

RhoA and Rac-Cdc42 activities were determined as described previously (Ren et al., 1999; Ren et al., 2000). Cells were replated on plates coated with FN (1 μ g/ml) and ephrin-A1-Fc or Fc (1 μ g/ml) for 15 minutes and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 10 mM MgCl₂, 150 mM NaCl, 1 mM PMSF, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin on ice for 5 minutes. Cell lysates were immediately incubated with GST-Rhotekin RBD or GST-PAK-CD coupled on glutathione-Sepharose-4B beads for 1 hour at 4°C. The beads were then washed and re-suspended in SDS sample buffer. The GTP-bound RhoA, Rac1 or Cdc42 was analyzed by immunoblotting using anti-RhoA, anti-Rac1 or anti-Cdc42 antibody, respectively. Blot densities were quantified using NIH Image software.

Yeast two-hybrid screening and cDNA isolation

The yeast two-hybrid screening using the EphA cytoplasmic domain as bait was performed as described previously (Pandey et al., 1994; Takeda et al., 1999). Briefly, an EphA1 bait plasmid containing the EphA1 cytoplasmic domain (AA570-976) fused to the GAL4 DNA-binding domain in pGBK-T7 (Clontech) was co-transformed into AH109 strain with a human placenta expression library fused to the GAL4 activation domain in pACT2 (Clontech). 54 clones were proved to be positive by confirmation using reporter gene (β -galactosidase activity) and re-transformation. Library plasmids recovered from the positive clones were used in co-transformation with either the EphA1 cytoplasmic bait or other control baits. A full-length human ILK cDNA was isolated from HeLa cell cDNAs by PCR using KOD plus polymerase (Takara) with 5'-ACCATGGACGACATTTCACCTAG-3' (forward) and 5'-CTCGAGCTTG-TCCTGCATCTTCTCAAG-3' (reverse) primers. The β -galactosidase liquid assay was performed as described previously (Takeda et al., 1999).

In vitro kinase assay

For the ILK kinase assay, ILK was immunoprecipitated with monoclonal anti-ILK antibody (Upstate), and washed twice with lysis buffer and once with kinase buffer [20 mM MOPS (pH 7.0), 10 mM MgCl₂, 5 mM MnCl₂]. Purified GST-GSK3 β (1 μ g) and 100 μ M ATP were added in 30 μ l of kinase buffer and incubated for 30 minutes at 30°C. Reactions were stopped by adding SDS-PAGE loading buffer. Phosphorylated substrates were separated by SDS-PAGE and detected by immunoblotting using anti-phospho-GSK3 β (Ser 9) antibody.

Statistical analysis

All values are presented as means \pm s.d. from at least three independent experiments. Statistical analysis was performed with Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

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