Selective Activation of Small GTPase RhoA by Tyrosine Kinase Etk through Its Pleckstrin Homology Domain*

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Etk/Bmx is a member of the Btk family tyrosine kinase, which contains an N-terminal pleckstrin homology domain. Etk has been shown to play a pivotal role in the regulation of various cellular processes including differentiation, apoptosis, and cell motility. Here we present evidence that Etk is a modulator of the small GTPase RhoA. Etk and RhoA both are translocated to the plasma membrane and can form a complex upon serum stimulation in C2C12 cells. Etk interacts with RhoA but not other closely related small GTPases such as Cdc42 and Rac1, suggesting a specific modulation of RhoA by Etk. Our results demonstrate that Etk activates RhoA and enhances Rho-mediated stress fiber formation and transcription activity in a pleckstrin homology domain-dependent manner. Furthermore, Etk disrupts the interaction between RhoA and Rho-GDI (guanine nucleotide dissociation inhibitor) and promotes the membrane translocation of RhoA. Our data suggest that Etk plays an important role in regulation of RhoA-mediated signaling.

Etk/Bmx, one of the Btk family kinases, has been shown to play a pivotal role in the regulation of various cellular processes including differentiation, apoptosis, and cell motility. Etk contains an N-terminal pleckstrin homology (PH)1 domain, a Src homology 3 (SH3) domain, a Src homology 2 (SH2) domain, and a C-terminal tyrosine kinase (SH1) domain (1, 2). The PH domains of Btk family kinases have been shown to bind to phospholipids as well as many protein partners including heterotrimeric G proteins, protein kinase C isoforms, STAT3, F-actin, Fas, and focal adhesion kinase (FAK). Recently, we reported that Etk mediates integrin signaling and promotes cell migration through the Etk PH domain and is regulated by FAK (3). Integrins and cytoskeleton reorganization are intimately connected (4, 5). The reorganization of the actin cytoskeleton is controlled by Rho family members and includes RhoA, Rac1, and Cdc42. These small GTPases can induce the formation of stress fiber, lamellipodia, and filopodia in fibroblasts, respectively (6, 7). The balance between Rac and Rho activity may have an important impact on the migratory behavior of epithelial cells and their derived tumor cells (8, 9). Increasing evidence suggests that Btk family kinases play a role in the regulation of actin polymerization. The PH domain of Btk is shown to bind to F-actin in vitro (10). Etk is colocalized with actin filaments upon extracellular stimulation (3). Itk, another Btk family member, is required for CD3-induced actin polymerization in Jurkat cells (11). Furthermore, it is reported that Btk family kinases may interact with guanine nucleotide exchange factors (GEFs) such as Vav and may act upstream of Rho GTPases (12). However, the mechanisms by which these kinases exert their effects on cytoskeletal reorganization are not yet understood.

The structures of small GTP-binding proteins revealed that they have two interconvertible forms, GDP-bound inactive and GTP-bound active (6, 13). Most small GTP-binding proteins are localized either in the cytosol or on membranes. In the cytosol, the small proteins are complexed with the guanine nucleotide dissociation inhibitors (GDIs) and maintained in the GDP-bound inactive form. The GDP-bound form is released from a GDI by a still unknown mechanism and is converted to the GTP-bound form with the aid of the action of guanine nucleotide exchange factors. The activated Rho proteins interact with cellular target proteins or effectors to trigger a wide variety of cellular responses, including the reorganization of the actin cytoskeleton and changes in gene transcription (14). Thereafter, the GTP-bound form is converted to the GDP-bound form by its intrinsic GTPase activity and GTPase activating proteins. The GDP-bound form then binds to a GDI and returns to the cytosol. It has been reported that GTP-RhoA binds to and activates Rho kinase, resulting in elevated myosin light chain phosphorylation (15). Lang et al. (16) reported that membrane-bound RhoA is the only target for protein kinase A and that cytosolic RhoA might be protected from protein kinase A phosphorylation by its binding to GDI.

In the present study, we explore the role of the Etk in the regulation of small GTPase RhoA signaling. Our data suggest that Etk acts upstream of RhoA and activates RhoA in a kinase activity-independent manner by releasing GDI from the RhoA-GDI complex through the interaction between the PH domain of Etk and RhoA.

MATERIALS AND METHODS

Cell Culture and Transfection—293, COS-1, HeLa, MDCK, and C2C12 cells were maintained in Dulbeco’s modified Eagle’s medium with 10% fetal bovine serum. Transfections were performed using FuGENE 6 (Roche Molecular Biochemicals) or LipofectAMINE 2000 (Invitrogen) according to the manufacturers’ instructions.
DNA Constructs and Antibodies—T7-tagged Etk and its mutants were cloned into pcDNA3 vector as described previously (1). HA-tagged Rho family constructs were kindly provided by Drs. Xiang-Dong Ren and Martin Schwartz (17). Anti-T7 antibody was purchased from Novagen. Anti-HA antibody was from Babco. Anti-RhoA and anti-Myc (9E10) were from Santa Cruz Biotechnology. Polyclonal Etk antibody was developed as described previously (1).

Immunoprecipitation and Western Blot—Transfected cells were lysed in the buffer (20 mM Tris/HC1, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na3VO4, 1 mg/ml apro
tinin, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation, and antibodies were added to lysates for 1 h at 4 °C. Antibodies were collected with protein A- or protein G-Sepharose beads, and protein complexes were washed three times at 4 °C with the lysis buffer. Immunoblotting was performed as described previously (1). Briefly, blots were incubated with the indicated primary antibodies at room temperature for 1 h and followed by detection with horseradish peroxidase-conjugated secondary antibody.

Immunofluorescence Staining—Cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde. Cells were stained with rhodamine-phalloidin for the visualization of actin stress fibers. For RhoA and Etk staining, cells were incubated with anti-RhoA, -Etk, or -HA antibody followed by incubation with rhodamine-conjugated anti-mouse and/or fluorescein isothiocyanate-conjugated anti-rabbit. Slides were examined with a confocal microscope. In some cases, transfected cells were identified by co-transfected GFP marker.

Rho Activity Assay—RhoA activity was assayed as described previously (8). GST-C21 was purified from Escherichia coli transformed with pGEX-2T-Rhotekin Rho binding domain (RBD) (kindly provided by Dr. John Collard). Bacteria were harvested in bacterial lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl2, 0.2 mM Na2S2O3, 5H2O, 2 mM dithiothreitol, 20% sucrose, and 10% glycerol) containing protease inhibitors and were lysed by sonication. After centrifugation (13,000 × g for 10 min), GST-C21 was collected by rocking at 4 °C for 45 min with glutathione-Sepharose 4B. The beads were washed three times with the lysis buffer and resuspended in

**FIG. 1.** Etk specifically interacts with RhoA. A, colocalization of Etk and RhoA in C2C12 cells. C2C12 cells were plated on coverslips. The cells were either maintained in complete medium containing 10% fetal bovine serum (+ Serum) or serum-starved for 6 h (− Serum) and then were fixed and stained with anti-RhoA and -Etk antibody. Yellow indicates the colocalization of Etk and RhoA. B, co-immunoprecipitation of Etk with RhoA. C2C12 were treated as above, and the cell lysates were immunoprecipitated with anti-RhoA antibody followed by immunoblotting with anti-Etk (top panel) or anti-RhoA antibody (bottom panel). The total cell lysate (T) was included as a control for immunoblotting. C, specific interaction between Etk and RhoA. HA-tagged wild-type Rho GTPases RhoA, Cdc42, Rac1, and their derivatives were co-expressed with T7-tagged Etk in COS-1 cells. Immunoprecipitations were performed with anti-Etk antibody followed by immunoblotting with anti-HA antibody. Bottom panels show the expression level of Rho GTPases and Etk in these experiments. D, interaction between Etk and RhoA is independent of its GTP/GDP binding status. Bacterially expressed GST-RhoA was immobilized on glutathione-Sepharose beads. The beads were then washed three times with nucleotide depletion buffer (NDB buffer: 20 mM Tris/HC1 pH 7.5, 1 mM dithiothreitol, 10 mM EDTA, 50 mM NaCl, 5% glycerol, and 0.1% Triton X-100) and incubated for 20 min at room temperature. To load the fusion protein with GDP or GTP, aliquots were washed three times with GDP loading buffer (NDB buffer containing 200 μM GDP (GDP-β-s, Roche Molecular Biochemicals) and GTP loading buffer (NDB buffer containing 20 μM GTP (5’-guanylylimidodiphosphate, Roche Molecular Biochemicals) and then were incubated for 25 min at room temperature. The GDP-loaded, GTP-loaded, and nucleotide-depleted GST-RhoA were mixed with cell lysates for 1 h at 4 °C followed by extensive washing with respective loading buffers. The bound proteins then were analyzed by SDS-PAGE followed by Western blotting with anti-Etk antibody and Coomassie Blue staining. Top panel, Etk associated with GST-RhoA; middle panel, Coomassie Blue staining of GST-RhoA; bottom panel, the level of Etk in the total cell lysate. IP, immunoprecipitate; IB, immunoblot.
fresh buffer. 24 h post-transfection, cells were rinsed with phosphate-buffered saline and lysed in buffer (50 mM Tris/HCl, pH 7.4, 1% glycero, 100 mM NaCl, 1% Nonidet P-40, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin). Lysates were clarified by brief centrifugation and then incubated with Sepharose-bound GST-C21 at 4 °C for 45 min. The beads were washed three times in cold lysis buffer, and the proteins were eluted by boiling in Laemmli buffer and separated by SDS-PAGE. Proteins were immunoblotted with anti-HA monoclonal antibody as described above. The precipitated RhoA was normalized to the RhoA present in total cell lysate.

**RESULTS**

**Colocalization of Etk and RhoA**—We reported previously that Etk is translocated to the plasma membrane upon extra-cellular matrix protein stimulation (3). Rho proteins have also been shown to move from cytosol to membranes upon activation (18–20). To look for the possible links between these two events, we examined the localization of Etk and RhoA in C2C12 cells in which both proteins are quite abundant. As shown in Fig. 1A, in the absence of serum both Etk and RhoA were primarily distributed in the perinuclear compartments. Upon serum stimulation, significant fractions of RhoA and Etk were translocated to the membrane. Moreover, they appeared to be colocalized with each other on the membrane as evidenced by the extensive yellow color when the RhoA and Etk images were merged. In contrast, minimal staining was observed on the membrane in the absence of serum. These observations suggest that Etk and RhoA are translocated and colocalized to membrane upon serum stimulation. This raises the possibility that Etk may be physically associated with RhoA, which is supported by the following co-precipitation experiments (Fig. 1B). A protein of ∼84 kDa immunoprecipitated with anti-RhoA antibody was recognized by the Etk antibody, suggesting that endogenous RhoA and Etk might form a complex in the presence of serum. To confirm this result, we also co-expressed various HA-tagged Rho GTPases with Etk in COS-1 cells. Fig. 1C shows that all three forms of RhoA were co-precipitated with Etk, regardless of its GTP/GDP binding status. This result was further confirmed by in vitro binding assays using nucleotide-depleted, GTP- or GDP-loaded purified GST-RhoA fusion proteins (Fig. 1D). Because the other related small GTPases, Cdc42 and Rac1, did not show appreciable interaction with Etk, the interaction between Etk and RhoA appears to be quite specific.

**Etk Binds to RhoA through Its PH Domain**—To determine the Etk sequence required for its interaction with RhoA, we expressed HA-tagged RhoA with wild-type Etk or various Etk deletion mutants (ΔPH, ΔSH3, ΔSH2, and ΔKin). Immunoprecipitations were performed with anti-Etk and followed by immunoblotting with anti-HA antibody. In Fig. 2A, RhoA was co-precipitated with wild-type Etk, ΔSH3, ΔSH2, and ΔKin but not with the ΔPH mutant. Furthermore, the PH domain of Etk alone was sufficient to immunoprecipitate RhoA, but the SH2 or SH3 domains alone were not (Fig. 2B). The same results were obtained from the in vitro binding assay. We mixed immunoprecipitated wild-type Etk or its mutants with purified GST-RhoA fusion protein. The bound proteins were fractionated by SDS-PAGE followed by immunoblotting with anti-RhoA antibody. Fig. 2C shows that wild-type Etk, EtkΔSH3, and EtkΔSH2 bound to the GST-RhoA protein, but EtkΔPH did not. These results indicate that the PH domain of Etk is required for its association with RhoA.

**Activation of RhoA by Etk**—To examine whether the interaction between Etk and RhoA affects RhoA activity, HA-tagged wild-type RhoA was co-transfected into COS-1 cells with T7-tagged Etk or its mutants. The active GTP-RhoA was then isolated from cell lysates by a GST pull-down assay using the fusion protein containing the binding domains of rhotekin (GST-C21) (8). As shown in Fig. 3A, co-expression of Etk with wild-type RhoA resulted in significant increases of GTP-bound RhoA. The kinase activity of Etk was dispensable for this effect because kinase-inactive Etk (EtkKQ) enhanced GTP-bound RhoA formation to similar levels as the wild-type Etk. Consistent with the co-precipitation data, deletion of the PH domain of Etk significantly reduced the level of GTP-RhoA in the cells, whereas deletion of the SH3 domain had little effect. This
suggests that the PH domain of Etk is required for RhoA activation.

Next, we examined the effect of Etk and its mutants on the formation of stress fibers, one of the phenotypic changes resulting from RhoA activation. Wild-type RhoA and GFP were co-transfected with Etk or its mutants into HeLa cells. The cells then were fixed and stained with rhodamine-phalloidin. There was a marked increase in actin stress fiber formation in the cells transfected with Etk or its mutant lacking the SH3 domain Etk-H9004SH3 but not with the PH deletion mutant (Etk-H9004PH) (Fig. 3B). This suggests that the Etk PH domain is required for the formation of actin stress fiber resulting from activation of RhoA by Etk. We also examined the effect of Etk on RhoA-mediated transcripational activity of the promoter containing the serum responsive element (SRE-L) (21). In agreement with its effects on stress fiber formation, overexpression of Etk in MDCK cells resulted in more than a 2-fold increase of RhoA-mediated transcripational activity of SRE-L containing promoter, whereas EtkΔPH had very little effect (Fig. 3C).

The Etk Interrupts Interaction between RhoA and GDI—It has been shown that Rho-GDI negatively regulates RhoA activity by sequestering RhoA from its GEFs such as Dbl and Vav (14, 22, 23). We reasoned that Etk might activate RhoA by disrupting the RhoA/Rho-GDI complex by interacting with RhoA through its PH domain. To test this possibility, HeLa cells were co-transfected with HA-tagged wild-type RhoA, Myc-tagged Rho-GDI, and T7-tagged Etk or Etk PH domain. The level of the RhoA/Rho-GDI complex in these cells was determined by the amount of Myc-tagged Rho-GDI co-immunoprecipitated with the HA-tagged RhoA. Fig. 4A shows that Myc-tagged Rho-GDI was effectively co-immunoprecipitated with HA-tagged RhoA, whereas the co-expression of wild-type Etk or Etk PH domain dramatically reduced the amount of Rho-GDI associated with RhoA without affecting the expression level of these proteins. These data suggest that the association of RhoA with Rho-GDI is disrupted by the Etk through its PH domain. Furthermore, we examined the effect of Etk on the subcellular location of RhoA in HeLa cells. As can be seen in Fig. 4B, when HA-tagged RhoA and Myc-
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Etk mutant lacking the PH domain (EtkΔPH) could no longer promote RhoA translocation. These observations provide evidence that Etk activates RhoA by disrupting the Rho-GDI interaction and promoting the translocation of RhoA to the membrane where it is converted to the GTP-RhoA by GEFs.

DISCUSSION

Small Rho GTPases function as GDP/GTP-regulated signaling relay switches and play important roles in cytoskeletal reorganization in response to diverse extracellular stimuli. Although many modulators and effectors of Rho GTPases have been identified in the past decade, only a handful of them seem to be selective or specific for a single small Rho GTPase (e.g., Rac-specific GEF Tiam1 and Cdc42-specific GEF Intersectin). In this report, we provided evidence that the PH domain-containing tyrosine kinase Etk is a selective upstream regulator for RhoA but not for the related Cdc42 or Rac1. Our data suggest a direct interaction between Etk and RhoA. The PH domain of Etk appears to be required for its association with RhoA and its effects on RhoA-mediated stress fiber formation and transcriptional activation. Moreover, the interaction between the Etk PH domain and RhoA resulted in its dissociation from the Rho-GDI complex, which is thought to be a prerequisite step in the activation of Rho GTPases by GEFs. Interestingly, the Dbl family GEFs all contain tandem DH-PH domains, and the PH domain of Dbl family GEFs has been proposed to be involved in the regulation of the localization and modulation of the activity of the DH domain (24, 25). We speculate that during the GTP loading process, the PH domain of Dbl GEFs also may be involved in excluding the GDI from the GTPase complex, which allows the DH domain to exert its catalytic activity. Further experiments are needed to explore this possibility. The PH domain of Etk has been shown to interact with a number of protein partners such as G protein βγ-subunits, the FERM domain of FAK, and STAT3. It is likely that the interaction between the Etk PH domain and these signaling molecules is dynamic and spatially restrained. Our preliminary data showed that both FAK and RhoA are detectable in Etk immunoprecipitates when three proteins are overexpressed in COS-1 cells. We do not see competition between RhoA and FAK for Etk binding. Overexpression of RhoA enhances the interaction of FAK with Etk, and the reverse is true also. This suggests that both FAK and RhoA could bind to Etk simultaneously and cooperatively. Our results further support the notion that the PH domain of Etk plays an important role not only in receiving such upstream signals as phospholipids, βγ-subunits of trimeric G proteins, and FAK but also in exerting effects on its downstream effectors such as STAT3 and RhoA. It is noteworthy that up-regulation of RhoA and Etk activities has been implicated in metastasis of several human cancers including prostate and breast. Overexpression of RhoA promotes invasion and metastasis of many tumor cell lines both in vivo and in vitro (26–28). Our study provides new insights into the mechanisms by which these signaling molecules regulate various cellular processes.

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