Interaction between Tyrosine Kinase Etk and a RUN Domain- and FYVE Domain-containing Protein RUFY1

A POSSIBLE ROLE OF ETK IN REGULATION OF VESICLE TRAFFICKING*

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Etk/BMX tyrosine kinase is involved in regulation of various cellular processes including proliferation, differentiation, motility, and apoptosis. Through a yeast two-hybrid screening for the effectors of Etk, a new gene family designated as RUFY was identified. The RUFY gene family (RUFY1 and RUFY2) contains an N-terminal RUN domain and a C-terminal FYVE domain with two coiled-coil domains in-between. They appear to be homologues of a recently identified mouse Rabip4 (Cormant, M., Mari, M., Galmiche, A., Hofman, P., and Le Marchand-Brustel, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1637–1642). RUFY proteins are localized predominantly to endosomes as evidenced by their co-localization with early endosome antigen marker (EEA1). Etk interacts with RUFY1 through its SH3 and SH2 domains. RUFY1 is tyrosine-phosphorylated and appears to be a substrate of Etk. The RUFY1 mutant lacking the phosphorylation sites failed to go to the endosomes. Furthermore, overexpression of Etk in COS-1 and B82L cells resulted in increased plasma membrane localization of the epidermal growth factor receptor and delayed its induced endocytosis in COS-1 cells. The effects of Etk were blocked by the FYVE domain of RUFY1. Interestingly, the FYVE domain of RUFY1 is targeted to the plasma membrane through an interaction between its proline-rich motif and the SH3 domain of Etk or possibly some other membrane-associated SH3 domain-containing protein(s), whereas the lipid binding activity of the FYVE domain is not required. Our data suggest that Etk may be involved in regulation of endocytosis through its interaction with an endosomal protein RUFY1.

Etk/BMX is a cytoplasmic tyrosine kinase that belongs to the Btk kinase family. Mutations of Btk, the prototype of this kinase family, have been linked to the human inherited disease X-linked agammaglobulinemia (1, 2). Etk was identified previously from a human prostate tumor xenograft (3, 4). Etk has been shown to play a pivotal role in a variety of cellular processes including proliferation, differentiation, motility, and apoptosis (4–8). As with other members of the Btk kinase family, Etk has multiple signaling modules including a PH domain, an SH3 domain, an SH2 domain, and a tyrosine kinase domain. Over the past decade it has been very well established that these protein modules involved in protein-protein interaction play a critical role in signal transduction (see Refs. 9 and 10). The PH domain is shown to bind PI3-kinase products such as PIP3 and some protein partners. The SH3 domain is involved in binding to proline-rich motifs and the SH2 domain interacts with phosphotyrosine residues in a sequence-specific context. To date, many protein-interacting partners for each module of BTK family kinases have been identified through various biochemical approaches or functional screenings such as the yeast two-hybrid system (see Ref. 11). Accumulating evidence suggests that activation of Etk requires two steps: 1) translocation of Etk to plasma membrane induced by the interaction between the PH domain of Etk and one of its ligands such as the PI3-kinase product PIP3 β subunits of the trimeric G protein, or the FERM domain of the focal adhesion kinase (FAK); and 2) subsequent phosphorylation of Tyr-565 in the catalytic domain by Src family kinases, which allows the kinase to be activated. Some of the downstream effectors that are required for Etk to exert its biological functions (such as Stat3) have been identified (5, 12), and we believe more are yet to be uncovered. In the present study we report that a novel gene family designated as the RUN and FYVE domain-containing (RUFY) protein family has been identified as the downstream effector of Etk. Previous studies demonstrated that the downstream effects of PI3-kinase signaling are in many cases mediated by proteins containing a PIP3 binding module (in turn) FYVE finger domain. The signature of the FYVE finger domain is the sequence CXXC(R/K)(R/K)HHCR, which comprises two zinc binding sites. Many FYVE domain-containing proteins are localized at endosomes and play an important role in endocytosis (13–17). Here we present evidence that Etk interacts with RUFY1 through the SH3 and SH2 domains and that tyrosine phosphorylation of RUFY1 by Etk appears to be important for its endosomal localization. Our study suggests that Etk may play a role in the regulation of endocytosis as a downstream effector of PI3-kinase.

EXPERIMENTAL PROCEDURES

DNA Constructs and Yeast Two-hybrid Screen—A yeast two-hybrid HeLa cell cDNA library was purchased from CLONTECH. The yeast two-hybrid screen was performed according to the manufacturer’s in-
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structures using LexA-Etk as bait. Positive clones were subjected to sequencing analysis. The full-length cDNA of RUFY1 was obtained by 5'-RACE from human prostate cancer cell lines LNCaP and CWR22R as described previously (4) and was subcloned into pCDNA3.1-FLAG and pcDNA3.1-GFP vector. Deletions and site mutagenesis were carried out using the PCR-based approach (18). All mutations were confirmed by sequencing. Etk constructs were described in previous studies (4, 7). The cDNA fragment encoding amino acids 216–418 of RUFY1 was inserted into the pGEX vector to generate the glutathione S-transferase fusion protein. The polyclonal antibody for RUFY1 was raised against the purified glutathione S-transferase fusion protein expressed in Escherichia coli. The cDNA of human Etk receptor was kindly provided by Dr. Heung-Jien Kung at the University of California at Davis and was subsequently cloned into pCDNA3.1-GFP vector.

Cell Culture, Transfection, Antibodies, and Reagents—COS-1, MDCK, and C2C12 cells were obtained from the ATCC and maintained in Dulbecco’s modified Eagle’s serum with 10% fetal bovine serum. B22L cells were cultured as previously described (19). Transfections were performed using FuGENE 6 (Roche Molecular Biochemicals) or LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Antibodies were purchased from the indicated companies: anti-p-tyrosine (Upstate Biotechnology), anti-EEA1 (BD Transduction Laboratories), anti-pMAPK (Cell Signaling Technology, Inc.), MAPK (Santa Cruz Biotechnology), anti-HEMA (Berkeley Antibody), anti-T7 (Novagen), anti-EGF (NeoMarker), and anti-FLAG (Sigma). EGF was obtained from Sigma.

Immunoprecipitation, Western Blot Analysis, and Northern Blot—Immunoprecipitation was performed as described previously (7). Briefly, the cells were lysed by modified radioimmune precipitation buffer. Insoluble material was removed by centrifugation. Antibodies were then added to lysates, incubated for 1 h at 4 °C, and collected with protein A- or protein G-Sepharose beads, and the immunocomplexes were washed three times with lysis buffer at 4 °C. The immunoprecipitates were resolved on SDS-PAGE followed by Western blotting. The bound primary antibody was visualized by enhanced chemiluminescent detection (Amersham Biosciences). The human multiple tissue blots were resolved on SDS-PAGE followed by Western blotting. The bound primary antibody was visualized by enhanced chemiluminescent detection (Amersham Biosciences). The full-length cDNA of this gene was obtained by 5'-RACE. Fig. 1A shows the predicted amino acid sequence of this gene and its alignments with the related proteins, which together form a gene family. The alignments reveal several conserved functional domains in this gene family including an N-terminal RUN domain and a C-terminal FYVE domain with two coiled-coil domains in-between (Fig. 1B).

Therefore, this gene is designated as RUFY1. RUFY2, a homologue of RUFY1, also has been identified by searching the human EST data base and was subsequently cloned from human prostate cancer cell line LNCaP and neuroblastoma cell line SH-SY5Y. Northern analysis of the human multiple tissue blot using a probe specific for RUFY1/2 indicated that RUFY1 is quite abundant in many tissues examined including brain, kidney, liver, lung, placenta, and testis (Fig. 1C). Expression of RUFY2 seems to be relatively restricted and can only be detected in brain, lung, and testis. RUFY1 appears to be the human ortholog of the recently identified mouse Rabip4 gene, a Rab4 effector involved in early endosomal trafficking (17). However, the tissue distribution of RUFY1 is quite different from Rabip4. For example, Rabip4 is highly expressed in mouse heart but not in testis (17), whereas neither RUFY1 nor RUFY2 could be detected in human heart, but both were highly expressed in testis (Fig. 1C). These data imply that mouse Rabip4 and human RUFY1/2 are regulated by different mechanisms and that one or more new RUFY family member(s) may remain to be uncovered.

Localization of RUFY1—Many FYVE domain-containing proteins have been shown to localize to endosomes (13–17). We examined whether RUFY1 is also targeted to endosomes. Full-length RUFY1 was fused with GFP and overexpressed in COS-1 cells. As shown in Fig. 2A, RUFY1 was primarily localized to the punctate structures that were EEA1-positive (indicated by arrows), suggesting that RUFY1-GFP goes to endosomes. It is noteworthy that the endosomes (indicated by arrows) in the cells overexpressing RUFY1 appear to be much larger than their counterparts (indicated by arrowheads) in untransfected cells. To define the sequence element responsible for endosomal targeting of RUFY1 we generated a series of deletion mutants (Fig. 2B). As shown in Fig. 2C, the mutants RUFY1(1–473) and RUFY1(1–418) lacking the FYVE domain, still went to endosomes, although the sizes of these endosomes were smaller in comparison to those in wild-type RUFY1-expressing cells. Two N-terminal deletion mutants, RUFY1(216–473) and RUFY1(216–418), also localized to endosomes. These observations suggest that neither the RUN domain nor the FYVE domain seems to be involved in endosome targeting of RUFY1. On the other hand, the deletion of the second coiled-coil domain (RUFY1acc) completely abolished endosomal localization of RUFY1. In addition, the mutant RUFY1(216–418) containing the two coiled-coil domains efficiently targeted to endosomes. Taken together, these data indicate that the two coiled-coil domains are the determinate factors for targeting RUFY1 to the endosomes.

Interaction between RUFY1 and Etk—To verify that RUFY1 indeed interacts with Etk as observed in the yeast two-hybrid system we raised a rabbit polyclonal antibody against the coiled-coil domains of RUFY1. As indicated in Fig. 3A, endogenous Etk was co-immunoprecipitated with RUFY1 by anti-RUFY1 antibody from C2C12 cell lysates when the cells were cultured in medium containing serum. The interaction between Etk and RUFY1 was diminished when the serum was with-
drawn from the medium, suggesting that this interaction is regulated by some extracellular stimulus present in the serum. The interaction between Etk and RUFY1 or RUFY2 also occurred when we co-expressed T7-tagged Etk and FLAG-tagged RUFY1 or RUFY2 together in COS-1 cells (Fig. 3B).

To understand how this interaction is regulated, we set out to define the domain(s) of Etk involved in binding RUFY1. As shown in Fig. 3C, deletion of the PH, SH3, or SH2 domain individually had little effect on Etk binding to RUFY1. However, deletion of both the SH3 and SH2 domains together (∆SH) dramatically disrupted the interaction. On the other hand, SH domain (SH3 and SH2 domain) alone is sufficient to bring down RUFY1. These data suggest that both SH3 and SH2 domains of Etk are involved in interaction with RUFY1.

Because the SH2 domain of Etk is involved in interaction with RUFY1, and this interaction is stimulated by serum, we reasoned that RUFY1 may be tyrosine-phosphorylated and that phosphorylation may mediate its interaction with the SH2 domain of Etk. Therefore, we examined the effect of Etk on RUFY1 in the absence of serum. Fig. 4A shows that co-expression of RUFY1 with the wild-type Etk but not with the kinase-

![Diagram](image-url)
inactive mutant EtkKQ results in elevated phosphotyrosine content of RUFY1. Mutation of the conserved Tyr-281 and Tyr-292, which are located in the linker region of the two coiled-coil domains, respectively, has a limited effect on tyrosine phosphorylation. However, mutation of both tyrosine residues completely abolished the tyrosine phosphorylation of RUFY1 induced by Etk. Interestingly, the localization of the RUFY1 mutant Y281F/Y292F was no longer localized at endosomes (Fig. 4B), suggesting that localization of RUFY1 is dynamically regulated by tyrosine phosphorylation. This is consistent with a recent report that the endosomal localization of Rabip4, the mouse homologue of RUFY1, is disrupted by the PI3-Kinase inhibitor Wortmannin (20). Because PI3-kinase is one of the upstream kinases of Etk, and Wortmannin has been shown to block IL6-induced activation of Etk (4), Etk very likely may be one of the downstream effectors of PI3-kinase regulating vesicle trafficking by phosphorylating the endosomal protein RUFY1. To demonstrate further that tyrosine phosphorylation of RUFY1 is indispensable for its localization at endosomes, COS-1 cells were transfected with RUFY1-GFP (left) or RUFY1Y281F/Y292F-GFP (right) for 24 h. The images were captured as described in the legend to Fig. 2B. A, Tyrosine phosphorylation of RUFY1. COS-1 cells were transfected with the indicated plasmids for 24 h. Immunoprecipitation and Western blot were performed as described under “Experimental Procedures.” Tyrosine phosphorylation of RUFY1 was determined by anti-phosphotyrosine Western blot of the anti-RUFY1 immunoprecipitates (top). T7 antibody was used to detect the expression of T7-tagged Etk and its mutants (middle), and anti-FLAG antibody was used to detect the expression of the FLAG-tagged RUFY1 and its mutants (bottom). B, cellular localization of Rufy1 mutant lacking tyrosine phosphorylation. COS-1 cells were transfected with RUFY1-GFP (left) or RUFY1Y281F/Y292F-GFP (right) for 24 h. The images were captured as described in the legend to Fig. 2B. C, interaction between the SH2 domain of Etk with tyrosine-phosphorylated Tyr-281 and Tyr-292. COS-1 cells were transfected with the indicated constructs for 24 h. The association between Etk and the coiled-coil domains of RUFY1 (RUFY1(216–418)) or its mutants with Y281F or Y294F substitution was determined by immunoprecipitation using anti-Etk antibody followed by Western blot using anti-FLAG antibody (top). The expression of FLAG-tagged RUFY1 mutants and T7-tagged Etk was monitored by Western blot using either anti-FLAG (middle) or anti-T7 (bottom), respectively. D, interaction between the SH3 domain of Etk with the proline-rich motif of RUFY1. Experiments were performed as above.
FIG. 5. Modulation of the localization of EGFR by Etk and the FYVE domain of RUFY1. A, localization of EGFR in B82L cells. B82L cells were transfected with pcDNA3.1-EGFR-GFP-FLAG with the indicated constructs for 24 h. HA-tagged FYVE domain constructs (encoding amino acids 529–600 of RUFY1) were used in this study. The images were obtained as described in the legend to Fig. 2B. B, correlation between EGF-induced MAPK activation and the efficiency of membrane localization of EGFR. Transfections were performed as above. Activation of MAPK by EGF was determined by Western blot using anti-phospho-MAPK antibody. Expression of MAPK, EGFR, and FYVE domain was monitored by Western blot using the indicated antibody. C, regulation of ligand-induced endocytosis of the endogenous EGFR by Etk. Expression vectors containing the indicated genes were cotransfected with GFP marker into COS-1 cells. The cells were serum-starved for 6 h before they were treated with 100 ng/ml EGF for 15 min. Cells were then washed with cold phosphate-buffered saline and fixed immediately. EGFR was detected by immunofluorescence staining with an anti-EGFR antibody. Transfected cells were identified by the expression of GFP marker. D, cellular localization of the FYVE domain of RUFY1 and its mutants. HA-tagged FYVE domain (FYVE) and its mutants FYVE(PP/AA), FYVE(HH/SS), or FYVE(R/A) were detected by staining with anti-HA antibodies and visualized by a rhodamine-conjugated secondary antibody as described in the legend to Fig. 2A.
phosphorylation of these two residues is involved in its interaction with Etk. We tested the binding activity of RUFY1(216–418) and its mutants lacking the phosphorylation sites (Tyr-281 or Tyr-292) with Etk and its mutants lacking the SH2 domain. As shown in Fig. 4C, either deletion of the SH2 domain of Etk or mutation of the phosphorylation site(s) led to the loss of interaction between the two proteins. These data suggest that the SH2 domain of Etk interacts with the tyrosine-phosphorylated RUFY1 and that both phosphorylation sites (Tyr-281 and Tyr-292) appear to be required for efficient interaction with Etk.

To understand the mechanisms by which the SH3 domain of Etk binds to RUFY1 we examined the amino acid sequences of both RUFY1 and RUFY2 and identified a conserved SH3 domain recognition motif PXXPXP located in the FYVE domains of the RUFY family proteins. The substitution of two proline residues (Pro-578 and Pro-590) to alanines abolishes its binding to the Etk mutant lacking the SH2 domain (Fig. 4D). Taken together, these data suggest that there are two possible interfaces between Etk and RUFY1. One is the interaction between the SH2 domain of Etk with phosphorylated Tyr-281 or Tyr-292, and another is the interaction between the SH3 domain of Etk with the proline-rich motif in the FYVE domain. Thus, both SH3 and SH2 domains of Etk are involved in interaction with RUFY1.

Modulation of EGFR Localization by Etk and the FYVE Domain of RUFY1—To examine whether the interaction between Etk and RUFY1 plays a role in regulation of endocytosis of the membrane proteins, we cotransfected the EGFR-GFP fusion construct with Etk and the FYVE domain of RUFY1 into the B82L cell line that lacks endogenous EGFR. As shown in Fig. 5A, when EGFR-GFP was co-expressed with a vector control, EGFR-GFP was primarily localized in endoplasmic reticulum and Golgi, and a small fraction of EGFR-GFP was present diffusely on the plasma membrane. While co-expressed with Etk the amount of plasma membrane localized EGFR-GFP was dramatically increased. Interestingly, the Etk-induced plasma membrane localization of EGFR-GFP was significantly inhibited by expression of the FYVE domain of RUFY1 but not the FYVE domain mutant FYVE(PP/AA) that lacks the proline-rich motif recognized by the Etk SH3 domain. In parallel, we also examined the effects of Etk and the FYVE domain of RUFY1 on EGF-induced MAPK activation in these cells. Fig. 5B shows that Etk enhanced EGF-induced MAPK activation in B82L cells, presumably by increasing the level of EGF receptor on the plasma membrane because expression of Etk alone in B82L cells has no effect on MAPK phosphorylation (data not shown). Overexpression of the FYVE domain dramatically diminished EGF-induced MAPK activation, whereas the mutant FYVE(PP/AA) did not. Thus, there is a correlation between the amounts of plasma membrane-localized EGFR and the efficiency of EGF-induced MAPK activation in B82L cells. These data suggest that Etk might be able to modulate the basal endocytosis rate of EGFR in B82L cells either by preventing early endosome formation or by promoting the EGFR-GFP-containing endosome recycling back to the membrane. To address this issue we examined the effects of Etk and the FYVE domain of RUFY1 on endocytosis of the endogenous EGFR in EGF-treated COS-1 cells. As shown in Fig. 5C, in the control-transfected cells EGFRs underwent extensive internalization after 15 min of EGF-treatment, which is evidenced by the dramatic increase of EGFR in the punctate endosomes. While in the Etk-transfected cells a large fraction of EGFR remained on the plasma membrane, and greatly reduced internalization could be detected in comparison with its counterpart in the untransfected cells. Co-expression of the FYVE domain of RUFY1 but not the FYVE(PP/AA) mutant can reverse Etk effects. Our data suggest that Etk may enhance plasma membrane localization of EGFR and delay its ligand-induced endocytosis. The interaction between Etk and the FYVE domain of RUFY1 seems to be a negative regulator in this process.

To understand how the FYVE domain of RUFY1 exerts its effects on localization of EGFR, we examined the localization of the FYVE domain of RUFY1. To our surprise the FYVE domain was primarily localized on plasma membrane, whereas the FYVE(PP/AA) mutant was in cytosol (Fig. 5D). Membrane localization of the FYVE domain did not require lipid binding activity because the mutation of several critical residues (H554H555 and R557) in the lipid binding pocket of the FYVE domain had little effect on membrane targeting, although the corresponding mutations in Rabip4 and EEA1 have been shown to abolish the lipid binding activity of their FYVE domains (20, 21). These data suggest that the plasma membrane targeting of the FYVE domain of RUFY1 depends on the interaction between its proline-rich motif and the SH3 domain of Etk, and this seems to be correlated with its ability to inhibit the membrane localization of EGFR.

DISCUSSION

In this report we demonstrated that RUFY1 is a downstream effector of Etk. Similar to many FYVE domain-containing proteins, RUFY1 is localized predominantly to endosomes. However, the FYVE domain is dispensable for its endosomal targeting, which is similar to the case of Hrs (22). The two coiled-coil domains appear to be the determinate factor for endosomal localization of RUFY1. Phosphorylation of the two tyrosine residues, Tyr-281 and Tyr-292, located in the linker region of the two coiled-coil domains by Etk seems to be critical for RUFY1 targeting to the endosomes. Phosphorylation of these tyrosine residues may have an impact on the conformation of the coiled-coil domains and their interactions with some endosomal proteins that are essential for localizing RUFY1 to endosomes. Tyrosine phosphorylation of RUFY1 is required for its endosomal targeting, which coincides with the PI3-kinase inhibitor Wortmannin and blocks the endosomal localization of Rabip4 (20), a mouse homologue of RUFY1. These observations strongly suggest a role of Etk in the regulation of vesicle trafficking as a downstream effector of PI3-kinase. This theory is corroborated further by our observation that Etk can modulate the membrane localization of EGFR in B82L cells. Our preliminary data suggest that Etk may also regulate the membrane presentation of some other cell surface molecules such as integrins and adhesion proteins.2 This is in agreement with the observation that the XID (x-linked immune deficiency) cells lacking functional Btk have a tendency to form aggregates (1), which likely is caused by the deregulation of some plasma membrane proteins in these cells. It is possible that Etk and its homologues may exert their influence through phosphorylation of and/or interaction with some vesicle-associated molecules such as RUFY family proteins.

We also showed that two interfaces between Etk and RUFY1 possibly exist; one is the interaction between the SH2 domain of Etk and the phosphorylated Tyr-281 or Tyr-292 of RUFY1, and another is the SH3 domain of Etk and the proline-rich motif present in the FYVE domain of RUFY1. Further studies are necessary to understand whether these interactions occur simultaneously, coordinately, sequentially, or even separated spatially.

It is quite intriguing to us that the FYVE domain of RUFY1 is able to localize to the plasma membrane rather than go to endosomes or stay in the cytosol as many previously studied

2 Y. Qiu, unpublished data.
FYVE domains do (13–16, 21–24). This raises the possibility that the FYVE domain of RUFY1 may play a critical role in localizing the protein to the plasma membrane through interactions with SH3 domain-containing molecules such as Etk and possibly some other membrane-anchored adaptor proteins. We did observe that a small fraction of RUFY1 could be detected on the plasma membrane in fixed cells by immunostaining. Therefore, localization of RUFY1 during membrane trafficking may be dynamic, and it very likely may shuttle between the plasma membrane and endosomes. Its plasma membrane localization may be transient and much more difficult to capture, although its endosomal localization may be relatively steady and obvious. The predominant localization of RUFY1 at endosomes may have been overrepresented in the currently used overexpression system. Future studies based on inducible-expression systems and live cell images will provide more insight into the dynamics of RUFY1 during trafficking. Nevertheless, our studies have provided strong evidence that Etk, as a downstream tyrosine kinase of PI3-kinase, is involved in regulation of vesicle trafficking by interacting with vesicle-associated proteins such as RUFY family proteins. To our knowledge, this is the first example that an endosomal protein is directly regulated by a tyrosine kinase.

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