Src Kinase Regulates the Activation of a Novel FGD-1-related Cdc42 Guanine Nucleotide Exchange Factor in the Signaling Pathway from the Endothelin A Receptor to JNK

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Small GTPases act as binary switches by cycling between an inactive (GDP-bound) and an active (GTP-bound) state. Upon stimulation with extracellular signals, guanine-nucleotide exchange factors (GEFs) stimulate the exchange of GDP to GTP to shift toward the active forms of small GTPases, recognizing the downstream targets. Here we show that KIAA0793, containing substantial sequence homology with the catalytic Dbl homology domain of the faciogenital dysplasia gene product (FGD1), is a specific GEF for Cdc42. We, therefore, tentatively named it FRG (FGD1-related Cdc42-GEF). Src kinase directly phosphorylates and activates FRG, as Vav family GEFs. Additionally, FRG is involved in the signaling pathway from the endothelin A receptor to c-Jun N-terminal kinase, resulting in the inhibition of cell motility. These results suggest that FRG is a member of Cdc42-GEF and plays an important role in the signaling pathway downstream of G protein-coupled receptors.

Rho family small GTPases comprise a large branch within the Ras family of low molecular weight guanine nucleotide-binding proteins (1–4). One of the well known roles of Rho family GTPases is the regulation of the actin cytoskeleton (5, 6). Rho family GTPases RhoA, Rac, and Cdc42 induce the formation of stress fibers, lamellipodia, and filopodia, respectively (5). Furthermore, Rho family GTPases have been implicated in the control of diverse physiological responses, such as cell proliferation and cell motility as well as in physiopathological processes such as transformation and metastasis (7, 8).

Rho family GTPases adopt either an active GTP-bound or an inactive GDP-bound conformational state (2–4). Their activity is controlled positively by guanine nucleotide exchange factors (GEFs), which catalyze the replacement of GDP with GTP, and negatively by GTPase-activating proteins, which accelerate the endogenous GTPase activity (4, 9). Dbl family proteins are GEFs for Rho family GTPases and share a Dbl homology (DH) domain adjacent to a pleckstrin homology (PH) domain structure (4, 9). The DH domain is responsible for catalytic activity (10), and the PH domain appears to be necessary for proper localization and full activation (11–13). Numerous members of Rho family GEFs have been identified in mammals (9). Some GEFs are specific for each Rho family GTPase, whereas others show a broad activity (9). For example, p115RhoGEF/Lsc and Tiam1 are specific GEFs for RhoA (14–17) and Rac1 (18), respectively, and Vav acts as the GEF for RhoA, Rac1, and Cdc42 (19–25).

The molecular mechanism by which GEFs are activated is largely unknown, although it has been shown that Vav family GEFs are directly tyrosine-phosphorylated and activated by Src kinase (20–22).

The G protein-coupled receptors (GPCRs) are the largest family of human cell-surface receptors (26–29). Some GPCRs activate c-Jun N-terminal kinase (JNK), a subfamily of mitogen-activated protein kinases (28). This pathway depends on Src kinase and Rho family GTPases, Src kinase often acting upstream of Rho family GTPases (28, 30–32). We previously showed that endothelin, a ligand of GPCRs, activates JNK via the signaling pathway dependent upon Src kinase and Rho family GTPases Rac1 and Cdc42 (33). In this signaling pathway, Src kinase acts as an upstream regulator of Rac1 and Cdc42. However, the detailed mechanism linking Src kinase to Rho family GTPases remains unclear (34).

To clarify the mechanism of GPCR-mediated activation of Rho family GTPases, we performed a BLAST search using Dbl big sister (Dbs), a GEF involved in GPCR-mediated activation of Rho family GTPases (35), as a query. As a result, we found a novel Rho family GEF gene, KIAA0793. The KIAA0793 protein shows sequence similarity to a human faciogenital dysplasia gene product (FGD1), which is a specific activator of Cdc42 (36) and appears to be a family of related Cdc42-GEFs including mouse Fgd2 and Fgd3 (37) and mouse Frabin (38).

In the present study, we show that KIAA0793 is a specific GEF toward Cdc42, thus termed FRG (FGD1-related Cdc42-GEF). FRG is directly phosphorylated by Src kinase, which increases the Cdc42-GEF activity of FRG. We also demonstrate that FRG is involved in the signaling pathway linking the endothelin A (ETA) receptor to JNK, leading to the inhibition of cell motility. Taken together, our results indicate that FRG is a novel member of Cdc42-GEFs and may act as a direct regulator linking the Src kinase and Rho family GTPases in the chemorepellent GPCR-JNK-signaling pathway.

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* The abbreviations used are: GEF, guanine nucleotide exchange factor; FGD1, faciogenital dysplasia gene product; JNK, c-Jun N-terminal kinase; DH, Dbl homology; FERM, band 4.1, ezrin, radixin, and moesin homology; ET, endothelin; G protein, heterotrimeric guanine nucleotide-binding protein; PH, pleckstrin homology; GPCR, G protein-coupled receptor; HA, hemagglutinin; CA, constitutively activated form; GST, glutathione S-transferase.

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EXPERIMENTAL PROCEDURES

Antibodies—A mouse monoclonal antibody M2 against a FLAG-pep- tide was obtained from Sigma-Aldrich. A mouse monoclonal antibody 12CA5 against the hemagglutinin (HA) epitope was purchased from Roche Diagnostics. A mouse monoclonal antibody IE4 against Aequorea victoria green fluorescence protein was obtained from Medical and Biological Laboratories, Co., Ltd. (Nagoya, Japan). A mouse monoclonal anti-phosphorylated tyrosine antibody 4G10 was purchased from Upstate Biotechnology. A rabbit polyclonal antibody against the mammalian FLAG epitope was kindly provided by Dr. M. Karin (University of California, San Diego, CA). All antibodies were used at a 1:1000 dilution.

Inhibitors—P1 and P2, inhibitors of Src kinase, were purchased from Biozel (Plymouth Meeting, PA) and Calbiochem-Novabiochem, respectively. Clostridium difficile toxin B, which inhibits Rho family small GTPases, was obtained from Calbiochem-Novabiochem. Endothelin-1 was purchased from Pepitoide, Inc. (Osaka, Japan).

Plasmids—The cDNA of KIAA0793, GenBank® accession number A5918336, was kindly provided by Drs. F. Miki and T. Nagase (Kazusa DNA Research Institute, Chiba, Japan). The region encoding the KIAA0793 (FRG) protein (1055 amino acids) was subcloned into the mammalian HA tag expression vector pCMV-2HA. The fragments of FRG (ΔDH) and FRG (ΔDHHP) lacking amino acids 501–729 and 501–820, respectively, were inserted into pCMV-2HA. The portions encoding the FERM domain (1–500), the proline-rich domain (501–500), the DH domain (501–729), and the second PH domain (821–1055) were amplified from cDNA of FRG as a template and ligated into the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells using the method of reverse transcription polymerase chain reaction and were inserted the mammalian FLAG-tag expression vector pCMV-FLAG.
each membrane was measured as before. Activities were normalized to the amounts of FRG polypeptides in the immunoprecipitates.

In Vitro Tyrosine Phosphorylation—Cells were lysed in 900 μl of lysis buffer A for a 10-cm dish, and the lysates were centrifuged, as described above. Aliquots (800 μg) of the supernatants were used for an in vitro phosphorylation reaction using recombinant c-Src (6 units) in 30 μl of kinase buffer B containing 20 μM ATP at 30 °C for 15 min and then chilled on ice. The tyrosine-phosphorylated FRG protein was washed twice with an exchange buffer and used for an exchange reaction for Cdc42.

Cell Motility Assay—Cell motility was measured using a 24-well Boyden chamber (BD Biosciences) according to the manufacturer’s protocol. Briefly, upper wells with polyethylene terephthalate filters (8-μm pore size) were coated with 10 μg/ml extra cellular matrix E-C-L (Upstate). Serum-starved cells (2 × 10⁵ cells in 500 μl of Dulbecco’s modified Eagle’s medium per well) were loaded into upper wells, which were immediately plated on the chamber containing 165 nM endothelin-1 (750 μl of Dulbecco’s modified Eagle’s medium per well). After incubation at 37 °C for 5 h, upper filters were stained with a Diff-Quick staining kit (Biochemical Sciences Inc., Sterling Height, MI) according to the manufacturer’s protocol. Cells that had not migrated were wiped away from the inner surface of the upper wells. Using an optical microscope, the number of migrated cells was counted in at least three independent experiments.

Statistical Analysis—Statistical analysis was performed using SAS StatView 5.0. Values shown represent the mean ± S.E. from at least three independent experiments.

RESULTS

KIAA0793 Shows a Significant Homology to Cdc42-specific GEFs—We performed a BLAST search using the DH domain of Db because Dbs was implicated in the signaling pathway linking GPCRs to Rho family GTPases (35). The search revealed the appearance of several uncharacterized proteins exhibiting a putative DH domain. One of them, KIAA0793, was of particular interest and further characterized. Fig. 1 shows the structural features of KIAA0793 in comparison with those of other Rho family GEFs. This molecule contains a tandem of DH and two PH domains, the DH domain being closely related to those of CDEP (chondrocyte-derived ezrin-like domain-containing protein) (43, 44) (54% identity, 71% similarity), Frabin (38) (30% identity, 47% similarity), Fgd2 (30% identity, 51% similarity), and Fgd3 (37) (26% identity, 46% similarity). KIAA0793 contains an N-terminal region exhibiting extensive homology to the FERM domain, also called band 4.1 homology domain of ezrin, radixin, and moesin (ERM) proteins, which function as the cross-linker between plasma membranes and actin filaments (45, 46). KIAA0793 exhibits an additional structural feature, a prolinerich region (amino acids 398–498) (47).

KIAA0793 Activates Cdc42 but Not RhoA and Rac1—To examine which Rho family GTPases are activated by the DH and PH domains of KIAA0793, the ability of KIAA0793 to induce the dissociation of [3H]GDP from recombinant RhoA, Rac1, and Cdc42 proteins was measured. It has been demonstrated that the isolated fragment containing DH and PH domains of Rho family GEFs acts as a limited catalytic domain toward Rho family GTPases (12, 48). As shown in Fig. 2A, the isolated DH and PH fragment of KIAA0793 promoted nucleotide exchange on Cdc42 but not on RhoA or Rac1. In agreement with the release of [3H]GDP from Cdc42, the fragment of KIAA0793 promoted the binding of [32P]GTP to Cdc42 but not to RhoA and to Rac1 in a time-dependent manner (Fig. 2B). These results suggested that KIAA0793 may be the GEF toward Cdc42.

To confirm further whether KIAA0793 acts as the Cdc42-specific GEF in vivo, we measured the GTP-bound form of endogenous and exogenous RhoA, Rac1, and Cdc42 in 293T cells expressing the isolated DH and PH fragment. The RhoA binding domain of mDia1 was used to precipitate GTP-bound RhoA from the cell lysates (40). The Rac1 and Cdc42 interactive binding domain of aPakCRIB was used to pull down activated GTP-bound Rac1 or Cdc42 from the cell lysates (41). Fig. 2C shows that transfection of the DH and PH fragment of
Fig. 2. The catalytic domain of KIAA0793 can activate Cdc42. A and B, cells were transfected with a mock plasmid (open square) or the plasmid encoding KIAA0793-DH and -PH domains (closed circle). A, release of guanine nucleotide from RhoA (upper), Rac1 (middle), and Cdc42 (lower) was assayed as described under “Experimental Procedures.” B, binding of guanine nucleotide for RhoA (upper), Rac1 (middle), and Cdc42 (lower) was assayed as described under “Experimental Procedures.” C, cells were transfected with a mock plasmid, the plasmid encoding KIAA0793-DH and -PH domains, and the plasmids encoding wild-type or active Rho family GT-Pases. The active form of Rho family GT-Pases was measured by a pull-down assay using recombinant mDia1RBD or cPa-kCRI. Expression of Rho family GT-Pases in the cell lysates was estimated by immunoblot analysis (IB).
KIAA0793 to cells activates Cdc42 but not RhoA and Rac1, suggesting that KIAA0793 acts as the Cdc42-specific GEF. As discussed above, the DH domain is responsible for the nucleotide exchange activity for Rho family GTPases, and FGD1 and Frabin have been shown to behave as the Cdc42-specific GEFs (36, 38). Therefore, this newly identified molecule KIAA0793 is tentatively named FRG (FGD1-related Cdc42-GEF).

JNK Activation by Endothelin Is Mediated by Src kinase and Cdc42—It has been shown that GPCR-mediated activation of JNK involves Src kinase and Rho family GTPases (8, 30–33). To confirm whether the ETA receptor activates JNK dependent on Src kinase and Rho family GTPases in 293T cells, we added endothelin to cells that were transfected transiently with the plasmids encoding the ETA receptor (A–C), JNK (A–C), and dominant negative mutants of Rho family GTPases (C) and pretreated without or with 10 μM PP1 (B), 10 μM PP2 (B), or 0.2 ng/ml toxin B (C) for 16 h before the addition of 100 nM endothelin. The JNK activity was measured by the amount of □P radioactivity incorporated into recombinant c-Jun. D, cells were transfected with the plasmid encoding the ETA receptor and pretreated without or with 10 μM PP1 for 16 h before the addition of 100 nM endothelin. The active form of Rho family GTPases was measured by a pull-down assay using recombinant mDia1RBD or αPakCRIB. Expression of Rho family GTPases in the cell lysates was estimated by immunoblot analysis (IB).

Fig. 3. Activation of JNK induced by the ETA receptor is mediated by Src kinase and Cdc42. A–C, cells were transfected with the plasmids encoding the ETA receptor (A–C), JNK (A–C), and dominant negative mutants of Rho family GTPases (C) and pretreated without or with 10 μM PP1 (B), 10 μM PP2 (B), or 0.2 ng/ml toxin B (C) for 16 h before the addition of 100 nM endothelin. The JNK activity was measured by the amount of □P radioactivity incorporated into recombinant c-Jun. D, cells were transfected with the plasmid encoding the ETA receptor and pretreated without or with 10 μM PP1 for 16 h before the addition of 100 nM endothelin. The active form of Rho family GTPases was measured by a pull-down assay using recombinant mDia1RBD or αPakCRIB. Expression of Rho family GTPases in the cell lysates was estimated by immunoblot analysis (IB).

JNK Activation by Endothelin Is Mediated by Src Kinase and Cdc42—It has been shown that GPCR-mediated activation of JNK involves Src kinase and Rho family GTPases (8, 30–33). To confirm whether the ETA receptor activates JNK dependent on Src kinase and Rho family GTPases in 293T cells, we added endothelin to cells that were transfected transiently with the plasmid encoding cDNA of the ETA receptor and HA-tagged JNK. Using an anti-HA antibody, the epitope-tagged JNK was immunoprecipitated from the cell lysates, and the in vitro kinase activity was assessed as the radioactivity incorporated into recombinant c-Jun. The peak of JNK activation was observed 20 min after the addition of endothelin (Fig. 3A).

Next we examined the involvement of Src kinase and Rho family GTPases in endothelin-induced JNK activation. As shown in Fig. 3B, pretreatment of cells with PP1 or PP2, specific inhibitors of Src kinase, inhibited the activation of JNK induced by endothelin. Pretreatment with C. difficile toxin B,
which glycosylates Rho family GTPases RhoA, Rac1, and Cdc42 and inhibits their intracellular functions, also blocked JNK activation elicited by endothelin (Fig. 3C). Additionally, the endothelin-induced activation of JNK was suppressed only by the dominant-negative mutant of Cdc42 but not by the dominant-negative mutants of RhoA and Rac1. These results suggest that endothelin may activate JNK through Src kinase and Cdc42.

To investigate whether Rho family GTPases are activated by endothelin, we measured the active form of Rho family GTPases in cells. As shown in Fig. 3D, stimulation of endothelin activates Cdc42 but not RhoA and Rac1. Pretreatment of cells with PP1 inhibited the endothelin-induced activation of Cdc42. Again, these results indicate that endothelin induces Cdc42 activation, which may be regulated by Src kinase.

**FRG Is Involved in the Signaling Pathway from the ETA Receptor to JNK**—To examine the involvement of FRG in the signaling pathway induced by endothelin, we co-transfected the plasmids encoding the ETA receptor with FRG (ΔDHPH) or FRG (ΔDH). When transfected into cells, FRG (ΔDHPH), which is the deletion mutant lacking the DH and PH domains, and FRG (ΔDH), which is the deletion mutant lacking the DH domain, were detectable with an anti-HA antibody (Fig. 4A). It is known that the mutant lacking DH and PH domains and the mutant carrying the mutation within the DH domain of Rho family GEF show a dominant inhibitory effect (49, 50). As shown in Fig. 4B, the expression of the deletion mutants of FRG suppressed endothelin-induced activation of Cdc42, suggesting that FRG is involved in the activation of Cdc42 after the stimulation of endothelin. Likewise, an in vitro kinase assay revealed that JNK activation induced by endothelin was inhibited by co-transfection of FRG (ΔDHPH) or FRG (ΔDH) (Fig. 4C). Taken together with the data of Fig. 4B, these results indicate that FRG may be a candidate of Rho family GEF for the signaling pathway from the ETA receptor to JNK.

**FERM and Proline-rich Regions of FRG May Be Responsible for the Endothelin-induced JNK Activation**—To identify the functional domains of FRG responsible for the signaling pathway from the ETA receptor to JNK, we utilized the variants including the FRG-FERM domain (FRG-FERM), the FRG-proline-rich domain (FRG-Proline), the FRG-DH and -PH domains (FRG-DHPH), and the FRG-second PH domain (FRG-2ndPH). Each fragment was detected with an anti-FLAG antibody in the transfected cells (Fig. 4A). As shown in Fig. 4D, the endothelin-induced activation of JNK was blocked by FERM and the proline-rich regions of FRG. On the other hand, the DH and PH domains of FRG induced an increase of JNK activity without endothelin stimulation (Fig. 4E), suggesting that FRG is involved in the endothelin-induced inhibition of the cell motility signaling pathway. These results indicate that FRG may be a candidate of Rho family GEF for the signaling pathway from the ETA receptor to JNK.

**Involvement of FRG in the Inhibition of Cell Motility Dependent on the JNK Pathway Induced by the ETA Receptor**—We previously reported that the endothelin inhibits cell motility through the JNK pathway (33). To examine whether FRG inhibits the cell motility, we transfected the plasmid encoding the catalytic DH and PH domains of FRG into cells. As shown in Fig. 4F, the DH and PH domains inhibited cell motility. In addition, MKK4K95R, a kinase-deficient variant that inhibits the transmission of signals from GPCRs to JNK (30–32), rescued the FRG-DH and -PH domain-induced inhibition of the cell motility. These results suggest that FRG suppresses cell motility through the JNK pathway.

As indicated before, the endothelin-induced activation of the JNK pathway involved FRG. Thus, we investigated the involvement of FRG in the endothelin-induced inhibition of the cell motility. As shown in Fig. 4G, co-transfection of FRG (ΔDHPH) or FRG (ΔDH) with the ETA receptor into cells blocked the inhibition of the cell motility induced by endothelin. Taken together with the results of Fig. 4F, these results indicate FRG is involved in the endothelin-induced inhibition of the cell motility through the JNK pathway.

Furthermore, to explore whether other portions of FRG are involved in the endothelin-induced inhibition of the cell motility, we transfected the variants of FRG, FRG-FERM, FRG-Proline, and FRG-2ndPH into cells. As shown in Fig. 4H, FRG-FERM rescued the inhibition of cell motility induced by endothelin, suggesting the involvement of the FERM region of FRG in the inhibition of the cell motility signaling pathway linking the ETA receptor to JNK.

**Src Kinase Directly Regulates the Activation of the Cdc42-GEF Activity in Cells and in Vitro**—As shown in Fig. 3D, we showed that Src kinase is required for the activation of Cdc42 in the pathway downstream of the ETA receptor. Thus, we investigated whether Src kinase activates the Cdc42-GEF activity in cells. We transfected the plasmid encoding CA-Src, a constitutively activated form of c-Src, with FRG into cells. Transfection of CA-Src with FRG activated the Cdc42-GEF activity of FRG (Fig. 6A). Additionally, the activation of the Cdc42-GEF activity by CA-Src was prevented by pretreatment with PP1.

Next we examined the possibility that Src kinase induces tyrosine phosphorylation of FRG in cells. As shown in Fig. 6A, CA-Src was able to induce tyrosine phosphorylation of FRG in cells. PP1 inhibited the CA-Src-induced tyrosine phosphorylation of FRG in cells. These results suggest that Src kinase contributes to the activation and tyrosine phosphorylation of FRG in cells.

The well known mechanism by which Rho family GEFs are activated is that of Vav family GEFs, which are directly tyrosine-phosphorylated and activated by Src kinase (20). Thus, we next examined whether the immunoprecipitated FRG is directly tyrosine-phosphorylated and activated by recombinant c-Src in vitro. As shown in Fig. 6B, in vitro tyrosine-phosphorylated FRG exhibited and enhanced the Cdc42-GEF activity of FRG. This result suggests that Src kinase directly phosphorylates FRG and activates the Cdc42-GEF activity of FRG in vitro.

Then we investigated whether the endothelin-induced activation of the Cdc42-GEF activity of FRG requires Src kinase. As shown in Fig. 6C, the endothelin-induced activation of the Cdc42-GEF activity of FRG was blocked by pretreatment with PP1 and was inhibited by PP2 (data not shown). Thus, it is likely that Src kinase regulates the activation of the Cdc42-GEF activity of FRG in cells.

Additionally, we examined whether endothelin causes tyrosine phosphorylation of FRG in cells. Fig. 6C indicated that endothelin stimulated the tyrosine phosphorylation of FRG. The endothelin-induced tyrosine phosphorylation was inhib-
FIG. 4. Involvement of FRG in the signaling pathway from the ETA receptor to JNK. A, structure and expression of variant mutants of FRG. Structures of the proteins encoded by the expression plasmids used in this study are shown (upper). Lysates from cells transfected with a vector or with the expression plasmid encoding FRG, FRG (ΔDHPH), FRG (ΔDH), the FRG-FERM domain (FRG-FERM), the FRG-proline rich domain (FRG-Proline), the FRG-DH and -PH domains (FRG-DHPH), and the FRG-second PH domain (FRG-2ndPH) were immunoprecipitated (IP) and then immunoblotted (IB) with an anti-HA antibody (middle) or an anti-FLAG antibody (lower). B, cells were transfected with the plasmids encoding the ETA receptor, FRG (ΔDHPH) and FRG (ΔDH). The active form of Cdc42 was measured by a pull-down assay using recombinant αPakCRIB. Expression of Cdc42 in the cell lysates was estimated by immunoblot analysis. C–E, cells were transfected with the plasmids encoding the ETA receptor (C–D), JNK (C–E), and variant mutants (C–E). The JNK activity was measured 20 min after the addition of 100 nM endothelin by the amount of 32P radioactivity incorporated into recombinant c-Jun. Asterisks indicate $p < 0.01$ (n = 5) as compared with a vector under endothelin stimulation (D). F, cells were transfected with the plasmids encoding FRG-DH and -PH domains and MKK4K95R. Forty-eight h post-transfection, cell motility was measured using a Boyden chamber. G and H, cells were transfected with the plasmids encoding the ETA receptor and the variant mutants of FRG. Forty-eight h post-transfection, cell motility was measured using a Boyden chamber. After incubation at 37°C for 5 h with (+) or without endothelin (−), cells attached to the filters were stained and analyzed under a microscope (G, upper photos). The number of stained cells was counted (G, lower figure, and H). Asterisks indicate $p < 0.01$ (n = 5) as compared with a vector under endothelin stimulation (H).
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Fig. 5. Activation of the Cdc42-GEF activity of FRG upon stimulation of the ETA receptor. A–B, cells were transfected with a mock plasmid (open squares) or the plasmids encoding the ETA receptor and FRG (open circles and close circles) and treated without (open circles) or with (close circles) 100 nM endothelin. A, release of guanine nucleotide from RhoA (upper), Rac1 (middle), and Cdc42 (lower) by immunoprecipitated FRG was assayed as described under “Experimental Procedures.” B, binding of guanine nucleotide for RhoA (upper), Rac1 (middle), and Cdc42 (lower) was assayed as described under “Experimental Procedures.”

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\textit{DISCUSSION}

Although there are many Rho family RAFs (9), it remains unclear how these Rho family RAFs are activated by extracellular signals. In the present study, we performed a BLAST search using the DH domain of Dbs, a RAF involved in the GPCR-Rho family GTPase signaling pathway, as a query. We selected one putative RAF, KIAA0793, which has homology with the FGD1-related family. It has been shown that FGD-1 and Frabin, the members of FGD1-related family, are the RAFs for Cdc42 (36, 38). Using \textit{in vivo} and \textit{in vitro} assays, we demonstrated that KIAA0793 is also a Cdc42-specific RAF. We, therefore, refer to this RAF as FRG (FGD1-related Cdc42-GEF). FRG is phosphorylated and activated by Src kinase, which increases the Cdc42-GEF activity of FRG. Moreover, FRG is involved in the signaling pathway linking the ETA receptor to JNK, leading to the inhibition of cell motility. On the basis of these findings, we summarized the proposed sig-
naling pathway in Fig. 7. It is noteworthy that CDEP, which shows a significant homology with FRG, acts as a RhoA-specific GEF (44). It is of interest to examine whether FRG may activate RhoA under a certain condition, such as upon stimulation of receptor other than GPCRs.

GPCRs or Gα/β has been reported to stimulate Rho family GEFs. For example, lysophosphatidic acid or sphingosine-1-phosphate activates G13 through their GPCRs (16). The GTP-bound Gα13 directly induces the activation of p115RhoGEF, which is a RhoA-specific GEF and belongs to a subfamily of the

Fig. 6. Src kinase-dependent activation of the Cdc42-GEF activity of FRG in cells and in vitro. A, cells were transfected with a mock plasmid (open squares) or the plasmids encoding CA-Src and FRG (open circles and closed circles) and pretreated without (open circles) or with (closed circles) 10 μM PP1. Binding of guanine nucleotide to Cdc42 was assayed as described under “Experimental Procedures.” HA-FRG was immunoprecipitated (IP) with an anti-HA antibody from the cell lysates and immunoblotted (IB) with an anti-phosphorylated tyrosine antibody (upper) or an anti-HA antibody (middle). Expression of CA-Src is also shown (lower). B, the immobilized FRG was incubated without (open squares) or with (closed circles) recombinant c-Src, and binding of guanine nucleotide to Cdc42 was assayed as described under “Experimental Procedures.” Tyrosine phosphorylation of FRG was analyzed using immunoblotting with an anti-phosphorylated tyrosine antibody (upper). Tyrosine-phosphorylated c-Src (middle) and expression of FRG (middle) are shown. C, cells were transfected with a mock plasmid (open squares) or the plasmids encoding the ETA receptor and FRG (open circles and closed circles) and pretreated without (open circles) or with (closed circles) 10 μM PP1 for 16 h before the addition of 100 nM endothelin (open circles and closed circles). Binding of guanine nucleotide to Cdc42 was assayed as described under “Experimental Procedures.” HA-FRG immunoprecipitated with an anti-HA antibody from the cell lysates was immunoblotted with an anti-phosphorylated tyrosine antibody (upper) or an anti-HA antibody (middle). Expression of Src (lower) and the ETA receptor (lower) is shown. D, cells were transfected with the plasmids encoding CA-Src and variant mutants of FRG, FRG-FERM (first panel), FRG-Proline (second panel), FRG-DHPH (third panel), and FRG-2ndPH (fourth panel) and pretreated without or with 10 μM PP1 for 16 h. FLAG-FRG mutants were immunoprecipitated with an anti-FLAG antibody from the cell lysates and immunoblotted with an anti-phosphorylated tyrosine antibody (upper). Expression of FRG mutants is shown (lower).
regulator of G protein signaling domain-containing GEF, such as PDZ-RhoGEF (51, 52) and LARG (53–56). Recently, it has been reported that PDZ-RhoGEF forms a complex with GoqGTP, which appears to regulate its GEF activity (57). Furthermore, Gβγ directly activates a novel type of the Rac-GEF P-Rex1 synergistically with phosphatidylinositol 3,4,5-triphosphate (58). However, it remains to be investigated whether these Rho family GEFs act downstream of GPCRs/Gq/G11/G12 (59). Recently, it has been reported that PDZ-RhoGEF forms a complex with Src family kinase (20–22). Phosphorylation or analysis revealed that the DH domain of Vav is autoinhibited by Src-dependent activation of Cdc42-GEF FRG by endothelin

because of the difference of the signaling pathway and/or conditions of the experiments. It will be interesting to examine whether FRG is activated by Src kinase in the signaling pathway of neurite remodeling.

In this study, we identified a new signaling molecule, FRG, which directly links between Src kinase and Rho family GTPase Cdc42 in the GPCR/JNKs signaling pathway. Recently, it was indicated that tyrosine kinases are involved in the G protein-induced activation of Rho family GEFs (60, 61). Further study is necessary to clarify how Src kinase regulates Rho family GEFs in the pathway linking GPCRs to Rho family GTPases. Additionally, such studies might promote our understanding of the general mechanism by which Src kinase regulates Rho family GEFs and thereby help to elucidate the cellular function of the pathway.

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