Cdc42-dependent regulation of cell migration through the G protein-coupled receptor

（G タンパク質共役受容体による Cdc42 依存的細胞遊走の制御）

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Contents

Abbreviations

CA, constitutively activated form

DH, Dbl-homology

EGF, epidermal growth factor

ERK, extracellular signal-regulated protein kinase

ET, endothelin

ETA, endothelin type A

FERM, band 4.1, ezrin, radixin, and moiesin homology

FGD1, faciogenital dysplasia gene product 1

FRG, Fgd-1-related Cdc42 guanine nucleotide exchange factor

GAP, GTPase-activating protein

GEF, guanine nucleotide exchange factor

GFP, green fluorescence protein

GPCR, G protein-coupled receptor

G protein, heterotrimeric guanine nucleotide-binding protein

GST, glutathione-S-transferase

HA, hemagglutinin

JNK, c-Jun N-terminal kinase

MAPK, mitogen-activated protein kinase

MBP, maltose-binding protein

SH, Src homology

PH, pleckstrin homology

General Introduction

Cell migration is essential process in multicellular organisms and plays key roles not only during development, but also throughout homeostasis such as wound repair and immune responses. It also occurs in chronic human diseases including cancer, atheroscleorosis, and inflammatory diseases. Therefore, it is critical to elucidate the molecular mechanisms of the migration processes.

It is believed that cell migration can be divided into four mechanistically separate steps; lamellipodial extension, formation of new adhesions, cell body contraction, and tail detachment (1) (Figure 1). All these steps involve the assembly, the disassembly, or the reorganization of the actin cytoskeleton. Each step must be coordinated to generate forward movement spatially and in time.

Generally, cell migration is regulated by extracellular cues acting either as attractants or repellents. These cues stimulate transmembrane receptors to initiate the intracellular signaling pathway. They elicit a large variety of intracellular responses that include changes in the actin cytoskeleton, vesicular transport, and gene expression. Thus, the migration processes involve different types of intracellular signaling molecules: serine/threonine kinases, tyrosine kinases, phosphatidyl inositide kinases, adaptor proteins, and small GTPases. Among them, Rho family small GTPases play a pivotal role in regulating cell migration (2, 3).

Rho GTPases are ubiquitously expressed, and at least 20 members have been identified in mammals, 7 in *Drosophila melanogaster*, 5 in *Caenorhabditis elegans*, and 15 in *Dictyostelium discoideum* (4) (Table 1). Their best-characterized function is to regulate the actin cytoskeleton. Tissue culture studies (carried out originally in fibroblasts) have shown that Rho regulates the assembly of contractile, actin:myosin filaments (called stress fibers), while Rac and Cdc42 regulate the polymerization of actin to form peripheral lamellipodial and filopodial protrusions

(called lamellipodia and filopodia), respectively (5) (Figure 2). Thus, these three Rho GTPases are thought to participate in controlling cell migration. Furthermore, Cdc42 is uniquely required for the establishment of cell polarity, and it has been recently shown that these Rho GTPases can affect the microtubule assembly (6).

Like the ohter GTPases, Rho GTPases act as molecular switches to control signal transduction by cycling between an inactive GDP-bound and an active GTP-bound conformational state (Figure 3). Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP for GTP to generate the activated form, which is then capable of recognizing downstream effectors to elicit a variety of intracellular processes (7, 8). On the other hand, GTPase-activating proteins (GAPs) negatively regulate the switch by enhancing its intrinsic GTPase activity (9). Guanine nucleotide dissociation inhibitors (GDIs) block the GTPase cycle by sequestering and localizing the GDP-bound form in cytosol.

The first mammalian GEF, diffuse B-cell lymphoma (Dbl), isolated as an oncogene in the NIH 3T3 focus formation assay (10), was found to contain a region of 180 amino acids that displayed significant sequence similarity to CDC24, which is the upstream activator of CDC42 in yeast (11). Dbl catalyzes nucleotide exchange on human Cdc42 *in vitro*, and conserved domain in Dbl and CDC24, now known as the DH (Dbl homology) domain, is necessary for the GEF activity (12). To date, at least 60 GEFs have been postulated in humans, 23 in *Drosophila melanogaster*, 18 in *Caenorhabditis elegans*, 6 in *Saccharomyces cerevisiae* (7).

Rho family GEFs possess the pleckstrin homology (PH) domain adjacent to the DH domain, and in most cases the DH-PH module is the minimal structural unit that can promote nucleotide exchange. The PH domain is also known to bind to phosphatydil inositol phosphates (PIPs) (13). It is likely that the PH domain affects the catalytic activity of the DH domain, and helps

translocate GEFs to an appropriate intracellular region. Some GEFs contain additional functional domains that include Src homology (SH) 2, SH3, serine/threonine or tyrosine kinases, Ras-GEF, Rho-GAP, Rab-GEF, Ran-GEF, PSD-95/Dlg/ZO-1 (PDZ), or additional DH and PH (7). These domains appear to couple GEFs to upstream receptors and/or downstream signaling molecules.

As far as I know, little is known about the mechanism of cell migration regulated by G protein-coupled receptor (GPCR). In chapter 1, I show that the adaptor protein Nck1, but not Grb2 or CrkII, mediates the inhibition of cell migration induced by the endothelin-1 (ET-1) and endothelin type A (ETA) receptor through Cdc42-dependent c-Jun N-terminal kinase (JNK) activation. In chapter 2, I show that a novel GEF (named FRG) containing the substantial sequence homology with DH domain of the faciogenital dysplasia gene product (FGD1), and that this is a specific GEF for Cdc42. FRG is also involved in the signaling pathway from the ETA receptor to JNK, leading to the inhibition of cell migration. Together, these findings provide a novel mechanism whereby GPCR regulates cell migration in mammalian cells.

Table 1 Rho family members in humans, *Drosophila melanogaster, C.elegans,* and *Dictyostelium discoideum*

Subfamily	Humans	*Drosophila*	*C.elegans*	*Dictyostelium*
Rho	RhoA, B, C	Rho1	Rho1	None
Rac	Rac1, 2, 3/1B	Rac1, 2	Rac1/CED10, Rac2	Rac1a, b, c, RacF1, 2, RacB
Cdc42	Cdc42, TC10 TCL, Chp1, 2	Cdc42	Cdc42	None
Mig2	RhoG	Mt1/Mig-like	Mig2	None
Rnd	Rnd1, 2, RhoE/Rnd3	None	None	None
RhoBTB	RhoBTB1, 2	RhoBTB	None	RacA
Others	RhoD, Rif, TTF/RhoH	RhoL/Rac3	None	RacC, D, E, RacG-J, L

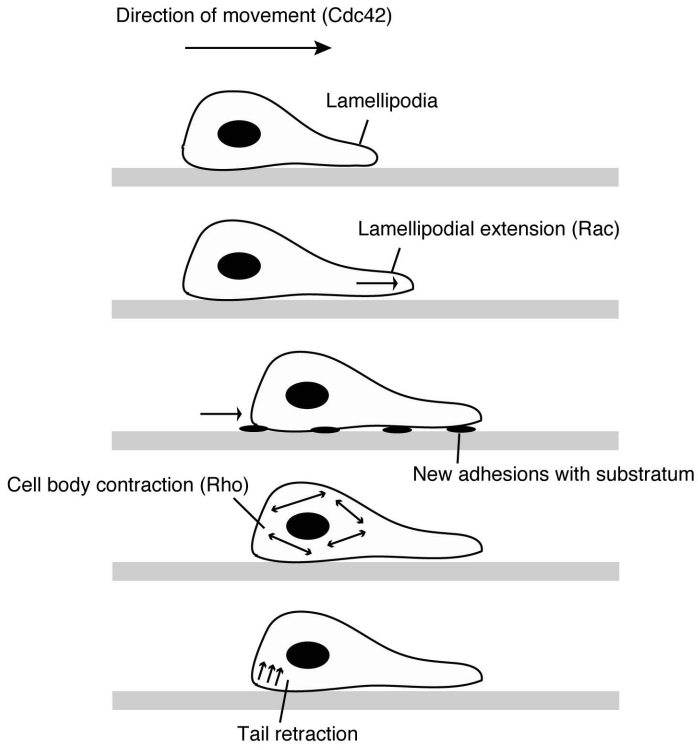

Figure 1. A model for the steps of cell migration. A migrating cell needs to perform a coordinated series of steps to move. Cdc42 regulates the direction of migration. Rac induces membrane extension, and Rho promotes cell body contraction. Finally, the tail of the cell detaches from the substratum and retracts.

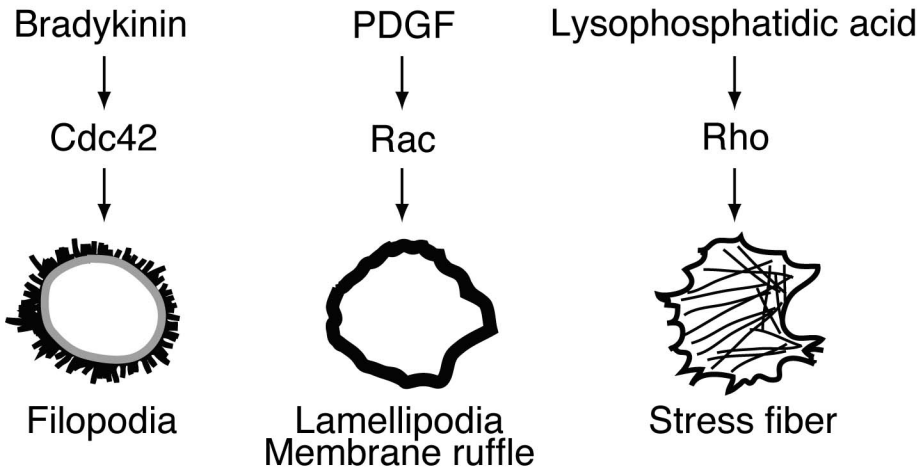

Figure 2. Rho, Rac, and Cdc42 control the assembly and organization of the actin cytoslkeleton in fibroblasts. Addition of bradykinin activates Cdc42, which leads to formation of filopodia. The growth factor PDGF induces Rac activation and lamellipodia. Lysophosphatidic acid activates Rho, leads to stress fiber.

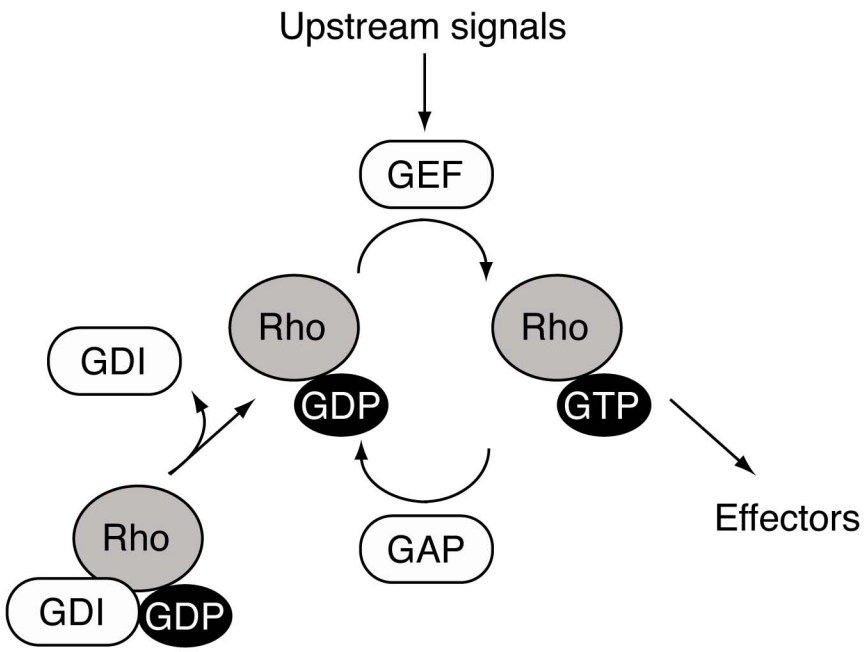

Figure 3. The Rho GTPase cycle. Rho GTPases cycle between an inactive GDP-bound form and an active GTP-bound form. The cycle is regulatled by GEFs, GAPs, and GDIs.

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Chapter 1

The adaptor protein Nck1 mediates ET-1/ETA receptor-regulated cell migration through the Cdc42-dependent c-Jun N-terminal kinase pathway

Introduction

Cell migration plays key roles in development and oncogenesis. During development, organized cell migration is essential for proper tissue formation (1). However, unregulated cell migration is observed in a pathological process, such as oncogenesis, which involves, specifically, invasion and metastasis. The elucidation of the molecular signaling mechanisms that positively and negatively regulate migration is critical in understanding the development process as well as these diseases.

In many cases of the migration process, cells show directed movement (called chemotaxis) toward soluble chemoattractants. A number of chemoattractants have been identified: chemokines, lipid mediators, growth factors, and cytokines (1, 2). Chemokines bind and activate their cognate chemokine receptors, which belong to a large family of G protein-coupled receptors (GPCRs) characteristic of the seven-transmembrane structure (3-6). In contrast, there is only limited information about the inhibitors of chemotaxis, i.e. chemorepellents, which are also the ligands of GPCRs. These include bioactive lipids, thrombin, metastin and μ–opioid peptide (7-10). However, the mechanism by which GPCRs inhibit migration is not fully understood so far.

Adaptor proteins, such as Nck1, Grb2, and CrkII, consist primarily of a single Src homology (SH) 2 domain and various numbers of SH3 domains and do not have other functional motifs (11, 12). The SH2 domain recognizes tyrosine-phosphorylated proteins, and the SH3 domain associates with the PXXP motif-containing proteins (13). These domains provide a site to couple the distinct molecules to the core machinery that regulates the cellular function and gives specificity to the signal transduction. Among them, Grb2 provides a well-characterized example

of how adapter proteins of this group function to transduce signals. Grb2 constantly associates with a guanine-nucleotide exchange factor (GEF) Sos of Ras GTPases via the SH3 domains (14). Following growth factor stimulation, Grb2 binds to tyrosine-phosphorylated receptor tyrosine kinases and/or other adaptor proteins, such as Shc, via its SH2 domain. As a result, Sos is translocated to the plasma membranes, leading to Ras-dependent activation of extracellular signal-regulated protein kinase (ERK), a subfamily of mitogen-activated protein kinases (MAPKs) (15, 16).

I demonstrated that the ET-1/ETA receptor activates Cdc42 of Rho GTPases, which in turn stimulates the signaling cascade of c-Jun N-terminal kinase (JNK), a subfamily of MAPKs (17). This signaling pathway is involved in the inhibition of cell migration (17). To further investigate the mechanism whereby the ET-1/ETA receptor inhibits cell migration, the effects of various dominant-negative mutants on cell migration were assayed on the transient transfection system using human epithelial 293T cells. Here, I show that the Nck1, but not Grb2 or CrkII, is a critical regulator in the chemorepellent signaling pathway coupling the ET-1/ETA receptor to Cdc42-dependent activation of JNK. Additionally, membrane-targeted Nck1 inhibited cell migration. These results suggest that Nck1 functions as a mediator of the chemorepellent signaling downstream of the ET-1/ETA receptor.

Materials and Methods

Materials--- Mouse monoclonal antibodies against active phosphorylated JNK (Thr^{183}/Tyr^{185}), active phosphorylated ERK (Thr^{202}/Tyr^{204}), and rabbit polyclonal antibodies against JNK and ERK were purchased from Cell Signaling Technology, Inc (Beverly, MA). A mouse monoclonal antibody against MBP and rabbit polyclonal antibodies against JNK1 and c-Src were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibodies against Cdc42 and Nck1 were purchased from BD Biosciences Transduction Laboratories (San Jose, CA). A mouse antibody against *Aequorea victoria* green fluorescence protein (GFP) was obtained from Medical and Biological Laboratories (Nagoya, Japan). A mouse monoclonal antibody against tubulin was purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse and anti-rabbit IgG antibodies conjugated with horseradish peroxidase were obtained from Amersham Biosciences (Buckinghamshire, UK). SP600125, U0126, SB203580, and PP1 were purchased from Biomol (Plymouth Meeting, PA), and *Clostridium difficile* toxin B was from Carbiochem-Novabiochem (San Diego, CA). Endothelin-1 and epidermal growth factor (EGF) were purchased from the Peptide Institute, Inc. (Osaka, Japan) and Roche Diagnostics Co. (Indianapolis, IN), respectively.

Plasmids --- Wild-type Nck1, Grb2 and CrkII were amplified from a mouse brain cDNA library and subcloned into the mammalian MBP-tag expression vector pCMV-MBP. Wild-type Nck1 was also ligated into the pEGFP-N3 (BD Biosciences Clontech, Franklin Lakes, NJ) to make pEGFP-Nck1-N3. To produce dominant negative mutants of Nck1 and CrkII, the conserved arginine residue of the FLVRES sequence in the SH2 domain was changed into lysine, making Nck1R308K and CrkIIR38K, or the first tryptophan residue of the characteristic tryptophan

doublet of the SH3 domain was changed to lysine, creating Nck1W38K, Nck1W143K, Nck1W229K, CrkIIW169K, and CrkIIW275K. These residues are identified as being essential for binding to their ligands (18). The amino acid substitutions were performed by the overlap extension method based on polymerase chain reaction (PCR) with mutant oligonucleotides. These mutants were ligated into the pCMV-MBP. Mutations in all three Nck1 SH3 domains (designed SH3All in this paper) were carried out by sequentially repeating the above procedure. The fragments of Grb2ΔSH2 lacking the SH2 domain (amino acids 60-158) and Grb2ΔSH3 lacking the SH3 domain (amino acids 1-60 and 159-218) of Grb2 were inserted into pCMV-MBP. For the myristoylated clones, the 14 amino-acid c-Src myristoylation signal was first synthesized by PCR (18). The PCR product was digested and cloned into pEGFP-N3 (BD Biosciences Clontech, Franklin Lakes, NJ) to make the pEGFP-myr-N3 plasmid. Nck1 was subcloned into pEGFP-myr-N3. pCMV-FLAG-RhoT19N, pCMV-FLAG-RacT17N, pCMV-FLAG-Cdc42T17N, and the *Escherichia coli* expression plasmid encoding the Cdc42-binding domain (CRIB) of αPak were constructed as described previously (19, 20). pUSE-CA-Src (a constitutively activated mutant of c-Src) was purchased from Upstate Inc. (Charlottesville, VA). pME-ETA receptor-EGFP was generously provided by Dr. T. Sakurai (Tsukuba University, Tsukuba, Japan) (21).

Cell culture and transfection --- Human epithelial 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 μg/ml kanamycin and 10% heat-inactivated fetal bovine serum. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Plasmid DNAs were transfected into cells by the calcium-phosphate precipitation method. Transfection efficiency typically exceeded 80% using the pEGFP-C1 (BD biosciences Clontech) as a control plasmid in 293T cells. The final amount of transfected DNA for 60-mm dish was adjusted to 15 μg by pCMV. The pME-ETA receptor-EGFP (0.3 μg) was cotransfected with the

dominant negative mutants of pCMV-MBP-adaptor proteins (1-3 μg) or pCMV-FLAG-Rho GTPases (1 μg) into 293T cells. In some cases, cells were transfected with 1 μg of pUSE-CA-Src, 1 μg of pEGFP-Nck1-N3, or 1 μg of pEGFP-myr-Nck1-N3. The medium was replaced 24 h after transfection, and cells were starved in a serum-free medium. Cells were pretreated with SP600125 (100 nM, 45 min), U0126 (10 μM, 1 h), SB203580 (10 μM, 1 h), PP1 (1 μM, 16 h), or ToxinB (0.2 ng/ml, 16 h) before stimulation with ET-1 (100 nM, 20-30 min) or EGF (10 ng/ml, 10 min).

siRNA preparation and transfection --- RNA oligonucleotides were synthesized by Dharmacon, Inc. (Lafayette, CO). GFP siRNA was used as a negative control. The siRNAs were transfected into 293T cells using Lipofectamine™ 2000 transfection reagent (Invitrogen Co., Carlsbad, CA), according to manufacturer's protocol.

Immunoprecipitation and immunoblotting --- After the addition of ET-1 or EGF, cells were lysed in 600 μl of a lysis buffer (20 mM HEPES-NaOH (pH 7.5), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 20 mM β-glycerophosphate, and 0.5% NP-40) per 60-mm dish (17). Immunoprecipitation was carried out as described previously (17). The immunoprecipitates or the expressed proteins in the cell lysates were denatured and then separated on SDS-polyacrylamide gels. The electrophoretically separated proteins were transferred to membranes, blocked, and immunoblotted as described previously (17). Images of protein bands in immunoblots were captured using Adobe Photoshop 5.0-J plug-in software and an EPSON GT-7300U scanner. The band intensity of kinases and Rho GTPases was semiquantified using NIH Image 1.61. The representatives of at least three separate experiments are shown in figures.

MAPK assay --- Immunoprecipitated JNK or cell lysates were immunoblotted with an anti-

phosphorylated JNK antibody or anti-phosphorylated ERK antibody (22). To compare the total amounts of MAPKs in the lysates, the immunoblottings were also performed using an anti-JNK or anti-ERK antibody. The levels of phosphorylated forms were normalized to the total amounts of kinases using NIH Image 1.61. Approximately, 10% of the total JNK were immunoprecipitated with anti-JNK antibody, and 20% of the immunoprecipitated JNK were phosphorylated after stimulation with ET-1. The representatives of four to six separate experiments are shown in figures.

Pull-down assay --- To detect endogenous active GTP-bound Cdc42 in the cell lysates, we performed pull-down assays using recombinant GST-tagged αPakCRIB. The levels of active GTP-bound Cdc42 were normalized to the total amounts of Cdc42 proteins.

Cell migration assay --- Cell migration was measured using a 24-well Boyden chamber (Becton Dickinson Labware, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, upper wells with polyethylene terephthalate filters (8 μm pore size) were coated with 10 μg/ml extracellular matrix E-C-L (Upstate Inc., Charlottesville, VA). Serum-starved cells (2 x 10^5 cells in 500 μl DMEM per well) were loaded into upper wells, which were immediately plated in a chamber containing 165 nM ET-1 (750 μl of DMEM per well). After incubation at 37 ºC for 5 h, the upper filters were stained with a Diff-Quick staining kit (Biochemical Sciences Inc., Sterling Height, MI). Using an optical microscope, the number of migrated cells was counted in at least three independent experiments.

Crude membrane preparation --- Briefly, the harvested cells were homogenized in an ice-cold buffer (5 mM Tris-HCl (pH 7.5), 250 mM sucrose, and 1 mM $MgCl_2$). Nuclei and unbroken cells were separated from the cell extract by a centrifuge (700 x g, 4 ºC, 10 min). The supernatant was sonicated and centrifuged (150,000 x g, 4 ºC, 30 min). The supernatant was used as the

postnuclear cytosol fraction, and the pellet was used as the crude membrane fraction (23).

Statistical analysis --- Values shown represent the mean ± S.E.M. from at least three separate experiments. A Student's t-test was carried out for intergroup comparisons with the control (*, $p<0.01$).

Results

The ET-1/ETA receptor induces the inhibition of cell migration via Cdc42/JNK --- Since Yamauchi *et al*. previously suggested that the ET-1/ETA receptor inhibits cell migration through the JNK signaling pathway (17), using the dominant negative mutant of JNK activator, MKK4K95R, I examined the effect of a JNK-specific inhibitor on cell migration. As shown in Figure 1A-C, ET-1/ETA receptor-induced inhibition of cell migration was blocked only by SP600125 (JNK inhibitor), but not by U0126 (MEK1/2 inhibitor) or SB203580 (p38 MAPK inhibitor). Next, I investigated the involvement of Rho GTPases in this signaling pathway. As shown in Figure 1D, the dominant negative mutant of Cdc42, but not of RhoA or Rac1, attenuated ET-1/ETA receptor-induced inhibition, indicating that Cdc42 functions downstream of the ET-1/ETA receptor. Taken together, these results indicate that the Cdc42/JNK pathway plays a key role in the inhibition of cell migration induced by the ET-1/ETA receptor.

The ET-1/ETA receptor induces the inhibition of cell migration through Nck1, but not through Grb2 or CrkII --- A growing number of studies have suggested that adaptor proteins play an important role in mediating the signaling pathway from receptor tyrosine kinases to Rho family-dependent regulation of actin cytoskeleton (24, 25). Cell migration is a complex cellular process that is regulated by a number of regulatory proteins and is driven by cytoskeletal reorganization. To explore the potential involvement of adaptor proteins in ET-1/ETA receptor-induced inhibition of cell migration, I transiently transfected the dominant negative mutants of these adaptor proteins into cells. The cells transfected with the plasmid encoding Grb2ΔSH2 or CrkIIR38K had no effect on ET-1/ETA receptor-induced inhibition of cell migration (Figures 2A and 2B). In contrast, various SH3 domain-deficient mutants (Nck1W38K, W143K, W229K, and

SH3All) and a SH2-deficient mutant (Nck1R308K) of Nck1 rescued the negative effect (Figure 2C). These results suggest that Nck1, but not Grb2 or CrkII, is necessary for the inhibition of cell migration induced by the ET-1/ETA receptor.

JNK activation induced by an ET-1/ETA receptor is mediated by Nck1, but not by Grb2 or CrkII --- To examine whether an ET-1/ETA receptor activates JNK through adaptor proteins, endogenous JNK was immunoprecipitated from the cell lysates and immunoblotted with an anti-phosphorylated JNK antibody, which recognizes the active state of JNK. As shown in Figure 3A, ET-1/ETA receptor-induced JNK activation was blocked by co-transfection with the negative mutants of Nck1 (Nck1W38K, W143K, W229K, SH3All, and R308K). However, co-transfection with dominant negative mutants of Grb2 (Grb2ΔSH3 and ΔSH2) or CrkII (CrkllW169K, W275K, and R38K) had no effect on JNK activation elicited by the ET-1/ETA receptor (Figures 3B and 3C). On the other hand, Grb2ΔSH3 inhibited EGF-induced ERK phosphorylation, and CrkIIR38K blocked EGF-induced JNK phosphorylation, in agreement with earlier findings (26, 27) (Figures 3D and 3E), whereas the dominant negative mutants of Nck1 had no effect on either EGF-induced JNK (Figure 3F) and ERK (data not shown) activation. These results indicate that Nck1, but not Grb2 or CrkII, is a specific component of the signaling pathway from the ET-1/ETA receptor to JNK.

The ET-1/ETA receptor increases the GTP-bound form of Cdc42 through Nck1, but not through Grb2 or CrkII --- Next, using pull down assay, I investigated whether the ET-1/ETA receptor activates Cdc42 through adaptor proteins. Following stimulation with ET-1, the GTP-bound active form of Cdc42 was dramatically increased, and this effect was blocked by the dominant negative mutants of Nck1 (Figure 4A). As shown in Figure 4B and 4C, the dominant negative mutants of Grb2 and CrkII had no effect on the ET-1/ETA receptor-induced activation of

Cdc42. Taken together, these results suggest that Nck1 mediates the ET-1/ETA receptor-induced inhibition of cell migration involving Cdc42 and JNK using Nck1.

The membrane-bound form of Nck1 stimulates the inhibition of cell migration by increasing the activities of Cdc42 and JNK--- To examine whether Nck1 can inhibit cell migration through the Cdc42/JNK signaling pathway, I made a membrane-bound mutant of Nck1. I fused the myristoylation signal sequence of c-Src to the N-terminal of Nck1 (Figure 5A). To verify the effectiveness of the myristoylation signal sequence, transfected cells were homogenized and fractionated into crude membrane, cytosol, and nucleus. Wild-type Nck1 was detected in the cytosol fraction, and myr-Nck1 was mainly found in the membrane fraction (Figure 5A). As shown in Figure 5B, myr-Nck1 significantly inhibited cell migration; however wild-type Nck1 did not. Additionally, myr-Nck1 increased the activities of JNK and the GTP-bound form of Cdc42 (Figures 5C and 5D). Taken together, these results suggest that Nck1 mediates the activation of Cdc42 and JNK and inhibits cell migration.

siNck1 inhibits the ET-1/ETA receptor-induced inhibition of cell migration through the JNK pathway --- To confirm the requirement of Nck1 in the pathway from the ET-1/ETA receptor to the inhibition of cell migration, I then carried out RNAi-mediated gene silencing using a synthetic 21-mer oligonucleotide RNA duplex (siRNA) of Nck1. As shown in Figure 6A, I designed a pair of oligonucleotides (siNck1-1 or siNck1-2) corresponding to the sequence of human Nck1. Transfection of siNck1-1 or siNck1-2 into cells suppressed the expression of endogenous Nck1, but not tubulin, in a dose-dependent manner (Figure 6A). Since I observed a prominent silencing effect of siNck1-1 on endogenous Nck1, I used siNck1-1 in the following experiments.

I investigated whether siNck1-1 affects the endogenous JNK activity stimulated by the ET-

1/ETA receptor. As shown in Figure 6B, ET-1/ETA receptor-induced JNK activation was inhibited by siNck1-1. In contrast, the control siRNA (siGFP) had no effect on the JNK activation. Additionally, the distinct signaling, i.e. the EGF-induced phosphorylation of ERK, was not affected by siNck1-1 (data not shown). In parallel with JNK activity, increase of the active form of Cdc42 induced by the ET-1/ETA receptor was inhibited by siNck1-1 (data not shown). Finally, I examined the effect of siNck1-1 on cell migration. As shown in Figure 6C, silencing of Nck1 partially rescued the ET-1/ETA receptor-induced inhibition of cell migration. These results strongly suggested that Nck1 has an essential role in the ET-1/ETA receptor-stimulated inhibition of cell migration through Cdc42 and JNK.

Src kinase suppresses cell migration via the Nck1/Cdc42/JNK pathway --- Yamauchi *et al.* previously demonstrated that Src kinase acts upstream of the Cdc42 and JNK in the ET-1/ETA receptor-signaling pathway (17). To investigate the involvement of Nck1 in the CA-Src-induced inhibition of cell migration, the dominant negative mutants of Nck1 were co-transfected with CA-Src. As shown in Figure 7A, the CA-Src-induced inhibition of cell migration was completely rescued by Nck1SH3All or Nck1R308K. Additionally, CA-Src-induced JNK (Figure 7B) and Cdc42 (data not shown) activation was also inhibited by these mutants.

Nagao *et al.* previously demonstrated that Gq-coupled receptor induces the JNK activation in a Src-dependent manner (28). Additionally, ET-1/ETA receptor-induced inhibition of cell migration was blocked by cotransfection of the Gαq inhibitors (regulator of G protein signaling (RGS) 4 or N-terminal domain of β-adrenergic receptor kinase 1 (βARKnt)) (data not shown). Taken together, these results indicate that ET-1/ETAreceptor regulates cell migration mediated through Nck1 with G protein, Src, Cdc42, and JNK (Figure 8).

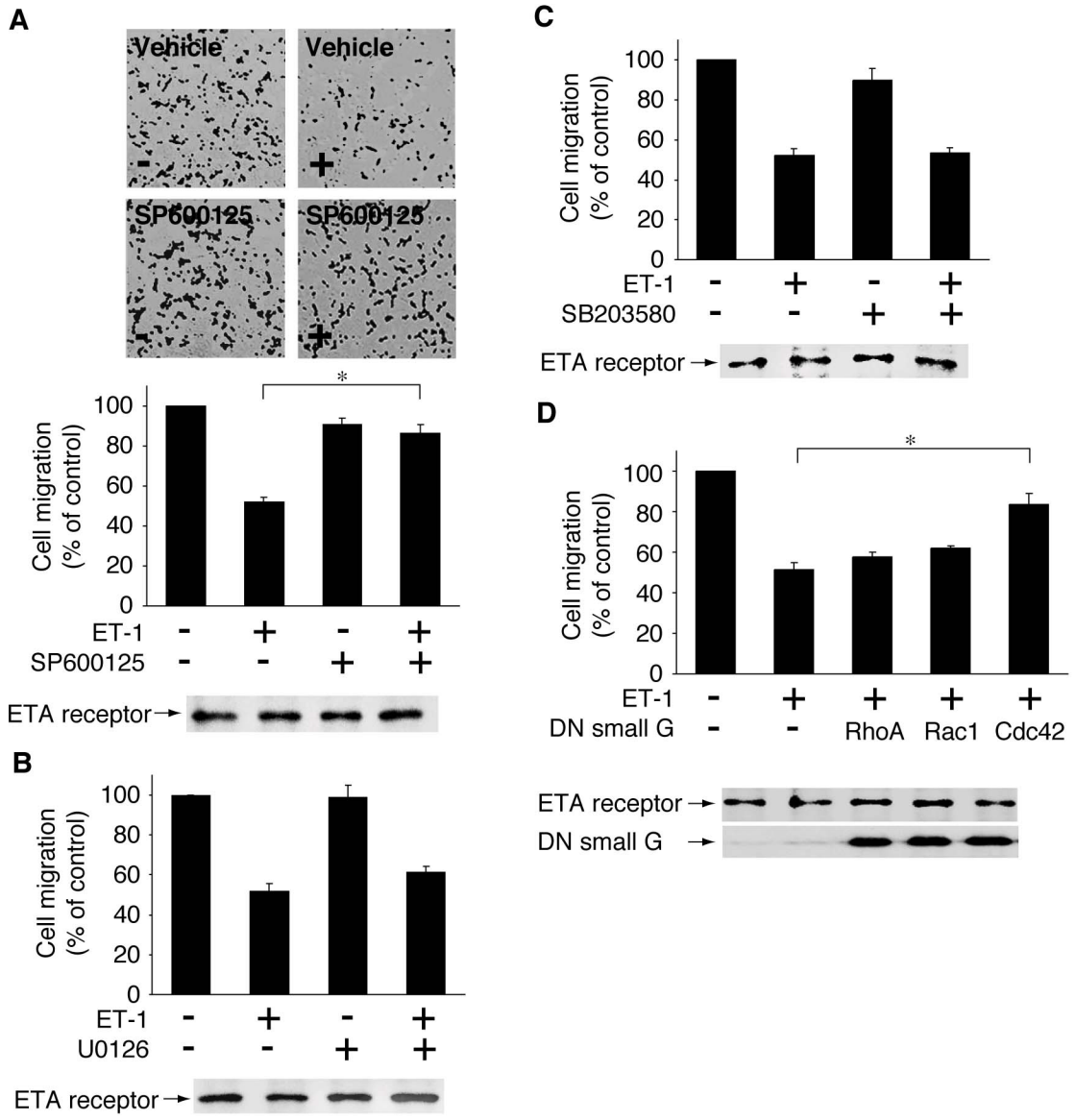

Figure 1. The ET-1/ETA receptor-induced inhibition of cell migration involves Cdc42/JNK. Cell migration was measured in epithelial 293T cells transiently transfected with the plasmid encoding ETA receptor (A-D) and dominant negative mutants of Rho GTPases (D) using a Boyden chamber. Cells were pretreated with or without SP600125 (100 nM, 45 min) (A), U0126 (10 mM, 1 h) (B), or SB203580 (10 mM, 1 h) (C). After incubation at 37oC for 5 h with (+) or without (-) ET-1 (165 nM), the cells attached to the filters were stained and analyzed under a microscope (A), and the number of stained cells was counted (A-D). Expression of ETA receptor (A-D) and the dominant negative mutants of Rho GTPases (D) was shown. Data were evaluated using the Student1s t-test. Asterisks indicate $p<0.01$.

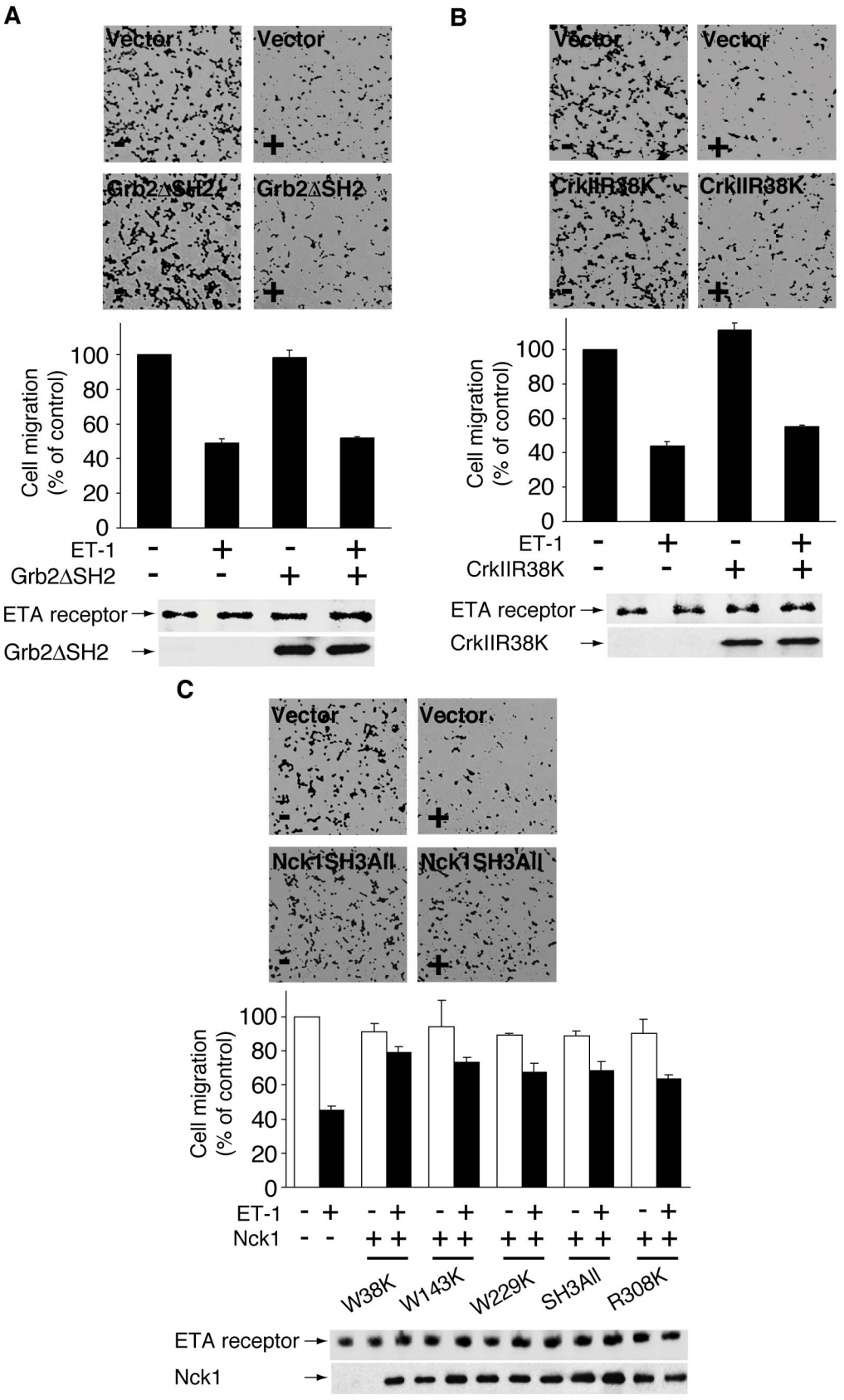

Figure 2. Involvement of adaptor protein Nck1 in the ET-1/ETA receptor-induced inhibition of cell migration. Cells were transfected with the ETA receptor (A-C) and dominant negative mutants of Grb2 (A), CrkII (B), or Nck1 (C). After incubation for 5 h, the cells attached to the filters were stained and counted. Expression of ETA receptor and the dominant negative mutants of adaptor proteins was shown.

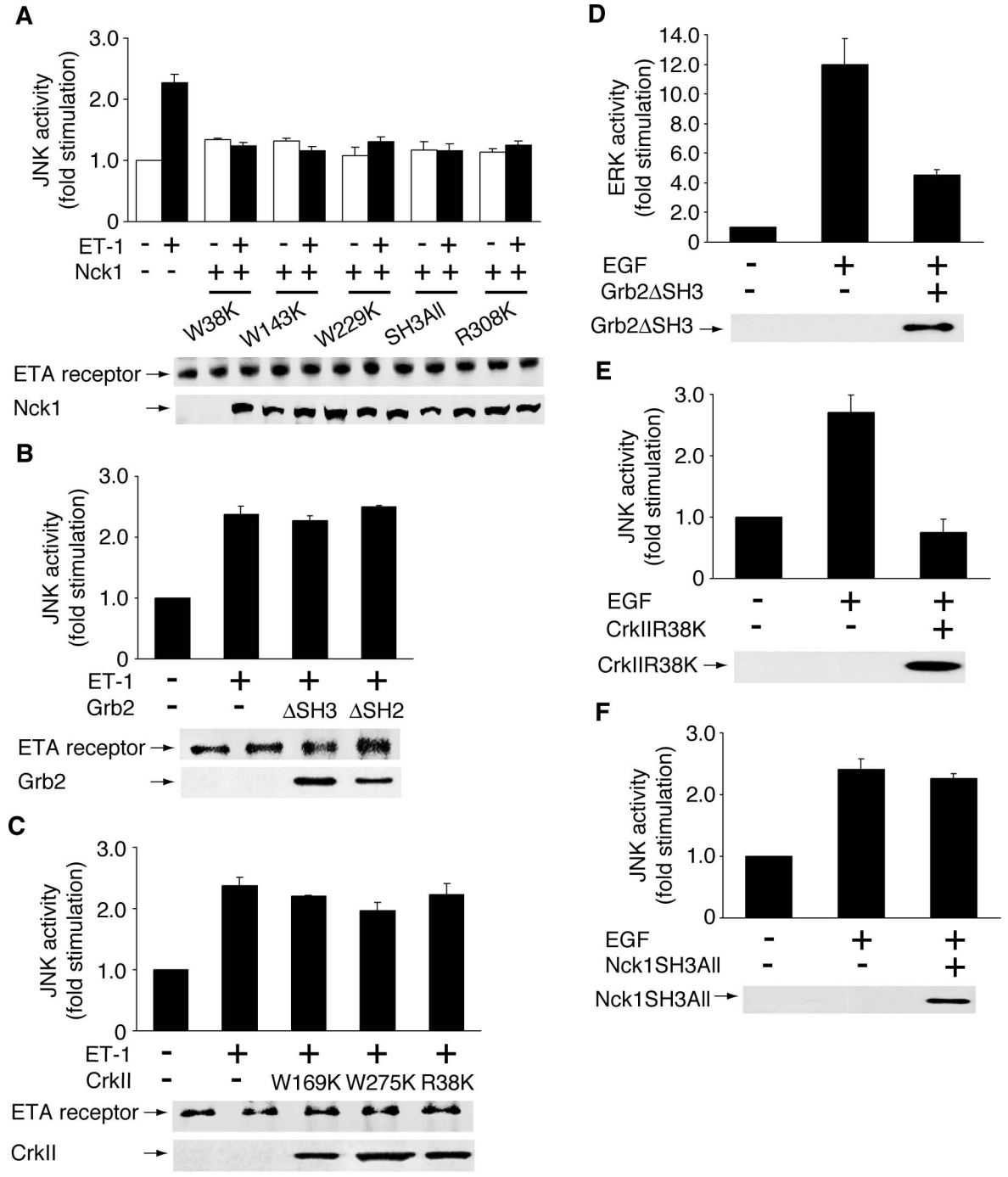

Figure 3. Nck1 is involved in ET-1/ETA receptor-induced JNK activation. JNK activity (A, B, C, E, and F) and ERK activity (D) were measured as described in Materials and Methods. (A-C) Cells cotransfected with the ETA receptor (A-C) and dominant negative mutants of Nck1 (A), Grb2 (B), or CrkII (C) were treated with ET-1 (100 nM, 20 min). Endogenous JNK was immunoprecipitated with anti-JNK antibody and blotted with anti-phosphorylated JNK antibody or anti-JNK antibody. The levels ofJNK phosphorylation were quantified and normalized against the total immunoprecipitated JNK levels (D-F). Cells transfected with the dominant negative mutants of adaptor proteins were treated with EGF (10 ng/ml, 10 min). Cell lysates were blotted with an anti-phosphorylated ERK or anti-ERK antibody. The levels of ERK or JNK phosphorylation were quantified and normalized against the total immunoprecipitated ERK or JNK levels. Expression of ETA receptor (A-C) and the dominant negative mutants of adaptor proteins (A-F) was shown.

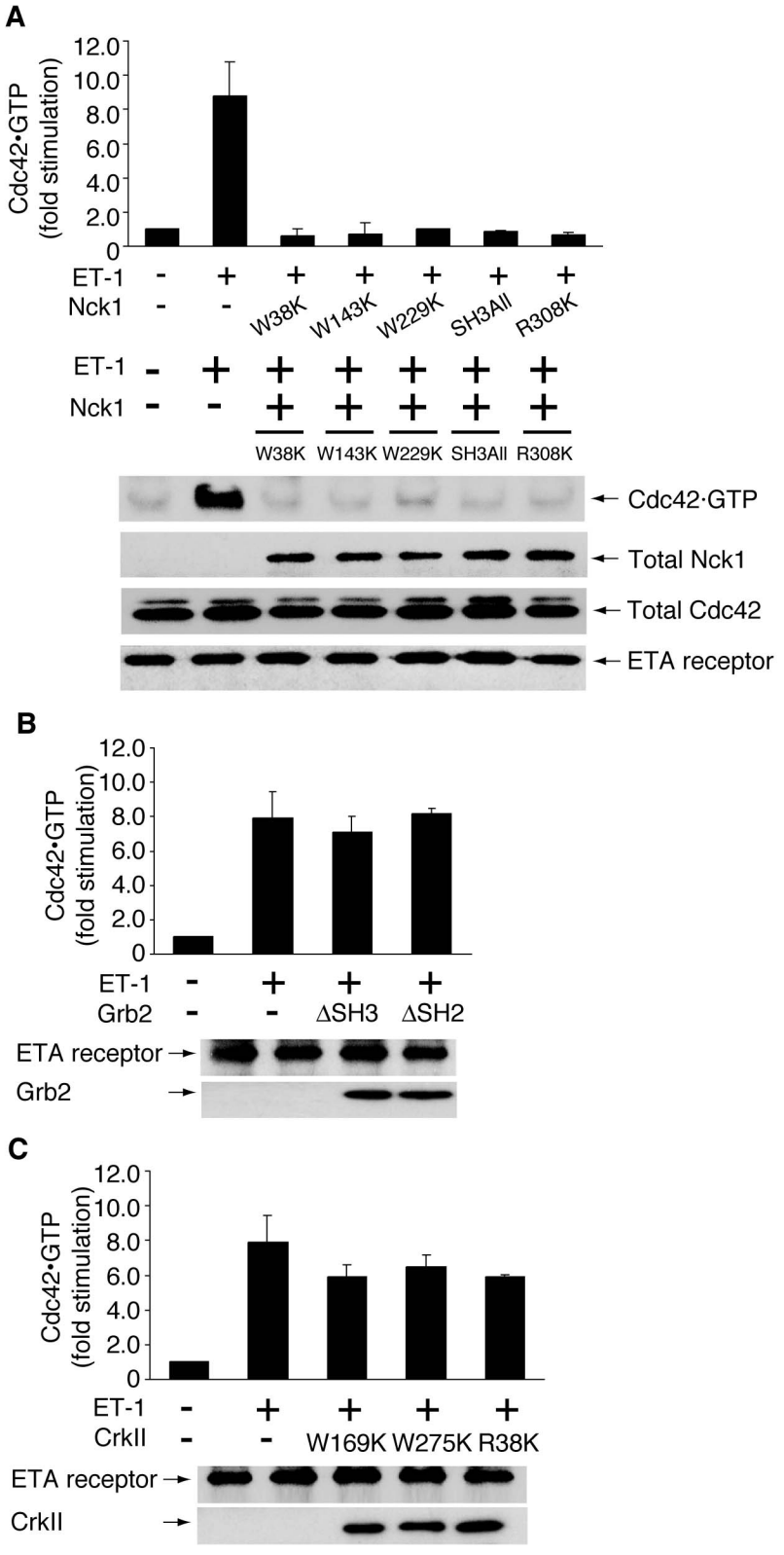

Figure 4. Nck1 mediates the ET-1/ETA receptor-induced activation of Cdc42. Cells were cotransfected with the ETA receptor (A-C) and dominant negative mutants of Nck1 (A), Grb2 (B), or CrkII (C). The amount of GTP-bound Cdc42 was assessed after the addition of ET-1 (100 nM, 30 min) with the pull-down assay using αPAK-CRIB (A-C). The total Cdc42 in the cell lysates was immunoblotted with anti-Cdc42 antibody. Expression of ETA receptor and the dominant negative mutants of adaptor proteins was shown.

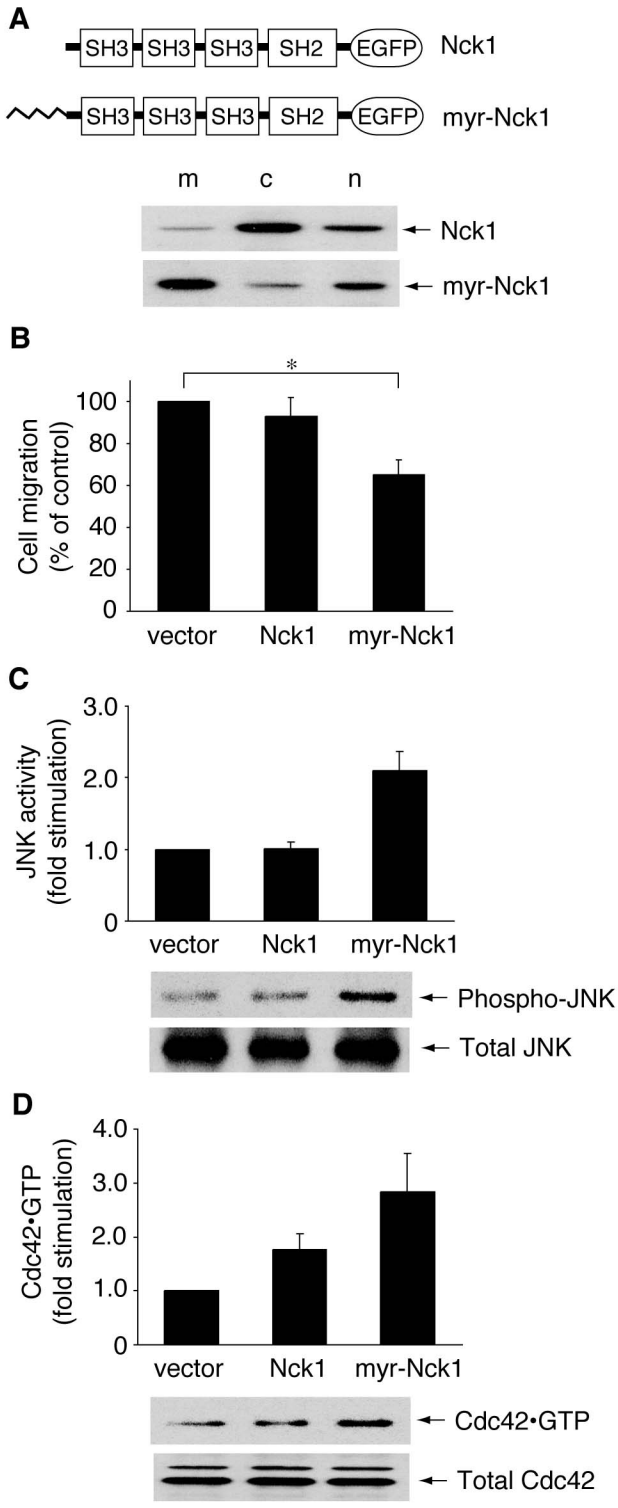

Figure 5. Membrane-bound Nck1 induces the inhibition of cell migration. (A) Structure and expression of wild-type or myristoylation signal-tagged Nck1 (myr-Nck1). For the membrane-bound construct, the 14 amino-acid c-Src myristoylation signal was linked to the N-terminal of the wild type of Nck1. Lysates from cells transfected with wild-type or myr-Nck1 were fractionated into crude membrane (m), cytosol (c), and nucleus (n). An equal amount (10 mg of proteins) of each fraction was blotted with an anti-Nck1 antibody. (B) Cells were transfected with wild-type or myr-Nck1. After incubation for 5 h, the cells attached to the filters were stained and counted. (C-D) Cells were transfected with wild-type or myr-Nck1. The amount of phosphorylated JNK (C) or GTP-bound Cdc42 (D) was measured after stimulation of ET-1. Data were evaluated using the Student's t-test. Asterisks indicate $p<0.01$.

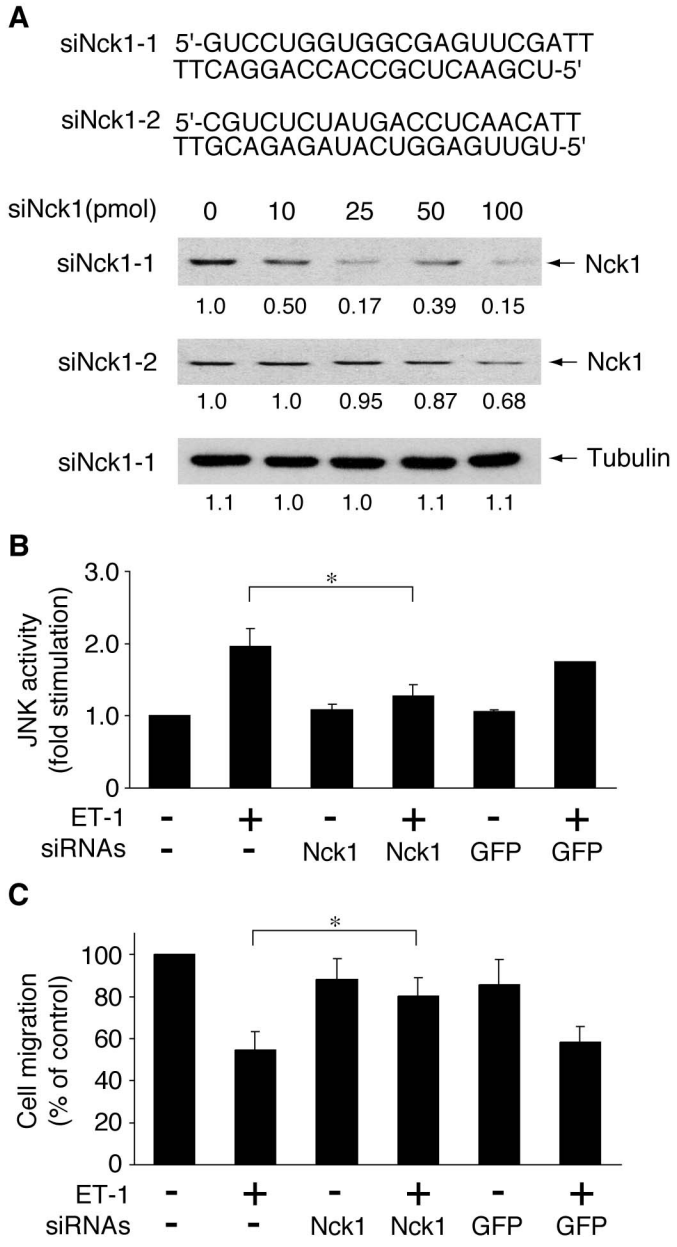

Figure 6. Effects of siNck1 on the ET-1/ETA receptor-induced inhibition of cell migration and Cdc42/JNK activation. (A) Sequence of the synthetic siRNA duplex targeting Nck1 (siNck1-1 or siNck1-2). The 2-nucleotide 3'-overhang of 2'-deoxythymidine is indicated as TT. The indicated amount of siRNAs was transfected using LipofectAMINE 2000 into cells. Cell lysates were immunoblotted with anti-Nck1 or anti-tubulin antibodies. (B) Cells were transfected with siNck1-1 or siGFP using Lipofectamine 2000. The phosphorylated JNK was detected after addition of ET-1. (C) Cells were transfected with siNck1-1 or siGFP. After incubation with ET-1, the migrating cells in the Boyden chambers were stained and counted. Data were evaluated using the Student's t-test. Asterisks indicate $p<0.01$.

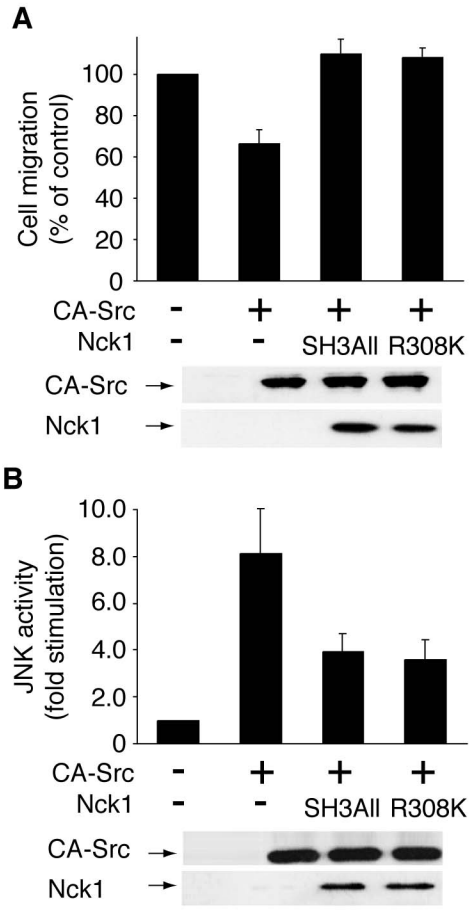

Figure 7. The Src kinase-induced inhibition of cell migration and activation of JNK involves Nck1. (A-B) Cells were cotransfected with CA-Src and dominant negative mutants of Nck1. After incubation for 5 h, the cells attached to the filters were stained and counted (A). The phosphorylated JNK was analyzed 20 min after stimulation with ET-1 (B). Expression of CA-Src and the dominant negative mutants of Nck1 was shown.

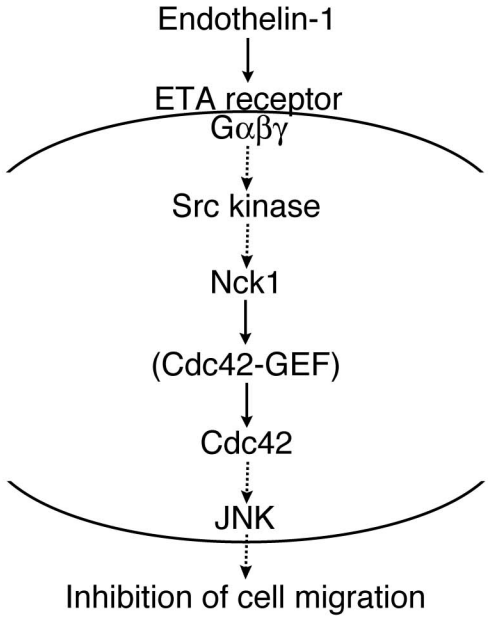

Figure 8. Schematic model for the signaling pathway coupling the ET-1/ETA receptorto the inhibition of cell migration. Details are described in the Results.

Discussion

During embryogenesis, complex patterns of cell migration are essential for proper tissue formation. Bladt *et al*. recently demonstrated that the inactivation of Nck genes (Nck1 and Nck2) results in profound defects in mesoderm-derived embryonic structures (29). They provided genetic evidence for a role of Nck proteins in cell migration during embryogenesis using fibroblasts derived from $Nck1^{-/-}$ $Nck2^{-/-}$ embryos. In addition, evidence indicates that endothelins and their receptors participate in the normal development of different neural crest lineages. Mice deficient in either ET-3/ETB receptor develop white spotted coats and an aganglionic megacolon due to the absence of neural crest-derived melanocytes and enteric neurons (30, 31). The phenotype of mice lacking the ET-1/ETA receptor-mediated signaling causes malformations in the heart and pharyngeal arch-derived structures (32, 33). These data imply that both ET/ET receptor systems may participate in cell migration, which is essential for the normal development.

Cardiovascular defects observed in the ET-1/ETA receptor-deficient mice (32), as well as the $Nck1^{-/-}$ $Nck2^{-/-}$ defects in mesodermal-derived notochord (29), are in common with defects in the development of mesodermal structures in embryogenesis, which imply their role in cell migration during early development. Thus, I am investigating whether Nck1 is involved in the inhibition of cell migration induced by the ET-1/ETA receptor. As a result, I show that Nck1 participates in a chemorepellent signaling pathway downstream of the ET-1/ETA receptor. As far as I know, this is the first report of the role of the adaptor protein in the regulation of cell migration downstream of GPCR. It is possible that the abnormal tissue morphogenesis observed in the ET-1/ETA receptor-deficient mice depends, at least in part, upon Nck1.

Cell migration includes multiple processes that are coordinately modulated by a number of regulatory proteins and driven by changes in the actin cytoskeleton (34, 35). Therefore, Nck1 could participate in the control of cell migration by binding and regulating signaling proteins involved in the rearrangement of the actin cytoskeleton. A genetic study on *Drosophila* indicates that Dreadlocks (Dock), which is structurally related to the mammalian Nck genes (36), links tyrosine kinases to the actin cytoskeleton (37). Furthermore, in mammalian cells, it is also likely that Nck1 functions to couple the phosphotyrosine signals to the actin cytoskeleton (25, 38). For example, the SH2 domain of Nck1 binds the receptor tyrosine kinases, such as platelet-derived growth factor, EGF, and Eph receptors, as well as the tyrosine-phosphorylated docking proteins p62Dok-1 and p130Cas, both the substrate of Src family tyrosine kinases (25, 38). On the other hand, two Nck1 SH3 binding proteins, N-WASP and PAK1, regulate the actin cytoskeleton through Rac1- and Cdc42-dependent or -independent mechanisms (25, 38). In the present study, I suggested that Nck1 links Src kinase to the Cdc42/JNK cascade, which may be involved in the reorganization of the actin cytoskeleton. It remains to be investigated whether these known binding partners with Nck1 are involved in the ET-1/ETA receptor signaling pathway in a Src/Cdc42/JNK-dependent manner.

Rho GTPases act as molecular switches between active (GTP-bound) and inactive (GDP-bound) states (39). Their activities are controlled positively by GEFs, which catalyze the replacement of GDP with GTP (40). GEFs may be the missing link between Nck1 and Cdc42 in the GPCR/JNK signaling pathway. Zhao *et al*. (41) showed that the second SH3 domain (SH3[2]) of Nck1 associates with PAK, NIK, and WIP through their conserved motif, PXXPXRXXS. As described in Chapter 2, I identified a new signaling molecule, FRG, which functions as a specific

GEF for Cdc42. I thus analyzed whether FRG contains this Nck1 SH3[2]-binding motif. It was shown that FRG possesses a nearly identical sequence to the Nck1 SH3[2]-binding motif. It would be interesting to examine whether FRG is a binding partner with the SH3 domain of Nck1 in the ET-1/ETA receptor-signaling pathway.

In this study, I demonstrated that the ET-1/ETA receptor inhibits cell migration through the Src/Nck1/Cdc42/JNK pathway. On the basis of these findings, I summarized the proposed signaling pathway in Figure 8. A challenge for the future will be to define the roles of Nck1 in regulating the potential for crosstalks among the various signaling pathways involving FRG in the control of cell migration. Such studies might promote our understanding of the GPCR-regulated mechanism of the early process of development as well as oncogenesis.

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Chapter 2

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

Introduction

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 GTPases is the regulation of the actin cytoskeleton (5, 6). Rho GTPases, RhoA, Rac, and Cdc42, induce the formation of stress fibers, lamellipodia, and filopodia, respectively (5). Furthermore, Rho GTPases have been implicated in the control of diverse physiological responses, such as cell proliferation and cell migration, as well as in physiopathological processes, such as transformation and metastasis (7, 8).

Rho 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 (GAPs), which accelerate the endogenous GTPase activity (4, 9). Dbl family proteins are GEFs for Rho 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 GEFs have been identified in mammals (9). Some GEFs are specific for each Rho GTPase, while 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 (MAPKs) (28). This pathway depends on Src kinase and Rho GTPases, Src kinase often acting upstream of Rho GTPases (28, 30-32). Yamauchi *et al*. previously showed that endothelin, a ligand of GPCRs, activates JNK via the signaling pathway dependent upon Src kinase and Rho 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 GTPases remains unclear (34).

To clarify the mechanism of GPCR-mediated activation of Rho GTPases, I performed a BLAST search using Dbl's big sister (Dbs), a GEF involved in GPCR-mediated activation of Rho GTPases (35), as a query. As a result, I found a novel Rho 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, I 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. I also demonstrate that FRG is involved in the signaling pathway linking the endothelin A (ETA) receptor to JNK, leading to the inhibition of cell migration. Taken together, my 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 chemorepellant GPCR-JNK signaling pathway.

Materials and Methods

Antibodies --- A mouse monoclonal antibody M2 against a FLAG-peptide was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). A mouse monoclonal antibody 12CA5 against the hemagglutinin (HA)-epitope was purchased from Roche Diagnostics Co. (Indianapolis, IN, USA). A mouse monoclonal antibody 1E4 against *Aequorea victoria* green fluorescence protein (GFP) was obtained from Medical and Biological Laboratories, Co., Ltd. (Nagoya, Japan). A mouse monoclonal anti-phosphorylated tyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY, USA). A rabbit polyclonal antibody against SRC2 and a mouse monoclonal antibody 26C4 against RhoA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal antibodies against Rac1 and Cdc42 were purchased from BD Biosciences (San Jose, CA, USA). Anti-mouse and anti-rabbit IgG antibodies conjugated with horseradish peroxidase were obtained from Amersham-Pharmacia, Inc. (Buckinghamshire, UK).

Inhibitors --- PP1 and PP2, inhibitors of Src kinase, were purchased from Biomol (Plymouth Meeting, PA, USA) and Carbiochem-Novabiochem (San Diego, CA, USA), respectively. *Clostridium difficile* toxin B, which inhibits Rho GTPases, was obtained from Carbiochem-Novabiochem. Endohelin-1 was purchased from Peptide Institute, Inc. (Osaka, Japan).

Plasmids --- The cDNA of *KIAA0793*, GenBank accession number AB018336, 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 (ΔDHPH) lacking amino acids 501-729 and 501-820 of FRG, respectively, were inserted into

pCMV-2HA. The portions encoding the FERM domain (1-300), the proline-rich domain (301-500), the DH and PH domains (491-827), 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 *Escherichia coli* GST-tag expression vector pET42a. pCMV-FLAG-RhoA, pCMV-FLAG-Rac1, pCMV-FLAG-Cdc42, pCMV-FLAG-RhoG14V, pCMV-FLAG-RacG12V, pCMV-FLAG-Cdc42G12V, pCMV-FLAG-RhoT19N, pCMV-FLAG-RacT17N, pCMV-FLAG-Cdc42T17N, and pCMV-FLAG-MKK4K95R were constructed as described previously (30, 39). pUSE-CA-Src (a constitutively activated mutant of c-Src) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The *Escherichia coli* expression plasmids encoding the RhoA-binding domain (RBD) of mDia1 and the Rac1- and Cdc42-binding domain (CRIB) of αPak were constructed as described previously (32, 40-41). pME-endothelin A (ETA) receptor-EGFP was generously provided by Dr. T. Sakurai (University of Tsukuba, Ibaraki, Japan) (42). pGEX2T-c-Jun (amino acids 1-221) and SRα-HA-JNK1 were kindly provided by Dr. M. Karin (University of California, San Diego, CA, USA). All DNA sequences were confirmed using DNA sequencer L-4200L (LI-COR).

Recombinant proteins --- Recombinant GST-tagged mDia1RBD, αPakCRIB, Rho family GTPases, and c-Jun proteins were purified using *E. coli* BL21 (DE3) pLysS, as described previously (32).

Cell culture and transfection --- Human kidney epithelial 293T cells were maintained in Dulbecco's modified Eagles medium (DMEM) containing 100 μg/ml kanamycin and 10% heat-inactivated fetal bovine serum. The cells were cultured at 37 ºC in a humidified atmosphere

containing 5% CO_2. Plasmid DNAs were transfected into cells by the calcium phosphate precipitation method. The final amount of the transfected DNA for a 60-mm dish was adjusted to 25 μg by an empty vector, pCMV. 293T cells were transfected with 1 μg of pCMV-2HA-FRG, 1 μg of pCMV-2HA-FRG-ΔDH, 1 μg of pCMV-2HA-FRG-ΔDHPH, 3 μg of pCMV-FLAG-FRG-FERM, 3 μg of pCMV-FLAG-FRG-Proline, 3 μg of pCMV-FLAG-FRG-DHPH, 3 μg of pCMV-FLAG-FRG-2nd PH, 3 μg of pCMV-FLAG-RhoA, 3 μg of pCMV- FLAG-Rac1, 3 μg of pCMV-FLAG-Cdc42, 3 μg of pCMV-FLAG-RhoG14V, 3 μg of pCMV-FLAG-RacG12V, 3 μg of pCMV-FLAG-Cdc42G12V, 1 μg of pCMV-FLAG-RhoT19N, 1 μg of pCMV-FLAG-RacT17N, 1 μg of pCMV-FLAG-Cdc42T17N, 3 μg of pCMV-FLAG-MKK4K95R, 3 μg of pUSE-CA-Src, 0.3 μg of pME-ETAR-EGFP, or 1 μg of SRα-HA-JNK1. The medium was replaced 24 h after transfection, and cells were starved in a serum-free medium for 24 h before the addition of 100 nM endothelin-1.

Immunoprecipitation and immunoblotting --- After the addition of endothelin-1, cells were lysed in 600 μl of lysis buffer A (20 mM HEPES-NaOH (pH 7.5), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 20 mM β-glycerophosphate, and 0.5% NP-40) for a 60-mm dish. The lysates were centrifuged at 15,000 rpm for 10 min at 4 °C. Aliquots (400 μg) of the supernatants were mixed with protein G-Sepharose CL-4B preabsorbed with 0.5 μg anti-FLAG or 0.5 μg anti-HA antibody. The immune-complexes were precipitated by centrifugation and washed twice with lysis buffer A and twice with kinase buffer A (20 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethane sulfonylfluoride, 0.1 μg/ml leupeptin, 0.1 mM EGTA, 10 μM Na_3VO_4, and 2 mM β-glycerophosphate) for JNK, kinase bufffer B (20 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethane

sulfonylfluoride, 0.1 μg/ml leupeptin, 0.1 mM EGTA, 10 μM Na_3VO_4, and 2 mM β-glycerophosphate) for Src kinase, or an exchange buffer (20 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethane sulfonylfluoride, 0.1 μg/ml leupeptin, and 3 mM EDTA). To compare the amounts of the immunoprecipitates or expressed proteins in the cell lysates in each transfection, the precipitates or aliquots of the cell lysates were boiled in a Laemmli sample buffer and then separated on 8 to 15% SDS-polyacrylamide gels. The electrophoretically separated proteins were transferred to PVDF membranes. The membranes were blocked with phosphate-buffered saline containing 0.1% Tween 20 and 10 mg/ml bovine serum albumin and immunoblotted with antibodies. The bound antibodies were detected using the ECL system (Amersham Biosciences, Buckinghamshire, UK) with anti-mouse or anti-rabbit IgG antibodies conjugated with horseradish peroxidase according to the manufacturer's protocol. Images of protein bands were captured using Adobe Photoshop 5.0 plug-in software and an EPSON GT-7000U scanner. The band intensity of kinases was semiquantified using NIH Image 1.61. The representatives of at least three separate experiments are shown in the figures.

Kinase assays --- After the addition of 100 nM endothelin-1 for 20 min, transfected cells were lysed in 600 μl of lysis buffer A for a 60-mm dish, and the lysates were centrifuged as described above. Aliquots (400 μg) of the supernatants were mixed with protein G-Sepharose CL-4B preabsorbed with anti-HA antibody. The activities of the proteins were measured as the [^{32}P]-radioactivity incorporated into recombinant c-Jun, using a BAS 2500 imaging analyzer (Fujifilm, Tokyo, Japan), as described previously (30-33, 38). Activities were normalized to the amounts of immunoprecipitated JNK, as estimated from these band intensities.

Pull-down assays of Rho family GTPases --- Cells were stimulated with or without 100 nM endothelin-1 for 15 min and then lysed in lysis buffer B (20 mM HEPES-NaOH (pH 7.5), 20 mM

$MgCl_2$, 150 mM NaCl, 1mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, and 0.5% NP-40). To detect active GTP-bound Rho family GTPases in the cell lysates, we performed pull-down assays using recombinant GST-tagged mDia1RBD (for RhoA) and αPakCRIB (for Rac1 and Cdc42), as described previously (32, 40-41). Aliquots (400 μg) of the supernatants mixed with glutathione-Sepharose 4B with 10 μg of GST-mDia1RBD or 20 μg of GST-αPakCRIB were precipitated by centrifugation, and complexes were boiled in a Laemmli sample buffer and then separated on 15% SDS-polyacrylamide gels. The separated proteins were immunoblotted using each antibody against Rho family small GTPases.

Exchange assays --- After the addition of endothelin-1 for 15 min, 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 the exchange assay. For releasing assays, the Rho GTPases • [^{3}H]GDP complex was obtained by incubation with an exchange buffer containing 125 ng/μl Rho GTPases, 250 ng/μl bovine serum albumin, 5 mM EDTA, and 0.3 μM [^{3}H]GDP (0.3 μCi/μl) at 30 ºC for 90 min. The reaction was stopped by adding 5 mM $MgCl_2$, and mixtures were immediately cooled on ice. The immunoprecipitated DH domains of FRG and the FRG protein were incubated in 30 μl of an exchange buffer containing 16 ng/μl Rho GTPases • [^{3}H]GDP, 33 ng/μl bovine serum albumin, and 3 μM cold GDP at 30 ºC for 0, 10, 20, and 30 min. The reactions were stopped by adding 1 ml of an ice-cold wash buffer (20 mM HEPES-NaOH (pH 7.5) and 10 mM $MgCl_2$) and filtered through nitrocellulose membranes. The membranes were immediately washed twice with an ice-cold wash buffer and air-dried. The radioactivity remaining on each membrane was measured by an LS6500 liquid scintillation counter (Beckman Coulter Inc., Palo Alto, CA, USA). For the binding assay, the immunoprecipitated DH domain of

FRG and the FRG protein were incubated in 30 μl of an exchange buffer containing 16 ng/μl Rho GTPases, 33 ng/μl bovine serum albumin, and 3 μM [^{32}P]GTP (0.1 μCi/μl) at 30 ºC for 0, 10, 20, and 30 min. The reactions were stopped by adding an ice-cold wash buffer and filtered through nitrocellulose membranes. The membranes were immediately washed twice with a wash buffer and air-dried. The radioactivity remaining on 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 U) in 30 μl of kinase buffer B containing 20 μM ATP at 30 ºC 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 migration assay --- Cell migration was measured using a 24-well Boyden chamber (Becton Dickinson Labware, Franklin Lakes, NJ, USA), 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 x 10^5 cells in 500 μl of DMEM per well) were loaded into upper wells, which were immediately plated on the chamber containing 165 nM endothelin-1 (750 μl of DMEM per well). After incubation at 37 ºC for 5 h, upper filters were stained with a Diff-Quick staining kit (Biochemical Sciences Inc., Sterling Height, MI, USA), 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.M. from at least three separate experiments. A Student's t-test was carried out for intergroup comparisons (*, $p<0.01$).

Results

KIAA0793 shows a significant homology to Cdc42-specific GEFs --- I performed a BLAST search using the DH domain of Dbs because Dbs was implicated in the signaling pathway linking GPCRs to Rho 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. Figure 1 shows the structural features of KIAA0793 in comparison with those of other Rho 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), FGD1 (36) (28% identity, 51% 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 proline-rich region (amino acids 398 to 498) (47).

KIAA0793 activates Cdc42, but not RhoA and Rac1 --- To examine which Rho GTPases are activated by the DH and PH domains of KIAA0793, the ability of KIAA0793 to induce the dissociation of [^{3}H]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 GEFs acts as a limited catalytic domain toward Rho GTPases (12, 48). As shown in Figure 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 [^{3}H]GDP from Cdc42, the fragment of

KIAA0793 promoted the binding of [^{32}P]GTP to Cdc42 but not to RhoA and to Rac1 in a time-dependent manner (Figure 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*, I 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 αPakCRIB was used to pull down activated GTP-bound Rac1 or Cdc42 from the cell lysates (41). Figure 2C shows that transfection of the DH and PH fragment of 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 GTPases (8, 30-33). To confirm whether the ETA receptor activates JNK dependent on Src kinase and Rho family GTPases in 293T cells, I 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 addition of endothelin (Figure 3A).

Next, I examined the involvement of Src kinase and Rho GTPases in endothelin-induced

JNK activation. As shown in Figure 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 GTPases RhoA, Rac1, and Cdc42 and inhibits their intracellular functions, also blocked JNK activation elicited by endothelin (Figure 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 GTPases are activated by endothelin, I measured the active form of Rho family GTPases in cells. As shown in Figure 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, I 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 (Figure 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 Figure 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 following 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) (Figure 4C). Taken together with the data of Figure 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, I 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 (Figure 4A). As shown in Figure 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 (Figure 4E), supporting the idea that the catalytic DH and PH domains directly activate Cdc42 (Figure 2). Other variants, FRG-FERM, FRG-Proline, and FRG-2ndPH, did not induce JNK activation (Figure 4E). The inhibitory effect of FERM and the proline-rich regions of FRG indicates that both regions might be involved in JNK activation induced by endothelin.

Involvement of FRG in the inhibition of cell migration dependent on the JNK pathway induced by the ETA receptor --- Yamauchi *et al*. previously reported that the endothelin inhibits cell migration through the JNK pathway (33). To examine whether FRG inhibits the cell migration, we transfected the plasmid encoding the catalytic DH and PH domains of FRG into cells. As shown in Figure 4F, the DH and PH domains inhibited cell migration. 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 migration. These results suggest that FRG suppresses cell migration through the JNK pathway.

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

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

The Cdc42-GEF activity of FRG was enhanced by the ETA receptor --- To investigate whether the GEF activity of FRG is activated upon the stimulation by endothelin, I measured the GEF activity of the immunoprecipitated FRG by dissociation of [^{3}H]GDP from recombinant RhoA, Rac1, and Cdc42. Upon the stimulation by endothelin, the Cdc42-GEF activity of FRG was specifically enhanced, while no change was observed in RhoA and Rac1 (Figure 5A). Additionally, as shown in Figure 5B, a Cdc42-GEF activity of FRG to induce the binding of [^{32}P]GTP was promoted by endothelin in a time-dependent manner. These results suggest that stimulation by endothelin enhances the Cdc42-GEF activity of FRG.

Src kinase directly regulates the activation of the Cdc42-GEF activity in cells and in vitro --- As shown in Figure 3D, I showed that Src kinase is required for the activation of Cdc42 in the pathway downstream of the ETA receptor. Thus, I investigated whether Src kinase activates the Cdc42-GEF activity of FRG in cells. I 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 (Figure 6A). Additionally, the activation of the Cdc42-GEF activity by CA-Src was prevented by pretreatment with PP1.

Next, I examined the possibility that Src kinase induces tyrosine phosphorylation of FRG in cells. As shown in Figure 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 GEFs are activated is that of Vav family GEFs, which are directly tyrosine-phosphorylated and activated by Src kinase (20). Thus, I next examined whether the immunoprecipitated FRG is directly tyrosine-phosphorylated and activated by recombinant c-Src *in vitro*. As shown in Figure 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, I investigated whether the endothelin-induced activation of the Cdc42-GEF activity of FRG requires Src kinase. As shown in Figure 6C, the endothelin-induced activation of the Cdc42-GEF activity of FRG was blocked by pretreatment with PP1, as was inhibited by PP2 (data not shown). Thus, it is likely that Src kinase mediates the activation of the Cdc42-GEF activity of FRG in cells.

Additionally, I examined whether endothelin causes tyrosine phosphorylation of FRG in cells. Figure 6C indicated that endothelin stimulated the tyrosine phosphorylation of FRG. The endothelin-induced tyrosine phosphorylation was inhibited by pretreatment with PP1. This result suggests that Src kinase is necessary for the endothelin-induced tyrosine phosphorylation of FRG in cells.

The N-terminal region and the DH and PH domains are responsible for the tyrosine phosphorylation --- To further elucidate which region of FRG is the site for tyrosine phosphorylation, I transfected the plasmid encoding CA-Src with the various truncated mutants of FRG into cells. As shown in Figure 6D, CA-Src was able to induce the tyrosine phosphorylation of FRG-FERM, FRG-Proline, and FRG-DHPH, while FRG-2ndPH was not tyrosine-phosphorylated by Src kinase. Additionally, the tyrosine phosphorylation of FRG-FERM, FRG-Proline, and FRG-DHPH by CA-Src was prevented by pretreatment with PP1. These results suggest that Src kinase may tyrosine-phosphorylate the N-terminal region and the DH and PH domains of FRG in cells.

Next, I investigated whether the FRG-FERM, FRG-Proline, and FRG-DHPH are directly phosphorylated by recombinant c-Src *in vitro*. The immunoprecipitated fragments of FRG-FERM, FRG-Proline, and FRG-DHPH were tyrosine-phosphorylated by Src kinase *in vitro* (Figure 6E). Taken together with those of Figure 6D, these results suggest that the FERM, proline-rich, and DH and PH domains of FRG provide the tyrosine phosphorylation sites and/or structural features required for the regulation of Cdc42 GEF activity. These possibilities are under current investigation.

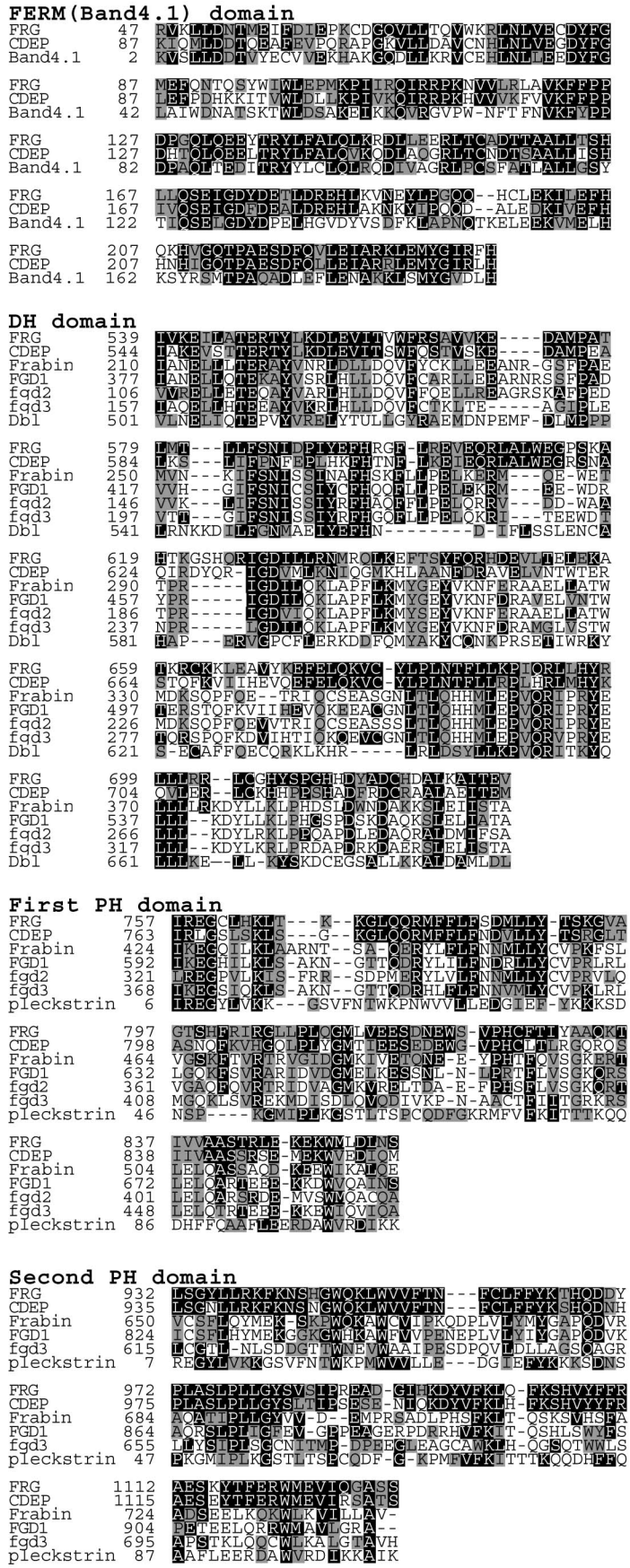

Figure 1. KIAA0793 contains several domains involved in signal transduction. Sequence comparison of KIAA0793 with proteins possessing FERM (Band 4.1), DH, and PH domains. Black boxes, identical residues; gray boxes, similar residues to KIAA0793.

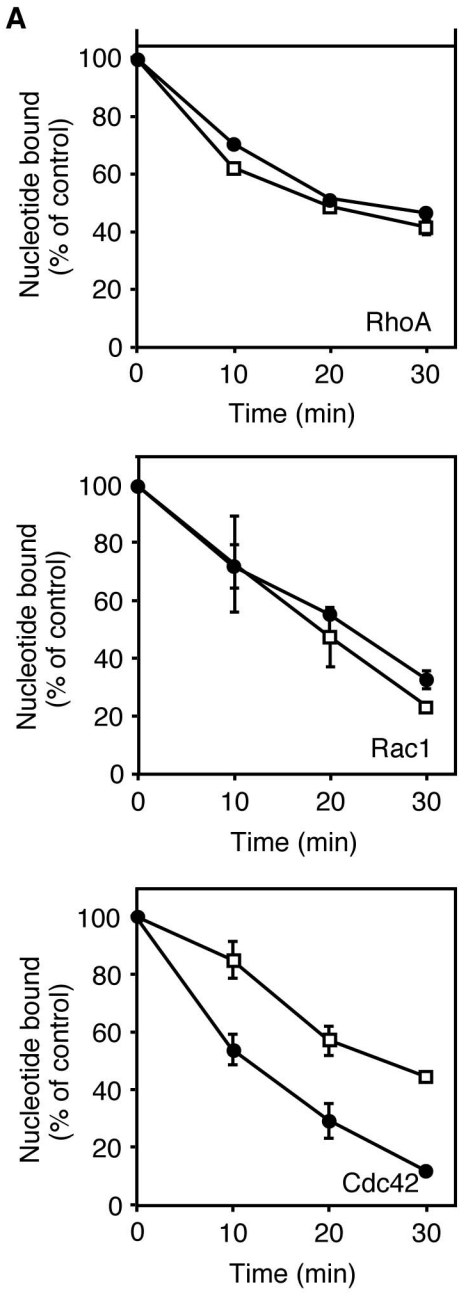

Figure 2A. The catalytic domain of KIAA0793 can induce the release of guanine nucleotide of Cdc42. (A) Cells were transfected with a mock plasmid (open square) or the plasmid encoding KIAA0793-DH and -PH domains (closed circle). Release of guanine nucleotide from RhoA (upper), Rac1 (middle), and Cdc42 (lower) was assayed as described in the Materials and Methods.

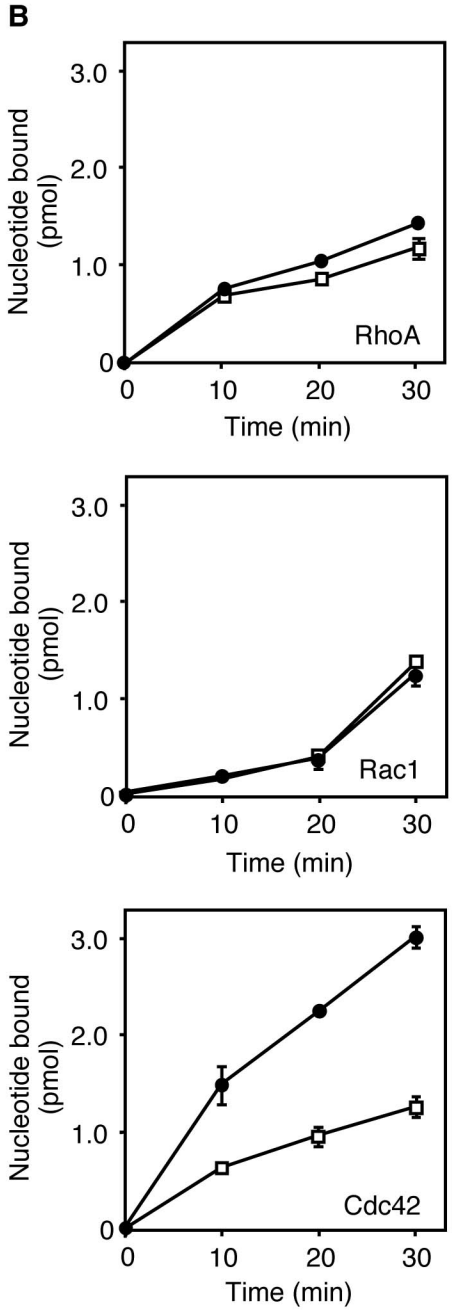

Figure 2B. The catalytic domain of KIAA0793 can stimulate the GTPγS binding to Cdc42. (B) Cells were transfected with a mock plasmid (open square) or the plasmid encoding KIAA0793-DH and -PH domains (closed circle). Binding of guanine nucleotide for RhoA (upper), Rac1 (middle), and Cdc42 (lower) was assayed as described in the Materials and Methods.

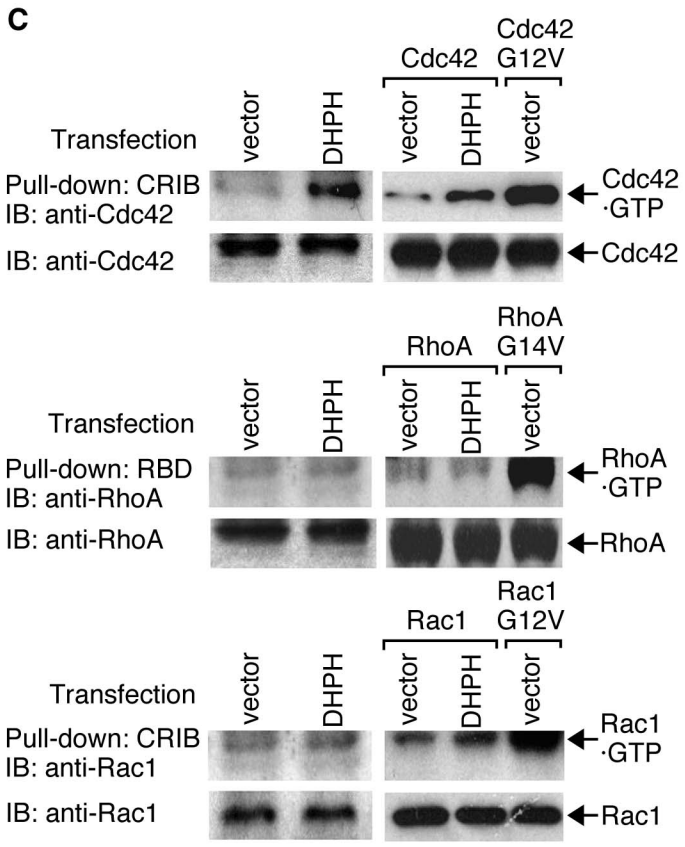

Figure 2C. The catalytic domain of KIAA0793 can increase the active formof Cdc42 *in vivo*. (C) Cells were transfected with a mock plasmid, the plasmid encoding KIAA0793 -DH and -PH domains, the plasmids encoding wild-type or active Rho GTPases. The active form of Rho GTPases was measured by a pull-down assay using recombinant mDia1RBD or αPakCRIB. Expression of Rho GTPases in the cell lysates was estimated by immunoblot analysis.

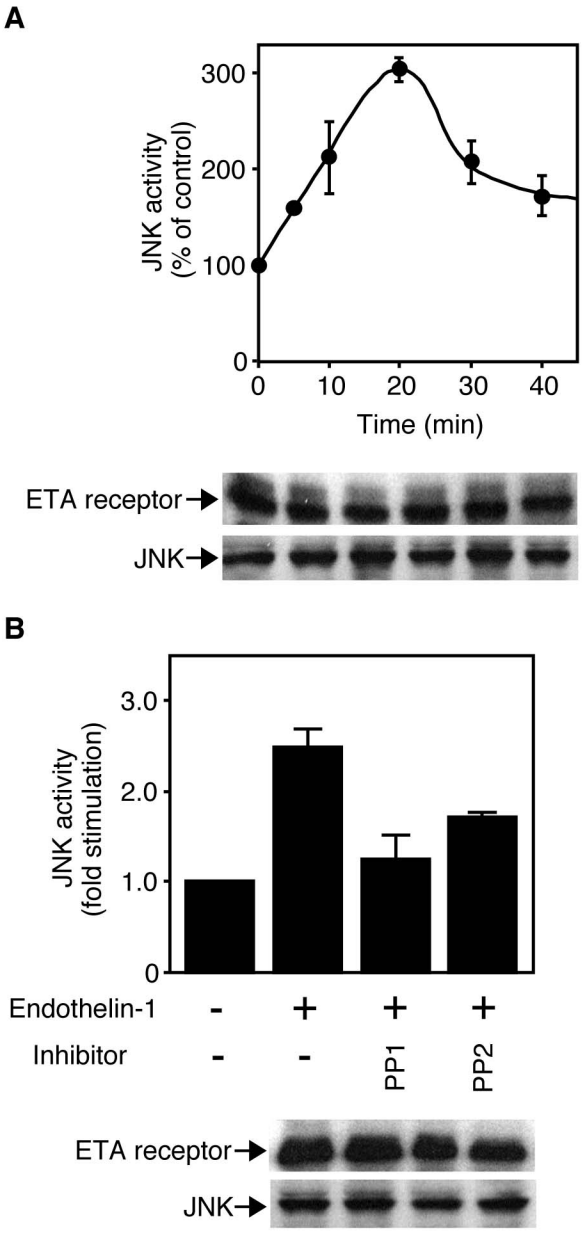

Figure 3A-B. Activation of JNK induced by the ETA receptor is mediated by Src kinase. (A, B) Cells were transfected with the plasmids encoding the ETA receptor and JNK and pretreated without or with 10 mM PP1 (B), or 10 mM PP2 (B) for 16 h before the addition of 100 nM endothelin. The JNK activity was measured by the amount of [^{82}P]-radioactivity incorporated into recombinant c-Jun.

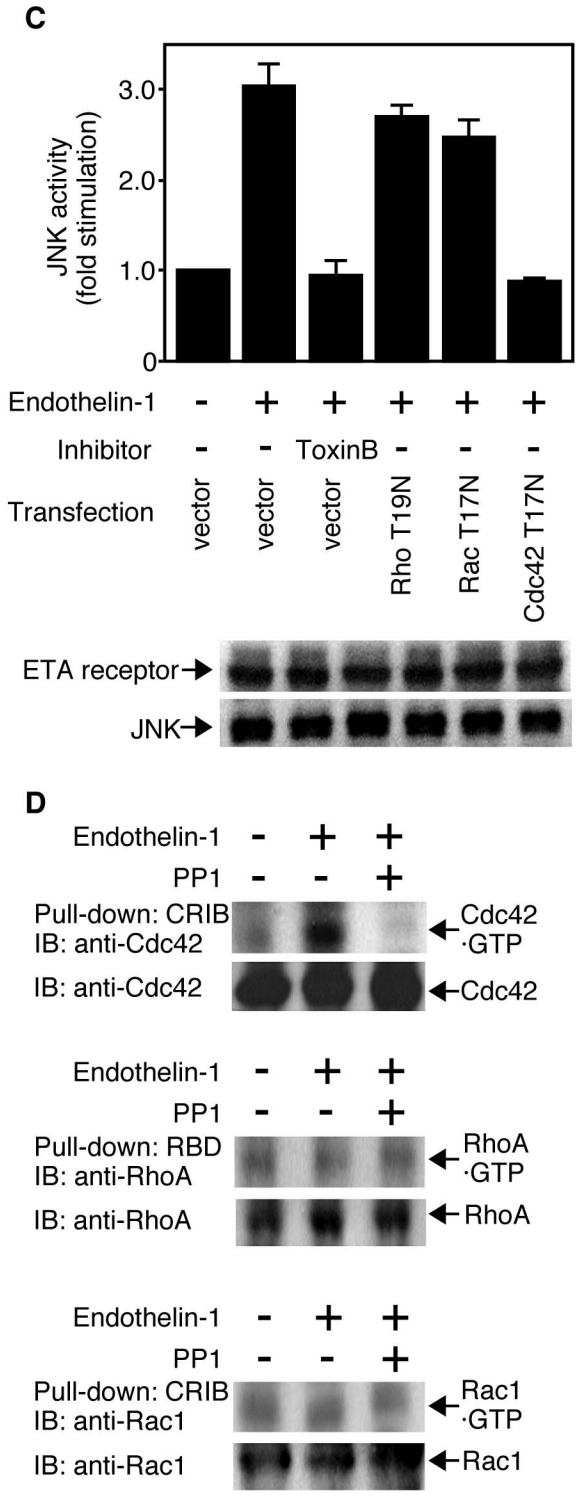

Figure 3C-D Activation of JNK induced by the ETA receptor is mediated by Src kinase and Cdc42. (C) Cells were transfected with the plasmids encoding the ETA receptor and JNK and pretreated without or with 0.2 ng/ml toxinB for 16 h before the addition of 100 nM endothelin. The JNK activity was measured by the amount of [^{32}P]-radioactivity incorporated into recombinant c-Jun. (D) Cells were transfected with the plasmid encoding the ETA receptor and pretreated without or with 10 mM PP1 for 16 h before addition of 100 nM endothelin. The active form of Rho GTPases was measured by a pull-down assay using recombinant mDia1RBD or αPakCRIB. Expression of Rho GTPases in the cell lysates was estimated by immunoblot analysis.

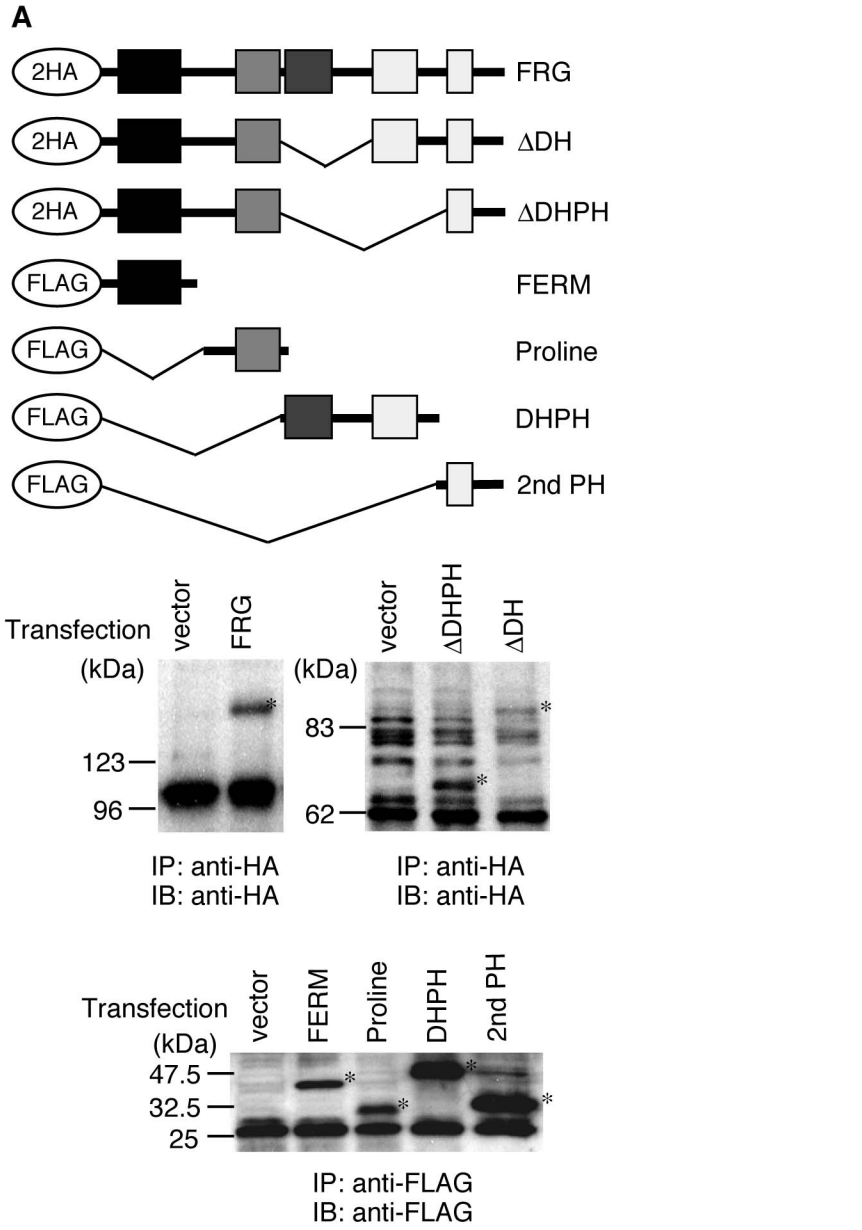

Figure 4A. Structure and expression of variant mutants of FRG. (A) Structures of the proteins encoded by the expression plasmids used in this study are shown (upper). Lysates from cells transfected with a vector or with the expression plasmid encoding FRG, FRG (ΔDHPH), FRG (ΔDH), the FRG-FERM domain (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 and then immunoblotted with an anti-HA antibody (middle) or an anti-FLAG antibody (lower).

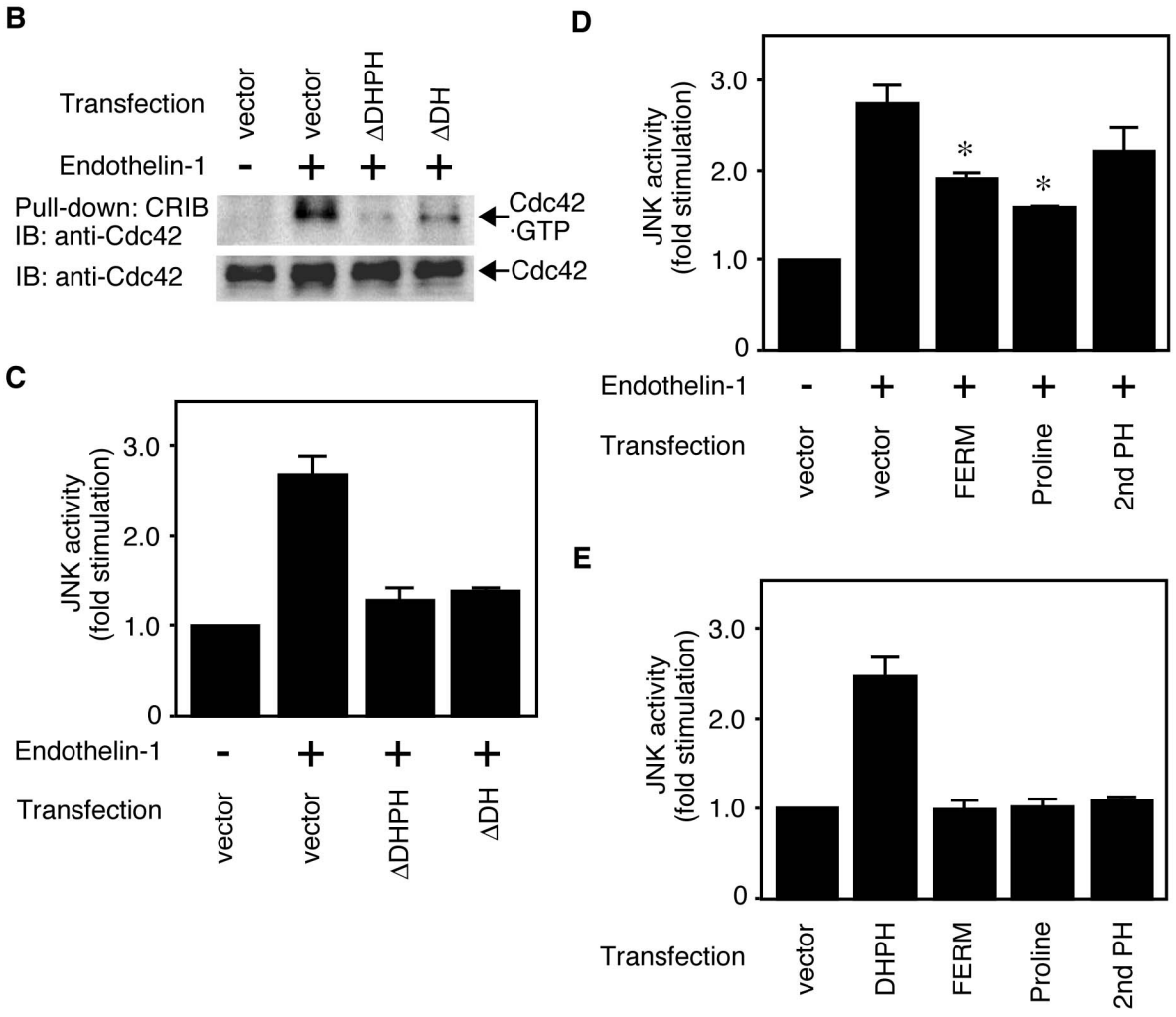

Figure 4B-E. Involvement of FRG in the signaling pathway from the ETA receptor to JNK. (B) Cells were transfected with the plasmids encoding the ETA receptor. The active form of Cdc42 was measured by a pull-down assay using recombinant αPakCRIB. Expression of Cdc42 in the cell lysates was estimated by 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 [^{32}P]-radioactivity incorporated into recombinant c-Jun. Asterisks indicate $p<0.01$ ($n=5$) as compared with a vector under endothelin stimulation (D).

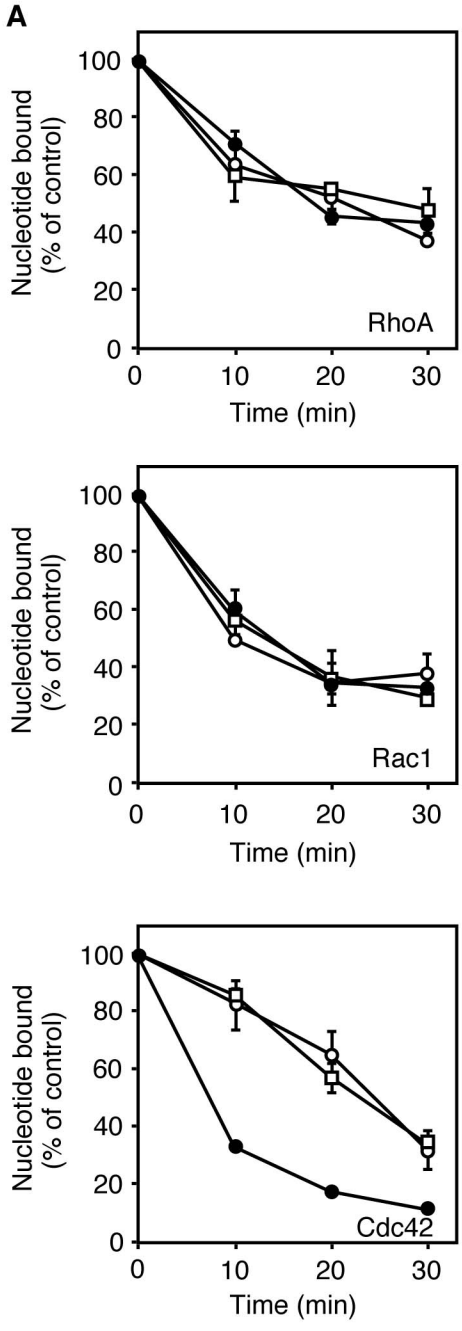

Figure 5A. Activation of the Cdc42-GEF activity of FRG upon stimulation of the ETA receptor. (A) Cells were transfected with a mock plasmid (open square) or the plasmids encoding the ETA receptor and FRG (open circle and close circle) and treated without (open circle) or with (close circle) 100 nM endothelin. Release of guanine nucleotide from RhoA (upper), Rac1 (middle), and Cdc42 (lower) by immunoprecipitated FRG was assayed as described in the Materials and Methods.

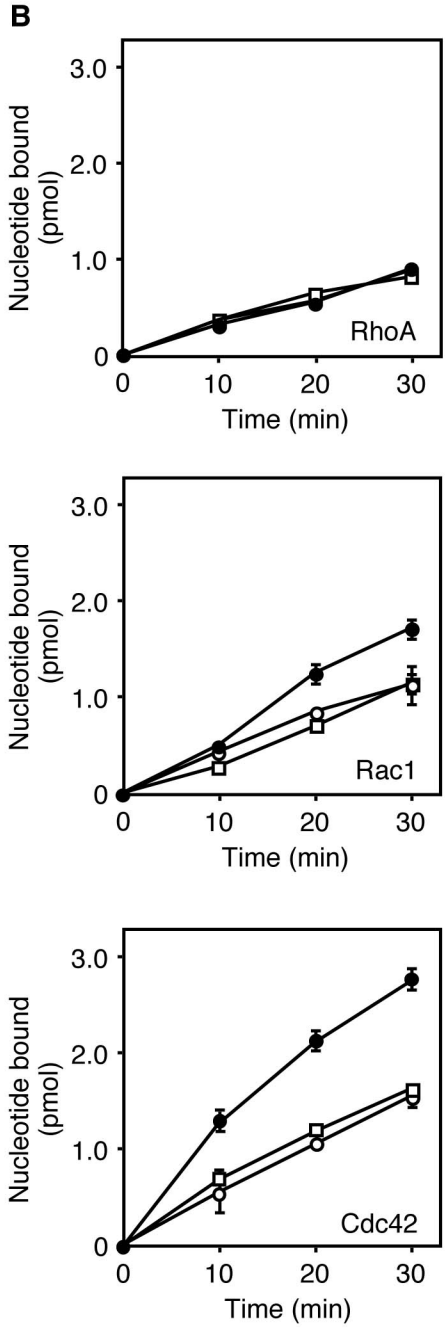

Figure 5B. Activation of the Cdc42-GEF activity of FRG upon stimulation of the ETA receptor. (B) Cells were transfected with a mock plasmid (open square) or the plasmids encoding the ETA receptor and FRG (open circle and close circle) and treated without (open circle) or with (close circle) 100 nM endothelin. Binding of guanine nucleotide for RhoA (upper), Rac1 (middle), and Cdc42 (lower) was assayed as described in the Materials and Methods.

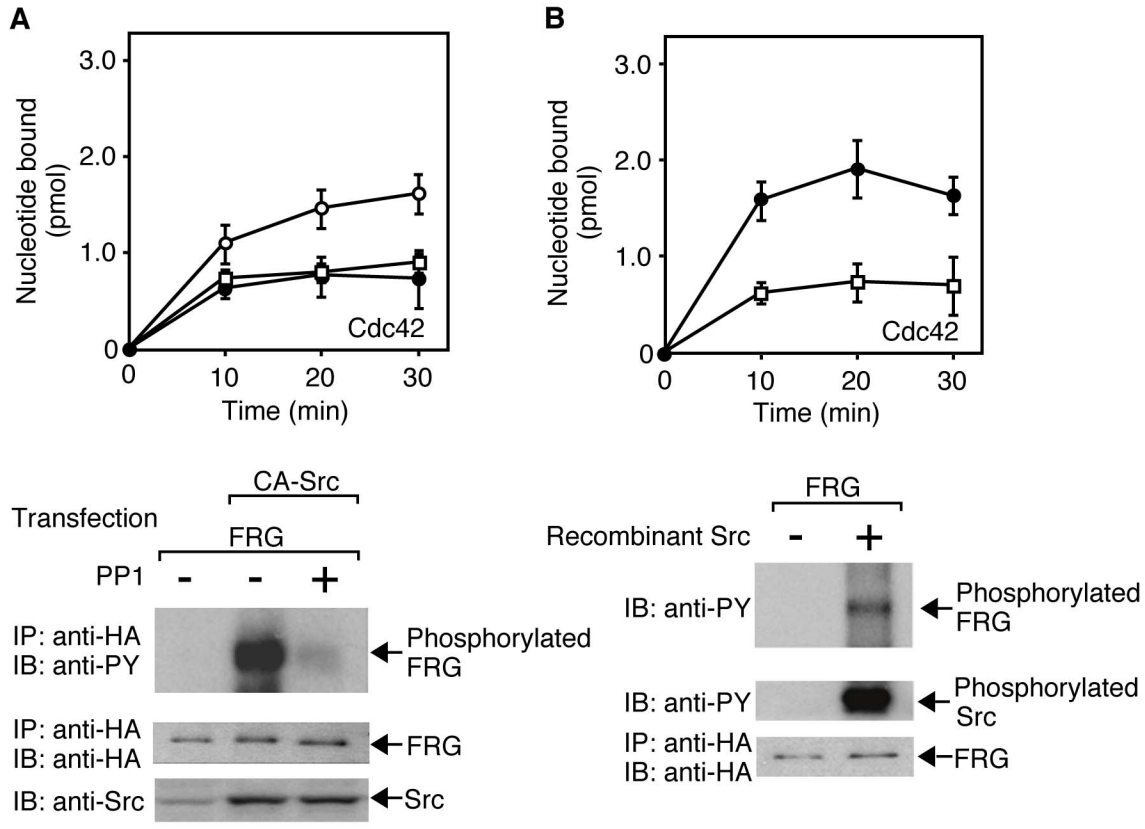

Figure 6A-B. Src kinase-dependent activation of the Cdc42-GEF activity of FRG in cells and in vitro. (A) Cells were transfected with a mock plasmid (open square) or the plasmids encoding CA-Src and FRG (open circle and closed circle) and pretreated without (open circle) or with (closed circle) 10 mM PP1. Binding of guanine nucleotide to Cdc42 was assayed as described in the Materials and Methods. HA-FRG was immunoprecipitated with an anti-HA antibody from the cell lysates and immunoblotted 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 square) or with (closed circle) recombinant c-Src, and binding of guanine nucleotide to Cdc42 was assayed as described in the Materials and Methods. 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.

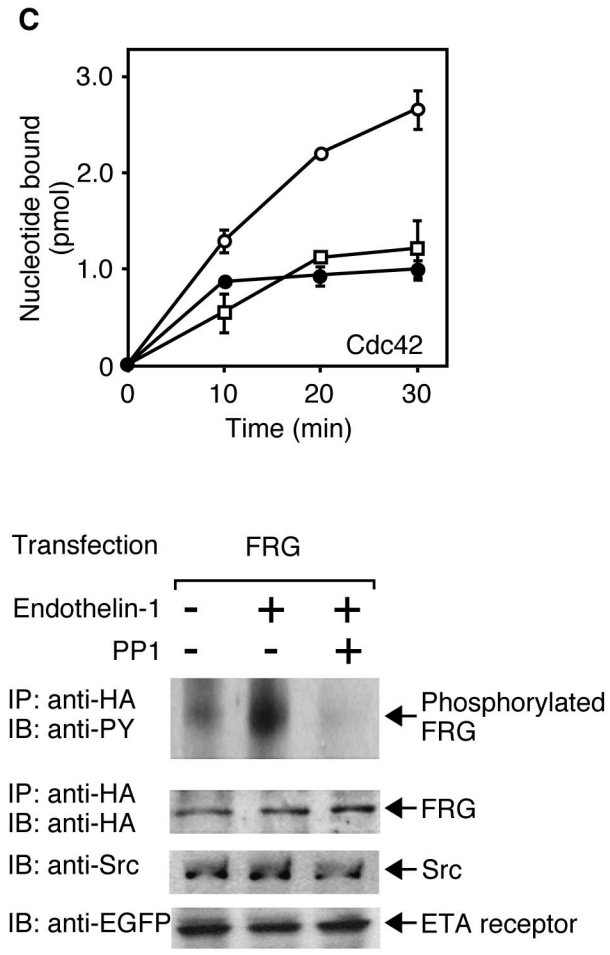

Figure 6C. Src kinase-dependent activation of the Cdc42-GEF activity of FRG in cells. (C) Cells were transfected with a mock plasmid (open square) or the plasmids encoding the ETA receptor and FRG (open circle and closed circle) and pretreated without (open circle) or with (closed circle) 10 mM PP1 for 16 h before the addition of 100 nM endothelin (open circle and closed circle). Binding of guanine nucleotide to Cdc42 was assayed as described in the Materials and Methods. 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 (middle) and the ETA receptor (lower) is shown.

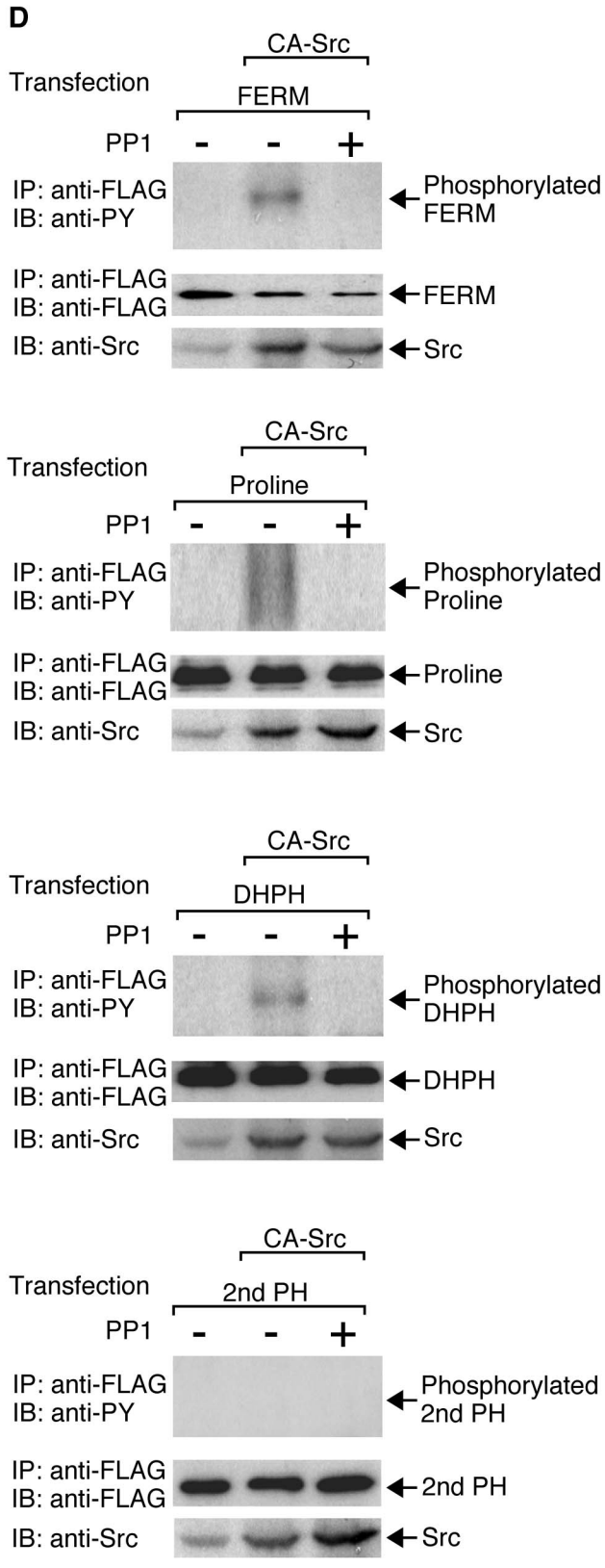

Figure 6D. Src kinase-dependent phosphorylation of FRG in cells. (D) Cells were transfected with the plasmids encoding CA-Src and variant mutants of FRG, FRG-FERM (first panel), FRG-Proline (second panel), FRG-DHPH (third panel), and FRG-2ndPH (fourth panel) and pretreated without or with 10 mM PP1 for 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) or an anti-FLAG antibody (middle). Expression of CA-Src is shown (lower).

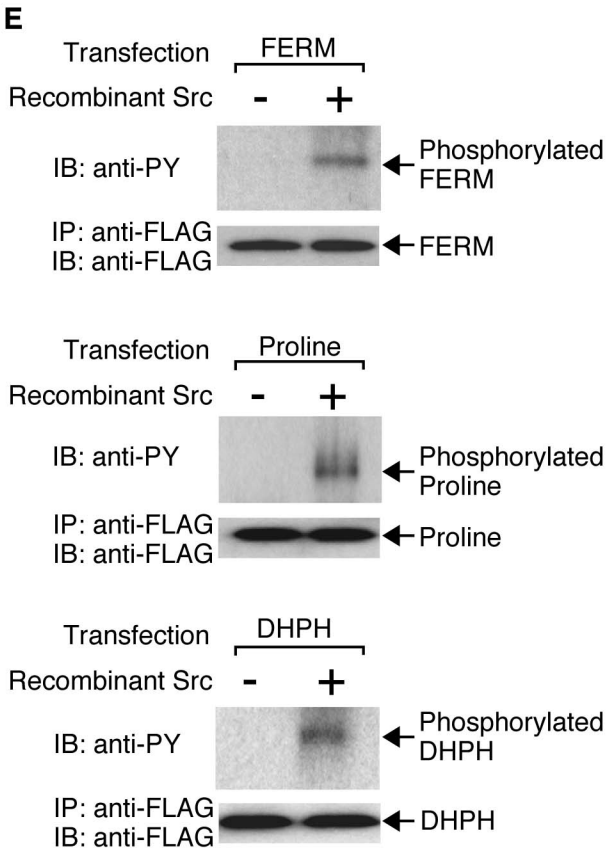

Figure 6E. Src kinase-dependent phosphorylation of FRG in vitro. (E) The immobilized FRG-FERM (first panel), FRG-Proline (second panel), and FRG-DHPH (third panel) were incubated with recombinant c-Src, and their tyrosine-phosphorylation was analyzed using immunoblotting with an anti-phosphorylated tyrosine antibody (upper). Expression of FRG mutants (lower) is shown.

Discussion

Although there are many Rho GEFs (9), it remains unclear how these Rho GEFs are activated by extracellular signals. In the present study, I performed a BLAST search using the DH domain of Dbs, a GEF involved in the GPCR-Rho GTPase signaling pathway, as a query. I selected one putative GEF, 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 GEFs for Cdc42 (36, 38). Using *in vivo* and *in vitro* assays, I demonstrated that KIAA0793 is also a Cdc42-specific GEF. I, therefore, refer to this GEF 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 migration. On the basis of these findings, I summarized the proposed signaling pathway in Figure 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 Gαβγ has been reported to stimulate Rho 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 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 Gαq•GTP, 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/Gαβγ in Src kinase-dependent or -independent manner.

Vav family GEFs, Vav, Vav2, and Vav3, are representative of Rho family GEFs that are directly tyrosine-phosphorylated and activated by tyrosine kinases of the Src family (20-23). However, it remains unknown whether Vav family GEFs are regulated generally downstream of GPCRs/Gαβγ. The activation of Vav by the N-formyl peptide receptor, a GPCR expressed in neutrophils and monocytes, has been reported. Ma *et al*. showed that Gβγ and phosphoinositide 3-kinaseγ activate the Rac1-GEF activity of Vav in a manner apparently independent of the tyrosine kinase (23). As far as I know, this is the first report of the Cdc42-specific GEF, which is directly phosphorylated and activated by Src kinase in the signaling pathway downstream of GPCR.

The DH and PH domains of FRG were tyrosine-phosphorylated by Src kinase *in vivo* and *in vitro* (Figure 6D-E). Structure analysis revealed that the DH domain of Vav is autoinhibited by its N-terminal region and that the Tyr174 in this region is phosphorylated by Src family kinase (21). Phosphorylation or deletion of this region results in the stimulation of GEF activity (22-23). Thus, tyrosine phosphorylation relieves autoinhibition by opening the Rho GTPase interaction surface of the DH domain (21-23). It is conceivable that tyrosine phosphorylation of the DH and PH domains of FRG may also play an essential role in the activation of GEF activity of FRG. However, the FERM and proline-rich domains of FRG were also tyrosine-phosphorylated by Src kinase (Figure 6D-E). Therefore, these regions are likely to provide the tyrosine phosphorylation sites required for the stimulation of the exchange activity of FRG. Further study should help reveal details of the regulatory mechanism of FRG by Src kinase.

Kubo *et al*. recently characterized KIAA0793 as a signaling molecule of neurite remodeling (59). They showed that KIAA0793 is a Rac1-specific GEF, while its activation mechanism has

not been described. It seems contradictory that I showed KIAA0793 as the Cdc42-specific GEF. This may be due to 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, I identified a new signaling molecule, FRG, which directly links between Src kinase and Rho GTPase Cdc42 in the GPCR/JNK signaling pathway (Figure 7). Recently, it was indicated that tyrosine kinases are involved in the G protein-induced activation of Rho GEFs (60, 61). Further study is necessary to clarify how Src kinase regulates Rho GEFs in the pathway linking GPCRs to Rho GTPases. Additionally, such studies might promote our understanding of the general mechanism by which Src kinase regulates Rho GEFs, and, thereby, help to elucidate the cellular function of the pathway.

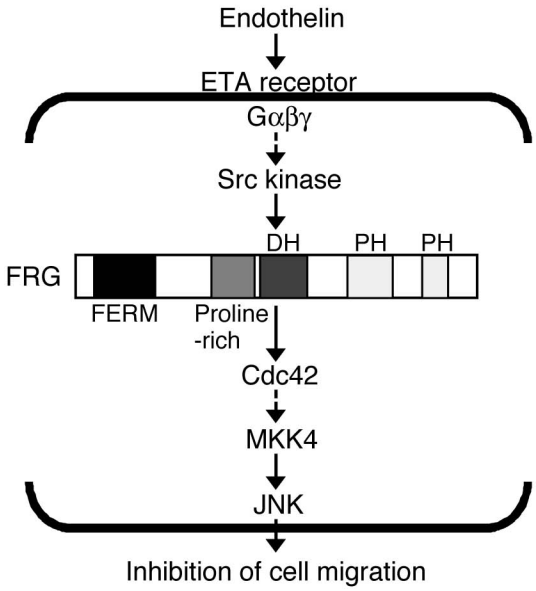

Figure 7. Schematic model for the signaling pathway from the ETA receptor to JNK. Details are described under Discussion.

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Conclusion

There are a number of studies on chemokines, the soluble chemoattractant factors, and their cognate chemokine receptors, which belong to the G protein-coupled receptors (GPCRs). On the other hand, there is only limited information about the GPCRs-mediated inhibition of cell migration and their intracellular signaling mechanisms. In Chapter 1, I show that the adaptor protein, Nck1, but not Grb2 or CrkII, mediates the inhibition of cell migration induced by the endothelin-1 (ET-1) and endothelin type A (ETA) receptor. The small interference RNA and dominant negative mutants of Nck1 inhibited the ET-1-induced inhibition. Although overexpression of wild-type Nck1 was detected in the cytosol and did not affect cell migration, expression of the myristoylation signal sequence-conjugated Nck1 was in the membrane and induced activation of Cdc42 and c-Jun N-terminal kinase (JNK), inhibiting cell migration. Taken together, these results indicate that the ET-1/ETA receptor transduces the signal of inhibition of cell migration through Cdc42-dependent JNK activation by using Nck1.

In Chapter 2, I show that KIAA0793, containing substantial sequence homology with the catalytic Dbl homology (DH) domain of the faciogenital dysplasia gene product (FGD1), is a specific GEF for Cdc42. I, therefore, tentatively named it FRG (FGD1-related Cdc42-GEF). Src kinase directly phosphorylated and activated FRG, as Vav family GEFs. Additionally, FRG was involved in the signaling pathway from the ETA receptor to JNK, resulting in the inhibition of cell migration. Together, these results demonstrate that FRG is a member of Cdc42-GEF and plays a key role in the signaling pathway downstream of GPCRs.

It is noteworthy to examine whether FRG is a binding partner with the SH3 domain of Nck1 in the ET-1/ETA receptor-signaling pathway. A challenge for the future will aid into defining the roles of Nck1 in regulating the potential for crosstalks among the various signaling pathways,

involving FRG in the control of cell migration. Such studies should promote our understanding of the GPCR-regulated mechanism of the early process of development as well as oncogenesis.

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