Proline-Rich Domain Plays a Crucial Role in Extracellular Stimuli-Responsive Translocation of a Cdc42 Guanine Nucleotide Exchange Factor, FGD1

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We previously demonstrated that FGD1, the Cdc42 guanine nucleotide exchange factor (GEF) responsible for faciogenital dysplasia, and its homologue FGD3 are targeted by the ubiquitin ligase SCF^FWDI upon phosphorylation of two serine residues in their DSGIDS motif and subsequently degraded by the proteasome. FGD1 and FGD3 share highly homologous Dbl homology (DH) and adjacent pleckstrin homology (PH) domains, both of which are responsible for GEF activity. However, their function and regulation are remarkably different. Here we demonstrate extracellular signal-responsive translocation of FGD1, but not FGD3. During the wound-healing process, translocation of FGD1 to the leading edge membrane occurs in cells facing to the wound. Furthermore, epidermal growth factor (EGF) stimulates the membrane translocation of FGD1, but not FGD3. As the most striking difference, FGD3 lacks the N-terminal proline-rich domain that is conserved in FGD1, indicating that proline-rich domain may play a crucial role in signal-responsive translocation of FGD1. Indeed, there is a faciogenital dysplasia patient who has a missense mutation in proline-rich domain of FGD1, by which the serine residue at position 205 is substituted with isoleucine. When expressed in cells, the mutant FGD1 with S^205/I substitution fails to translocate to the membrane in response to the mitogenic stimuli. Thus we present a novel mechanism by which the activity of FGD1, a GEF for Cdc42, is temporally and spatially regulated in cells.

Key words FGD1; FGD3; guanine nucleotide exchange factor; proline-rich domain; Cdc42; Rho family G protein

The Rho family small guanosine 5’-triphosphate (GTP)-binding proteins (G-proteins), consisting mainly of the Rho, Rac, and Cdc42 subfamilies, regulates various actin cytoskeleton-dependent cell functions, including cell shape change, cell migration, cell adhesion, and cytokinesis.1–3 Like all G-proteins, Rho family proteins act as binary switches by cycling between an inactive (GDP-bound) and an active (GTP-bound) conformation state.

Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP for GTP to generate active forms of Rho proteins. A family of candidate GEFs for Rho proteins has been identified based on the homology with CDC24, a protein genetically as an upstream activator of CDC42 in yeast.4 Dbl, isolated as an oncogene in a focus formation assay using NIH 3T3 cells transfected with DNA from a human diffuse B-cell lymphoma, was identified as the first mammalian Rho GEF showing significant sequence similarity to CDC24.5 The domain conserved in both Dbl and CDC24 is now recognized as the Dbl homology (DH) domain and almost 60 DH-domain-containing proteins have been found.6 The activities of GEFs must be tightly regulated and each member of the GEF family is likely to have a unique mechanism for activation and inactivation.

In our previous reports, we revealed that FGD1, a GEF for Cdc42 and known as the faciogenital dysplasia (or Aarskog-Scott syndrome; AAS) gene product, and FGD3, a homologue of FGD1, underwent proteasomal degradation through the ubiquitin ligation by the ubiquitin ligase SCF^FWDI.7,8 FGD3 contains adjacent DH and pleckstrin homology (PH) domains, both of which are highly homologous to those of FGD1. Pasteris et al. reported that DH+PH domains of FGD3 induced long finger-like protrusions, filopodia, as DH+PH domains of FGD1 did, suggesting that both FGD1 and FGD3 share the similar GEF activity to induce Cdc42 activation.9 However, we demonstrated that the inducible expression of the full-length FGD1 led to the formation of striking filopodia structures whereas that of full-length FGD3 induced broad sheet-like protrusions, i.e., remarkable lamellipodia structures.8 These results indicate that FGD1 and FGD3 play different roles in cells but undergo the same destruction pathway.

Although higher sequence similarity between FGD1 and FGD3 is observed in their DH and adjacent PH domains (i.e., amino acid residue identity is 70.0% and 60.6%, respectively),9 there exist striking differences in their sequence in other regions; for example, most prominently, FGD3 lacks the N-terminal proline-rich domain conserved in FGD1.

Proline-rich sequences are known to bind to specific protein interaction modules such as Src homology 3 (SH3), WW, and Ena/VASP homology 1 (EVH1) domains.10–12 These different modules recognize different types of proline-rich sequences and play important roles in various signal transduction pathways. Phenotypic and molecular analyses revealed that a patient with missense mutation occurred in the proline-rich domain in FGD1 showed typical clinical features as AAS.13 In this mutant FGD1, the serine residue at position 205 was replaced with isoleucine. Although the DH+PH domains necessary for the GEF activity remain intact in FGD1 (S^205/I), impaired interaction with other molecules via proline-rich domain may result in the loss of function of this mutant FGD1. It is likely that the activity of FGD1 may be turned on at an appropriate intracellular compartment through the interaction with the upstream signaling modules that are activated in a temporally and spatially regulated manner. In this study, we examined the extracellular stimuli-regulated function of FGD1. Immunostaining analyses revealed that FGD1 translocates to the leading edge membrane in migrating cells. The translocation of FGD1
also occurs in cells exposed to epidermal growth factor (EGF). By fractionating cells, we demonstrated that FGD1 translocates to the membrane fraction in cells under mitogenic condition while FGD3 and FGD1 (S205/I) do not. These results indicate that FGD1 is targeted to the membrane upon the interaction with proline-binding modules that are activated at the specific compartment in the membrane.

MATERIALS AND METHODS

Reagents Tetracycline (Tc) and anti-FLAG M2 Ab were obtained from Sigma-Aldrich. Fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse immunoglobulin G (IgG) was the product of Jackson ImmunoResearch Laboratories. Alexa Fluor 594 phalloidin was obtained from Molecular Probes. ProtoExtract Subcellular Proteome Extraction Kit was purchased from Calbiochem.

Cell Culture HeLa Tet-Off-derived stable cell lines that inductively overexpress either FGD1(SA) or FGD3(SA), in which the destruction sequence DSGIDS is replaced with DAGIDA were maintained in 5% fetal calf serum (FCS)/Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 μg/ml G418, 200 μg/ml hygromycin, and 2 μg/ml Tc as described previously. To induce FGD1(SA) or FGD3(SA), cells were trypsinized and then cultured in the absence of Tc for 72 h.

Immunostaining Immunostaining analyses were carried out as follows: cells cultured on glass coverslips were fixed with 4% paraformaldehyde, then permeabilized with 0.25% Triton X-100 and blocked with 10% skim milk in PBS. The cells were incubated with anti-FLAG M2 antibody, followed by the double staining with FITC-conjugated goat anti-mouse IgG and Alexa Fluor 594 phalloidin for 1 h. Fluorescence images were captured by CCD camera (QICAM FAST1394; QIMAGING) mounted on a Leica DM IRB microscope using IP Laboratory imaging software (Scanalytics).

Subcellular Fractionation Cells seeded on 35-mm dishes were harvested and subcellular fractionation was carried out using ProtoExtract Subcellular Proteome Extraction Kit. Briefly, cells were washed twice with ice-cold Wash Buffer and then cytosolic proteins were extracted with 0.4 ml ice-cold Extraction Buffer I. Subsequently, membranes and membrane organelles were solubilized with 0.4 ml ice-cold Extraction Buffer II without impairing the integrity of nucleus and cytoskeleton. Next, nuclear proteins were enriched with 0.2 ml ice-cold Extraction Buffer III. Finally, components of the cytoskeleton were solubilized with 0.2 ml room temperature Extraction Buffer IV.

Immunoblotting Extracts of cytosolic, membrane, nuclear and cytoskeletal fractions were resolved by SDS-PAGE and then subjected to immunoblotting analyses. The immunocomplexes on the polyvinylidene difluoride membranes were visualized using enhanced chemiluminescence detection.

Site-Directed Mutagenesis and Establishment of Stable Cell Lines Expressing FGD1(SA+S205/I) In order to replace Ser205 in FGD1 with an isoleucine, we carried out site-directed mutagenesis by PCR using the following primers designed in inverted tail-to-tail direction to amplify vector together with the target sequence: 5'-ACTAGTTCT-GCAGCATATCA-3' (sense) and 5'-GATGCGCTCAC-CTCTGGGGAC-3' (anti-sense). For the template cDNA, pBluescript SK(+)−FLAG-FGD1(SA) was used. The amplified sequence was verified by dyeoxy sequencing, and then FLAG-FGD1(SA+S205/I) cDNA was subcloned into pTRE vector (Clontech) and resultant plasmid was termed pTRE−FLAG-FGD1(SA+S205/I).

HeLa Tet-Off cells purchased from Clontech were cotransfected with pTRE-FLAG-FGD1(SA+S205/I) and pTK-Hyg. The hygromycin-resistant clonal cell lines were tested for Tc-regulated FLAG-tagged FGD1 expression.

RESULTS

In our previous study, we demonstrated that FGD1 and FGD3 are targeted by the ubiquitin ligase SCF<sup>FWD1</sup> upon phosphorylation of two serine residues in the conserved DSGIDS motif and then subsequently degraded by the proteasome. Sequence similarity between FGD1 and FGD3 is also observed in their DH and adjacent PH domain (i.e., amino acid residue identity is 70.0% and 60.6%, respectively). In contrast, there are striking differences in their sequence in other region; most prominently, FGD3 lacks the N-terminal proline-rich domain conserved in FGD1 (Fig. 1A). We previously established the HeLa Tet-Off-derived stable cell lines that inductively overexpress either FGD1(SA) or FGD3(SA), in which the destruction sequence DSGIDS is replaced with DAGIDA and found that cells expressing FGD1(SA) showed representative filopodia structure whereas cells expressing FGD3(SA) showed broad and sheet-like protrusions called lamellipodia, demonstrating that FGD1 and FGD3 induce different types of actin cytoskeleton reorganization.

In the present study, we further examined the difference of intracellular distribution of FGD1 and FGD3. Confluent monolayer of cells expressing FGD1(SA) or FGD3(SA) were wounded and further cultured for 30 min. Cells were then immunostained to detect the FLAG epitope. As shown in Fig. 1B, translocation of FGD1 to the leading edge membrane was prominently observed in FGD1(SA)-expressing cells that faced to the wound, whereas it was not obvious in FGD3(SA)-expressing cells. Interestingly, mitogenic stimulus-dependent translocation of FGD1, but not FGD3, was also observed. As reported previously, control FGD1(SA)-expressing cells showed long, finger-like protrusions called filopodia (Fig. 1C, “actin”). The treatment of cells with EGF induced membrane ruffles that were significantly stained with anti-FLAG antibody (Fig. 1C, “FLAG”). In contrast, EGF did not change the intracellular distribution of FGD3 (Fig. 1D).

We next examined the intracellular distribution of FGD1 and FGD3 by the use of subcellular fractionation kit. FGD1 was mainly detected in cytosolic fraction during the culture without FCS for 24 h while a small amount of FGD1 was also found in cytoskeletal fraction (Fig. 2A). In contrast, the amount of FGD1 in membrane fraction was significantly increased when cells were kept cultured in the presence of 5% FCS (Fig. 2A). Unlike FGD1, FCS did not affect the distribution of FGD3 that were detected in both cytosol and membrane fractions (Fig. 2B). These results suggest that FGD1 but not FGD3 will change its intracellular distribution in re-
response to the mitogenic stimuli. Indeed, translocation of FGD1 in the membrane fractions was clearly demonstrated in a time-dependent manner after cells were treated with EGF (Fig. 3A). In contrast, FGD3 did not change its distribution whether or not cells were treated with EGF (Fig. 3B).

Since FGD3 lacks the N-terminal proline-rich region while FGD1 contains it, we next examined the role of proline-rich domain of FGD1 in mitogenic stimuli-responsive translocation to the membrane.

FGD1 was originally identified and characterized by positional cloning in a family in which the phenotype of the developmental disorder was associated with an X;8 translocation.14,15) Genetic analyses revealed that different types of point mutations occurred in FGD1 are also responsible for AAS.16,17) In one of such mutations, we focused on the mutation in proline-rich domain of FGD1 (S205/I).

Orrico et al. identified the S205/I mutation in FGD1 from one of the patients with typical AAS phenotype.17) Proline-rich regions often contain serine or threonine residues, suggesting that proline-rich sequence-directed protein kinase may bind and phosphorylate these residues. As shown in Fig. 4A, not only in human FGD1 but also in FGD1 from other species, the serine residue at position 205 (position 36 in the case of rat) is conserved. Therefore, we established a HeLa Tet Off-FGD1 cell line that inducibly expresses a mutant mouse FGD1 protein in which the serine residue at position 205 is replaced with isoleucine in addition to DAGIDA replacement. As shown in Fig. 4B, several cells expressing FGD1 (SA/H11001 S205/I) showed two or three broad sheet-like lamellae at opposite sides of cells, as in the case of FGD3-expressing cells. Furthermore, in contrast to the case of wild type FGD1, FGD1 (SA/H11001 S205/I) was detected in cytosolic fraction even in the presence of 5% FCS during the culture (Fig. 4C). These results demonstrated that S205 conserved in proline-rich regions often contain serine or threonine residues, suggesting that proline-rich sequence-directed protein kinase may bind and phosphorylate these residues.
rich domain is critical for the membrane translocation of FGD1 in response to the mitogenic stimuli.

DISCUSSION

The Rho-family small G-proteins are defined by the presence of a Rho-type GTPase-like domain and to date 22 human genes have been described.\(^{18}\) For outnumbering Rho proteins, more than 60 GEFs and approximately 80 GTPase-activating proteins (GAPs) are encoded in human genome.\(^{6,19}\) Therefore, the activities of individual Rho family proteins in cells should be regulated by these overabundant GEFs and GAPs in upstream signals-dependent manners.

In our previous study, we revealed that two homologous GEFs, FGD1 and FGD3, conserved the same destruction se-
sequence “DSGISD” that lead to the down-regulation of both molecules by GSK-3β/SCF/FGD3, the proteasome-regulated pathway.\textsuperscript{7,8} When expressed as truncated forms consisting of only DH + PH domains that are necessary for the GEF activity, both FGD1 and FGD3 induced similar morphological changes; i.e., large numbers of filopodial extensions were formed out of cell periphery while neither stress fiber nor lamellipodia were observed.\textsuperscript{9,20} However, we found that FGD1 induces remarkable filopodial extensions while FGD3 induces thin and sheet-like protrusions known as lamellipodia when expressed as the full length GEFs.\textsuperscript{5,9}

In our previous report, we described that the broad sheet-like protrusions recognized as lamellipodia in FGD3(SA)-expressing cells contained many microspikes.\textsuperscript{5} Interestingly, at the tips of several microspikes, FGD3 was clearly stained whereas actin filaments were not stained at all.\textsuperscript{5} Interconversion between microspikes, filopodia and retraction fibers may be occurring at lamellipodial protrusions induced by FGD3. These broad sheet-like lamellae are often observed at opposite side of a cell. The “lamellipodium” is sometimes referred as the “leading edge” or the “leading lamella.”\textsuperscript{21} In the case of FGD3(SA)-expressing cells having two broad sheet-like lamellae oriented in opposite directions, it is therefore difficult to know which of them represents the “leading lamella.” In this study, we demonstrated that FGD1 was strongly stained at the membrane ruffles formed in cells stimulated with EGF or in cells facing to the wound-healing front (Figs. 1B, C). Translocation of FGD1 to the leading edge membranes was induced in an extracellular stimulus-dependent manner (Figs. 2A, 3A). Therefore, we can assume that FGD1, which responds to the “motogenic” stimuli and participates in leading lamella formation. It should be noted that the S\textsuperscript{205} residue conserved in proline-rich domain of FGD1 plays an important role in the function of FGD1, since the S\textsuperscript{205} /I mutation affected the patients with the characteristic AAS phenotype. Indeed, S\textsuperscript{205} /I mutation significantly impaired the signal-responsive translocation of FGD1 (Fig. 4C).

Hou et al. described that FGD1 directly interacted with SH3 domains of mouse cortactin and actin-binding protein 1 (mAbp1) through the SH3-binding region located in FGD1 proline-rich domain.\textsuperscript{23} Using yeast two-hybrid and in vitro binding techniques, they identified the FGD1 SH3-binding region as the amino acid sequence P\textsuperscript{157}PKQVPPPK\textsuperscript{165}.\textsuperscript{23} They also showed that full length FGD1, but not the truncated FGD1 lacking proline-rich region (residues 1—358), translocated to the ruffling membranes in response to PDGF.

Since the N-terminal proline-rich region (1—358) also contains S\textsuperscript{205}, we should emphasize that S\textsuperscript{205} as well as the SH3-binding region (residues 157—165) may be critical in order to translocate FGD1 to membrane ruffles.

We previously focused on the phosphorylation of two serine residues in the sequence of DS\textsuperscript{283}GIDS\textsuperscript{287}, which leads to the destruction of FGD1.\textsuperscript{7} We should next focus on the phosphorylation of S\textsuperscript{205}, which may result in the stabilization of FGD1 at specific intracellular compartments in response to the “motogenic” stimuli.

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REFERENCES