Role of FGD1, a Cdc42 Guanine Nucleotide Exchange Factor, in Epidermal Growth Factor-Stimulated c-Jun NH₂-Terminal Kinase Activation and Cell Migration

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FGD1 encodes a guanine nucleotide exchange factor for Cdc42. Mutations in the *FGD1* gene are responsible for an X-linked disorder known as Aarskog-Scott syndrome (AAS). While most mutations were found in the catalytic region, which consists of Dbl homology (DH) domain and adjacent pleckstrin homology (PH) domain, a missense mutation in the proline-rich domain is also found in a patient with typical clinical features as AAS. In this mutant FGD1, the serine residue at 205 is replaced with isoleucine. We recently demonstrated that FGD1 translocated to the membrane in response to extracellular stimuli such as epidermal growth factor (EGF) whereas FGD1 with S²⁰⁵/I substitution did not. Here we show that the proline-rich domain is critical for FGD1-induced directionally persistent cell migration. When inducibly expressed in HeLa Tet-Off cells, FGD1 stimulates directional migration whereas FGD1 with S²⁰⁵/I substitution does not affect it. We further demonstrate that FGD1 augments EGF-stimulated c-Jun NH₂-terminal kinase (JNK) activation. In the presence of JNK inhibitor SP600125, motility of FGD1-expressing cells is significantly impaired, indicating a critical role of JNK in cell migration. However, FGD3, an FGD1 homologue lacking the proline-rich domain, and FGD1 with S²⁰⁵/I substitution augment EGF-stimulated JNK activation similarly to FGD1, suggesting that the proline-rich domain is not involved in the regulation of JNK. Finally, we show that FGD1, but not FGD1 with S²⁰⁵/I substitution, is phosphorylated in response to EGF, suggesting that the phosphorylation of S²⁰⁵ may trigger the FGD1 translocation to the leading edge membrane and enable cells to undergo directional migration.

Key words FGD1; guanine nucleotide exchange factor; cell migration; c-Jun NH₂-terminal kinase; Aarskog-Scott syndrome; Cdc42

Cell migration is an essential process involved in development, wound healing, tumor invasion, and metastasis. In migrating cells, re-organization of the actin cytoskeleton occurs dynamically to provide the force for cell motility. The Rho family small guanosine 5'-triphosphate (GTP)-binding proteins (G-proteins), consisting mainly of the Rho, Rac, and Cdc42 subfamilies, are key molecular switches that regulate 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 (guanosine 5'-diphosphate (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, whereas GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity of Rho proteins to inactivate the switch. In theory, the active state of Rho proteins may be obtained through stimulation of a GEF or inhibition of a GAP, however, most evidence indicates that GEFs are the critical mediators for the activation of Rho proteins.⁴⁾

FGD1, a GEF for Cdc42, was originally identified and characterized by positional cloning in a family in which the phenotype of the developmental disorder (known as the faciogenital dysplasia or Aarskog-Scott syndrome; AAS) was associated with an X; 8 translocation.^{5,6)} Further genetic analyses revealed that different types of point mutations occurred in FGD1 are also responsible for AAS.⁷⁾ While most mutations were found in the DH domain or adjacent PH domain of FGD1, Orrico *et al.* reported that a patient with a missense mutation in the proline-rich domain of FGD1 showed typical clinical features as AAS.⁸⁾ In this mutant

FGD1, the serine residue at position 205 is replaced with isoleucine and the DH-PH modules necessary for the GEF activity remain intact.

In our previous reports, we revealed that FGD1 and FGD3, a homologue of FGD1, underwent proteasomal degradation through the polyubiquitination by the ubiquitin ligase SCF^{FWD1}.^{9,10)} FGD1 and FGD3 share strikingly similar Dbl homology (DH) domains and adjacent pleckstrin homology (PH) domains, both of which (*i.e.*, DH-PH modules) are responsible for guanine nucleotide exchange, however, there exist remarkable differences in their biological functions.¹⁰⁾ Whereas FGD1 induced long finger-like protrusions, FGD3 induced broad sheet-like protrusions when the level of GTP-bound Cdc42 was significantly increased by the inducible expression of FGD3.¹⁰⁾ Furthermore, FGD1 and FGD3 reciprocally regulated cell motility; when inducibly expressed in HeLa Tet-Off cells, FGD1 stimulated cell migrations whereas FGD3 inhibited it.¹⁰⁾

Although higher sequence similarity between FGD1 and FGD3 is observed in their DH-PH modules, there exist significant differences in their sequence in other regions; 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.^{11–13)} We have recently revealed that FGD1 translocated to the membrane fraction in response to epidermal growth factor (EGF) whereas FGD3 lacking the proline-rich domain did not.¹⁴⁾ Furthermore, FGD1 with a missense mutation in the proline-rich domain [FGD1(S²⁰⁵/I)] also failed to translocate to the membrane in response to extracellular stimuli.¹⁴⁾ Since a patient with same missense mutation in

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FGD1 showed typical clinical features as AAS,⁸⁾ the proline-rich domain may play a crucial role in the temporally and spatially regulated activation of FGD1.

Recent studies have demonstrated that mitogen-activated protein kinases (MAPKs), including the c-Jun NH₂-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), are involved in the regulation of cell migration.¹⁵⁾ For example, JNK is thought to regulate cell migration by phosphorylating paxillin, DCX, c-Jun, and microtubule-associated proteins.¹⁵⁾ In addition, inhibition of JNK by either the chemical inhibitor SP600125 or the dominant-negative mutant JNK1AF, significantly impairs the rate of migration of several different cell types.¹⁵⁾ Although overexpression of the DH-PH module of FGD1 was shown to induce JNK activation,^{16,17)} roles of JNK in FGD1-mediated cell migration remain obscure.

In this study, we examined the migratory behavior of cells inducibly expressing FGD1, FGD3, or FGD1 with S²⁰⁵/I substitution in the proline-rich domain. As we reported previously,¹⁰⁾ FGD1 stimulated cell migration whereas FGD3 inhibited it. Interestingly, cell motility was not significantly affected by the inducible expression of FGD1 with S²⁰⁵/I substitution. We also examined the activation profiles of MAPKs in cells inducibly expressing FGD1, FGD3, or FGD1 with S²⁰⁵/I substitution. Neither MAPKs were activated by FGD1 alone, however, EGF-stimulated JNK activation was significantly augmented in FGD1-expressing cells whereas the levels of JNK activation induced by tumor necrosis factor (TNF) or interleukin-1 (IL-1) were not affected by FGD1. Similar results were obtained in cells expressing FGD3 or FGD1 with S²⁰⁵/I substitution. Since JNK inhibitor SP600125 significantly inhibited FGD1-induced cell migration, JNK is necessary to increase intrinsic cell motility. On the other hand, directionally persistent cell migration induced by FGD1 might be achieved in the presence of its proline-rich domain. Furthermore, FGD1, but not FGD1 with S²⁰⁵/I substitution, was shown to be serine-phosphorylated in response to EGF. It is possible that phosphorylation of serine residue at position 205 in the proline-rich domain may play a key role to initiate directionally controlled FGD1-signaling.

MATERIALS AND METHODS

Reagents and Antibodies Recombinant EGF was obtained from Wako Pure Chemical Industries. Recombinant human IL-1 β and recombinant human TNF- α were purchased from R&D Systems. An antibody (Ab) specific for FLAG epitope (M2) was purchased from Sigma-Aldrich, St. Louis, MO, U.S.A. Phospho-SAPK/JNK (Thr183/Tyr185) Ab, SAPK/JNK Ab, phospho-p38 MAP Kinase (Thr180/Tyr182) Ab, p38 MAP Kinase Ab, phospho-p44/42 (Thr202/Tyr204) MAP Kinase Ab, p44/42 MAP Kinase Ab, phospho-SEK1/MKK4 (Thr261) Ab, phospho-MKK7 (Ser271/Thr275) Ab, and phospho-c-Jun (Ser63) II Ab were obtained from Cell Signaling Technology, Beverly, MA, U.S.A. Anti-phosphoserine (clone 4A4) Ab was the product of Millipore. Anti-actin Ab was purchased from Santa Cruz Biotechnology. SP600125 (JNK inhibitor II) was purchased from MERCK, Darmstadt, Germany.

Cell Culture HeLa Tet-Off-derived stable cell lines that inducibly overexpress FGD1(SA), FGD3(SA) or FGD1(SA+

S²⁰⁵/I) were maintained in 5% FCS/Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μ g/ml G418, 200 μ g/ml hygromycin, and 2 μ g/ml tetracycline (Tc,) as described previously.^{10,14)} To induce FGD1(SA), FGD3(SA) or FGD1(SA+S²⁰⁵/I), cells were trypsinized and then cultured in the absence of Tc for 72 h.

Wound-Healing Assay HeLa Tet-Off-derived cell lines were cultured for 48 h in the presence or absence of 2 μ g/ml Tc. Then cells were harvested and seeded on 35-mm dishes coated with type I collagen and allowed to grow to confluence in the presence or absence of Tc. Monolayers of confluent cultures were lightly scratched with a 1000- μ l disposable plastic pipette tip (#111 purchased from Quality). After having been washed with DMEM, the wound regions were allowed to heal in the medium described in the legend to Fig. 1 and Fig. 3.

Immunoblotting and Immunoprecipitation Cells were washed with phosphate buffered saline (PBS) and cell extracts were prepared by using either sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer or RIPA buffer as described below. After normalization of protein content by the protein assay, samples were resolved by SDS-PAGE and subjected to immunoblotting analysis. Immunocomplexes on the polyvinylidene difluoride membranes were visualized by enhanced chemiluminescence detection (GE Healthcare). For immunoprecipitation analyses, cells were lysed in RIPA buffer (25 mM Hepes/NaOH [pH 7.4], 0.3 M NaCl, 10% glycerol, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2 mM dithiothreitol (DTT), 20 mM β -glycerophosphate, 10 mM *p*-nitrophenyl phosphate, 20 mM NaF, 0.5 mM Na-O-vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 3 μ g/ml pepstatin, and 15 μ g/ml bestatin) to minimize the coimmunoprecipitation of other proteins. Cell extracts prepared in RIPA buffer were precleared with protein G-Sepharose beads. The resultant precleared supernatants were incubated with the relevant antibodies and protein G-Sepharose beads, and the immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting.

RESULTS

In our recent study, we have demonstrated the extracellular stimuli-responsive translocation of FGD1.¹⁴⁾ During the wound-healing process, translocation of FGD1 to the leading edge membrane was observed in cells facing to the wound,¹⁴⁾ suggesting that the polarized intracellular signaling is triggered by FGD1 that translocates to the leading edge membrane. We previously established the HeLa Tet-Off-derived stable cell lines that inducibly overexpress FGD1(SA), FGD3(SA), or FGD1(SA+S²⁰⁵/I), respectively.^{10,14)} FGD1(SA) and FGD3(SA) bear Ser to Ala amino acid substitution within the SCF^{FWD1} putative consensus binding site.¹⁰⁾ In addition, a Ser to Ile substitution is made at position 205 in FGD1(SA+S²⁰⁵/I).¹⁴⁾ These HeLa Tet-Off-derived stable cell lines were subjected to wound-healing assays. As shown in Fig. 1A, removal of Tc led to the significant induction of FGD1(SA), FGD3(SA), or FGD1(SA+S²⁰⁵/I), and their induction levels detected by anti-FLAG Ab

Fig. 1. Proline-Rich Domain of FGD1 Is Critical for Directionally Persistent Cell Migration

(A) HeLa Tet-Off-FGD1(SA), HeLa Tet-Off-FGD3(SA), and HeLa Tet-Off-FGD1(SA+S^{205/I}) cells were incubated with or without 2 μ g/ml tetracycline (Tc) for 72 h, and then lysed in SDS-PAGE sample buffer. The resultant whole cell lysates were subjected to SDS-PAGE followed by the immunoblotting to detect FLAG epitope. Using the same lysates, immunoblotting was carried out to detect β -actin levels as a loading control. (B) Representative images of the wound-healing process of HeLa Tet-Off-FGD1(SA) cells. Cells were cultured in the presence or absence of 2 μ g/ml Tc for 48 h. Then cells were harvested and seeded on 35-mm dishes coated with type I collagen and allowed to grow to confluence in the presence or absence of Tc. Monolayers of confluent cultures were lightly scratched as described in Materials and Methods. After having been washed with DMEM, the wound regions were incubated in DMEM for 12 h. Phase-contrast images of four frames at 0 h and 12 h were collected (scale bar, 0.3 mm). (C) Motility of HeLa Tet-Off-FGD1(SA) cells was assessed as the wound closure at 12 h after wounding. After collecting the images of four frames at 0 and 12 h, the widths of the wound were measured at four points per frame. By subtracting the width at 12 h from the corresponding widths at 0 h, the closure distances were calculated. Wound closures were then estimated as the averages of these closure distances. (D) Representative images of the wound-healing process of HeLa Tet-Off-FGD3(SA) cells. Cells were subjected to the wound-healing analysis as described in (B). (E) Motility of HeLa Tet-Off-FGD3(SA) cells was assessed as the wound closure at 12 h after wounding as described in (C). (F) Representative images of the wound-healing process of HeLa Tet-Off-FGD1(SA+S^{205/I}) cells. Cells were subjected to the wound-healing analysis as described in (B). (G) Motility of HeLa Tet-Off-FGD1(SA+S^{205/I}) cells was assessed as the wound closure at 12 h after wounding as described in (C).

were comparable. Similarly to the results obtained by using non-coated dishes in our previous study,¹⁰ wound closure of FGD1(SA)-expressing cells on the dishes coated with type I collagen occurred more faster than that of cells, in which the expression of FGD1(SA) was suppressed by Tc (Figs. 1B, C). In contrast, wound closure was significantly inhibited by the expression of FGD3(SA) (Figs. 1D, E), as we reported previously using non-coated dishes.¹⁰ When we examined the wound closure of HeLa-Tet-Off-FGD1(SA+S^{205/I}) cells, significant difference was not observed whether or not FGD1(SA+S^{205/I}) is expressed (Figs. 1F, G).

We next examined the effect of FGD1(SA), FGD3(SA), and FGD1(SA+S^{205/I}) on the activation status of MAPKs. FGD1(SA) significantly augmented the EGF-stimulated JNK activation (Fig. 2A, IB: p-JNK1/2). In contrast to the previous studies by others using the DH-PH module of FGD1,^{16,17} neither MAPKs were activated by FGD1(SA) alone (Fig. 2A, lane 6 vs. lane 1). The levels of the c-Jun phosphorylation were well correlated with that of JNK activation (Fig. 2A, IB: p-c-Jun). The activity of JNK is increased upon phosphorylation by the MAPK kinase, MKK4 or MKK7. While MKK7 is a specific activator of JNK, MKK4 can also phosphorylate p38.¹⁸ FGD1(SA) remarkably enhanced EGF-

stimulated MKK4 activation whereas it did not change the activities of MKK7 (Fig. 2A, IB: p-MKK4 and p-MKK7). Surprisingly, EGF-stimulated p38 activation was not affected at all in FGD1(SA)-expressing cells, in which MKK4 activation was significantly augmented (Fig. 2A, IB: p-p38). EGF-stimulated ERK activation was also not affected whether or not FGD1(SA) was expressed (Fig. 2A, IB: p-ERK1/2). Interestingly, TNF- or IL-1-stimulated JNK activation was not augmented at all by the inducible expression of FGD1(SA) (Figs. 2B, C). These results indicate that EGF-induced activation of MKK4/JNK pathway is specifically augmented by the expression of FGD1. In FGD3(SA)-expressing cells, EGF-stimulated activation of JNK, but not that of p38 or ERK, was also augmented (Fig. 2D). Similarly, FGD1(SA+S^{205/I}) also enhanced EGF-stimulated JNK activation (Fig. 2E). Thus the proline-rich domain of FGD1 is not necessary for the augmentation of EGF-stimulated JNK activation.

We next examined the role of JNK in the cell motility of FGD1-expressing cells. As shown in Fig. 3A, EGF-stimulated c-Jun phosphorylation in FGD1(SA)-expressing cells was inhibited by the JNK inhibitor SP600125 in a dose-dependent manner. Using 10 μ M SP600125, we demonstrated that the wound closure of FGD1(SA)-expressing cells was

Fig. 2. FGD1 Augments EGF-Stimulated JNK Activation Independently of Its Proline-Rich Domain

(A) Activation of MAPKs in response to EGF was examined in HeLa Tet-Off-FGD1(SA) cells. Cells were cultured in the presence or absence of 2 μ g/ml Tc for 48 h, and further incubated in the serum-starved medium containing 0.1% BSA with or without Tc for 24 h. Cells were then stimulated with 20 ng/ml EGF for indicated times and lysed in SDS/PAGE sample buffer. The resultant whole cell lysates were subjected to immunoblotting analyses. The active status of JNK (p-JNK1/2), MKK4 (p-MKK4), MKK7 (p-MKK7), c-Jun (p-c-Jun), ERK (p-ERK1/2), and p38 (p-p38) were examined by using phospho-SAPK/JNK (Thr183/Tyr185) Ab, phospho-SEK1/MKK4 (Thr261) Ab, phospho-MKK7 (Ser271/Thr275) Ab, phospho-c-Jun (Ser63) II Ab, phospho-p44/42 (Thr202/Tyr204) MAP Kinase Ab, and phospho-p38 MAP Kinase (Thr180/Tyr182) Ab, respectively. The expression levels of JNK (JNK1/2), ERK (ERK1/2), and p38 were examined using SAPK/JNK Ab, p44/42 MAP Kinase Ab, and p38 MAP Kinase Ab, respectively. The induction of FGD1(SA) was confirmed by the immunoblotting to detect FLAG epitope. (B) TNF-stimulated JNK activation was examined in Tet-Off-FGD1(SA) cells. Cells were cultured in the presence or absence of 2 μ g/ml Tc for 72 h, followed by the stimulation with 2 ng/ml TNF for the indicated times. Cells were then lysed in SDS/PAGE sample buffer. The resultant whole cell lysates were subjected to immunoblotting analysis to detect phospho-JNK. Using the same lysates, immunoblotting was carried out to detect JNK levels as a loading control. The induction of FGD1(SA) was confirmed by the immunoblotting to detect FLAG epitope. (C) IL-1-stimulated JNK activation was examined in Tet-Off-FGD1(SA) cells. Cells were cultured in the presence or absence of 2 μ g/ml Tc for 72 h, followed by the stimulation with 2 ng/ml IL-1 β for the indicated times. Cell lysates were prepared and immunoblotting analyses were performed as described in (B). (D) Activation of MAPKs in response to EGF was examined in Tet-Off-FGD3(SA) cells as described in (A). (E) Activation of MAPKs in response to EGF was examined in Tet-Off-FGD1(SA+S^{205/I}) cells as described in (A).

Fig. 3. Suppression of JNK Significantly Impairs FGD1-Induced Cell Migration

(A) Effect of JNK inhibitor SP600125 on the EGF-stimulated c-Jun phosphorylation was examined in HeLa Tet-Off-FGD1(SA) cells. Cells were cultured in the absence of Tc for 48 h, and further incubated in the serum-starved medium containing 0.1% BSA for 24 h. Then cells were pretreated with the indicated concentrations of SP600125 for 4 h, followed by the stimulation with 20 ng/ml EGF for 10 min. Cells were then lysed in SDS/PAGE sample buffer. The resultant whole cell lysates were subjected to immunoblotting analysis to detect phospho-c-Jun. Using the same lysates, immunoblotting was carried out to detect β -actin levels as a loading control. (B) HeLa Tet-Off-FGD1(SA) cells were cultured in the presence or absence of 2 μ g/ml tetracycline (Tc) for 48 h. Then cells were harvested and seeded on 35-mm dishes coated with type I collagen and allowed to grow to confluence in the presence or absence of Tc. Monolayers of confluent cultures were lightly scratched as described in Materials and Methods. After having been washed with DMEM, the wound regions were incubated in DMEM containing 20 ng/ml EGF, 10 μ M SP600125, and 0.1% BSA for 24 h. Phase-contrast images of four frames at 0 h and 24 h were collected and cell motility was assessed as described in Fig. 1C.

Fig. 4. EGF Stimulates Serine Phosphorylation of FGD1(SA), but Not FGD1(SA+S^{205/I})

(A) HeLa Tet-Off-FGD1(SA) cells were cultured in the presence or absence of 2 μ g/ml Tc for 48 h, and further incubated in the serum-starved medium containing 0.1% BSA with or without Tc for 24 h. Cells were then stimulated with 20 ng/ml EGF for indicated times and lysed in RIPA buffer. Resultant cell extracts were immunoprecipitated with anti-FLAG Ab as described in Materials and Methods. Immunoprecipitates were then subjected to SDS-PAGE followed by the immunoblotting using anti-phosphoserine Ab. The PVDF membrane was stripped and reprobed with anti-FLAG Ab. (B) HeLa Tet-Off-FGD1(SA) cells were cultured in the presence or absence of 2 μ g/ml Tc for 48 h, and further incubated in the serum-starved medium containing 0.1% BSA with or without Tc for 24 h. Cells were then stimulated with 20 ng/ml EGF for 5 min and lysed in RIPA buffer. Resultant cell extracts were immunoprecipitated with anti-phosphoserine Ab as described in Materials and Methods. Immunoprecipitates were then subjected to SDS-PAGE followed by the immunoblotting to detect FLAG epitope. Separately, whole cell extracts (WCE) were directly subjected to immunoblotting analysis to detect FLAG epitope. (C) HeLa Tet-Off-FGD1(SA+S^{205/I}) cells were cultured in the presence or absence of 2 μ g/ml Tc for 48 h, and further incubated in the serum-starved medium containing 0.1% BSA with or without Tc for 24 h. Cells were then stimulated with 20 ng/ml EGF for indicated times and lysed in RIPA buffer. Resultant cell extracts were immunoprecipitated with anti-FLAG Ab as described in Materials and Methods. Immunoprecipitates were then subjected to SDS-PAGE followed by the immunoblotting using anti-phosphoserine Ab. The PVDF membrane was stripped and reprobed with anti-FLAG Ab.

significantly suppressed (Fig. 3B). Thus JNK pathway is indispensable for basic cell movement.

As we described in our previous report,¹⁴⁾ not only in human FGD1 but also in FGD1 from other species (for example, cattle, mouse and rat), the serine residue at position 205 (position 36 in the case of rat) is conserved. It is possible that the phosphorylation of this serine residue plays a key role during the process of extracellular stimuli-responsive translocation of FGD1. As shown in the top panel of Fig. 4A, anti-phospho-serine Ab detected a 130-kDa band in the immunoprecipitates from FGD1(SA)-expressing cells that were treated with EGF. This band was confirmed as the FLAG-tagged FGD1(SA) band after reprobing the same polyvinylidene difluoride (PVDF) membrane with anti-FLAG Ab (Fig. 4A, bottom). The EGF-induced phosphorylation of FGD1(SA) was further confirmed by the immunoprecipitation with anti-phospho-serine Ab followed by the im-

muno- blotting with anti-FLAG Ab (Fig. 4B). In contrast, the corresponding 130-kDa band was not detected by the immunoblotting using anti-phospho-serine Ab, when immunoprecipitates from FGD1(SA+S^{205/I})-expressing cells, which were treated with EGF for the indicated times, were examined (Fig. 4C). Although the phospho-peptide mapping to confirm the exact phosphorylation site has not been performed yet, FGD1 might be phosphorylated on the serine residue at position 205 in response to external guidance factors such as EGF, and then translocate to the leading edge membrane, where signaling pathways through Cdc42 and JNK are activated to induce directed migration (Fig. 5).

DISCUSSION

In mammalian cells, Rho family proteins are positioned at the center of a complex signaling network, involving their

Fig. 5. Proposed Mechanism for the Transmission of FGD1 Signals That Lead to Directionally Persistent Cell Migration

External guiding factors such as EGF stimulate FGD1 phosphorylation on the serine residue at 205, thereby triggering the interaction between FGD1 and adaptor molecules. Simultaneously, FGD1 translocates to the membrane where leading edge is formed. Polyphosphoinositides (PIPs) contribute to stabilize FGD1 at the leading edge membrane. FGD1 localized at the leading edge membrane activates the polarized signaling through Cdc42, enabling cells to undergo directed migration. JNK activation is augmented by FGD1, leading to the elevation of intrinsic cell motility. GSK-3 β constitutively phosphorylates FGD1 on the serine residues at 283 and 287, resulting in the subsequent destruction through the ubiquitin-proteasome system. FGD1 domains shown are "Pro-rich" (proline-rich domain), "DH" (Dbl homology domain), "PH" (pleckstrin homology domain), and "FYVE" (domain present in Fab1, YOTOB, Vac1, and EEA1).

stimulation by a variety of upstream activators as well as their interaction with a diverse set of downstream effectors.^{1–3} For outnumbering Rho proteins, more than 70 GEFs and approximately 80 GAPs are encoded in human genome,^{4,19,20} enabling cells to regulate the activity of Rho proteins through multiple different pathways. In the case of GEFs, most members have been genetically identified by the criteria as to whether or not they possess the DH domain and the PH domain, which is adjacent and C-terminal to the DH domain. In most cases, the DH-PH module is the minimal structural unit that can promote nucleotide exchange.⁴ However, it is important to characterize the full length GEFs; because most GEFs also contain regulatory domains that are responsible for turning on or turning off the DH-PH-module-driven GEF activity.⁴

FGD1 and FGD3 have highly homologous DH-PH modules, which can induce a large number of striking filopodia extensions in Swiss 3T3 fibroblasts,^{21,22} indicating that both of them act as GEFs for Cdc42. However, when expressed as a full-length form, FGD1 induces long-finger like protrusions (*i.e.*, filopodia) and FGD3 induces broad sheet-like protrusions (*i.e.*, lamellipodia).¹⁰ To date, Rac is considered to serve as a regulator of lamellipodia formation; whereas Cdc42 is considered to regulate filopodia formation.¹ In our previous study, we showed that overexpression of the dominant negative version of Rac inhibited the lamellipodia formation, while striking filopodia-like protrusions became obvious in FGD3-expressing cells.¹⁰ Since Cdc42, Rac, and Rho have been known to constitute a hierarchical cascade wherein Cdc42 activates Rac,¹ FGD3 may act as a Cdc42 GEF, and induces lamellipodia formation through Cdc42-induced activation of Rac. The "lamellipodium" is sometimes

referred as the "leading edge" or the "leading lamella."²³ It should be noted that more than two broad sheet-like lamellae oriented in different directions are frequently observed in a cell expressing FGD3(SA).¹⁰ It is therefore difficult to know which of them represents the leading edge.

To migrate efficiently, cells must possess an asymmetric morphology with defined leading and trailing edges: *i.e.*, by forming and stabilizing actin-rich protrusions that maintain the orientation of the leading edge, cells can achieve directionally persistent migration.²⁴ Among the members of Rho family G-proteins, Cdc42 is thought to be a master regulator of cell polarization that influences directional migration.²⁵ Polarized intracellular signaling orients protrusion of the leading edge, integrin-mediated adhesion to the underlying substrate, and contraction/detachment at distinct regions of the cell to orchestrate cell motility.²⁴

Previous immunostaining study demonstrated that FGD1 is localized at the membrane ruffles formed in cells stimulated with EGF or in cells facing to the wound-healing front,¹⁴ suggesting that extracellular stimuli-dependent translocation enables FGD1 to activate polarized signaling through Cdc42 and induce the formation of leading edge. Subcellular fractionation revealed that FGD1 is mainly localized in cytosol, when cells were cultured in the absence of serum.¹⁴ However, FGD1 translocates to the membrane fraction, when cells are stimulated with EGF.¹⁴

Interestingly, FGD3 is localized in both cytosol and membrane fractions under the serum-starved culture condition and does not change its intracellular distribution even though cells are subsequently exposed to the mitogenic stimuli.¹⁴ Similarly to FGD1, FGD3 possesses the PH-FYVE-PH module, which may serve as the binding unit toward polyphosphoinositides (Fig. 5). Therefore, FGD3 that lacks the proline-rich domain may be localized at membrane subdomains wherever polyphosphoinositides are enriched and cause Cdc42-mediated Rac activation there. Then cells extend more than two broad sheet-like lamellae oriented in different directions and do not become polarized. In addition, Rac activation is known to stabilize cell-cell contact.²⁶ Thus it is likely that FGD3 fails to provide cell polarity; instead it increases the stability of cell-cell contact, thereby directed cell migration may be suppressed.

As shown in Figs. 1F and G, the mutant FGD1 with S²⁰⁵/I substitution neither stimulated nor inhibited the wound closure. It should be emphasized that this mutant FGD1 is kept localized in the cytosol even though cells are cultured under the mitogenic condition.¹⁴ Since S²⁰⁵/I substitution was originally found in the FGD1 from an AAS patient, S²⁰⁵ plays a crucial role in FGD1-mediated signaling. Indeed, FGD1(SA), but not FGD1(SA+S²⁰⁵/I), was phosphorylated in response to EGF (Figs. 4A–C), suggesting that S²⁰⁵ may be the target residue that turns to be phosphorylated to lead to the directionally persistent cell migration. Kim *et al.* reported that the amino acid residues 158–165 [KPQVPPKP] in the proline-rich domain of FGD1 binds directly to the SH3 domain of cortactin, which promotes actin assembly by actin-related protein Arp2/3 complex.²⁷ They suggested that FGD1 causes the ligation of cortactin at the leading edge of migrating cells, thereby stimulates Arp2/3-mediated actin polymerization.²⁷ The phosphorylation of S²⁰⁵ may occur upstream of the FGD1-cortactin binding, since FGD1(SA+S²⁰⁵/I), which

conserves the intact cortactin binding motif, fails to be targeted to the membrane and does not trigger the directional migration.

Besides Cdc42 and cortactin, JNK is known to be activated by FGD1.^{16,17} However, the FGD1 encompassing the DH-PH module was used in those studies.^{16,17} As shown in Fig. 2, JNK activation was not directly induced by the over-expression of full length FGD1. Interestingly, JNK activation induced by EGF, but not that by TNF or IL-1, was significantly enhanced in cells expressing FGD1(SA) (Fig. 2A). Similarly, the levels of EGF-stimulated JNK activation were elevated in cells expressing FGD3(SA) or FGD1(SA+S²⁰⁵/I) (Figs. 2D,E), suggesting that the proline-rich domain of FGD1 is not required for the augmentation of EGF-stimulated JNK activation. JNK is generally thought to play roles in inflammation, differentiation, apoptosis and insulin resistance.²⁸ However, accumulating evidence also implicates the JNK pathway in regulation of cell migration.¹⁵ Indeed, wound-healing of FGD1(SA)-expressing cells was significantly suppressed by the JNK inhibitor SP600125 (Fig. 3B), indicating the critical role of JNK in cell migration. On the other hand, JNK activation is not sufficient for cells to promote directional migration, since FGD3(SA) and FGD1(SA+S²⁰⁵/I), both of which augment EGF-stimulated JNK activation, failed to facilitate wound closure (Figs. 1D—G). In cells expressing FGD3(SA) or FGD1(SA+S²⁰⁵/I), enhanced JNK activation may lead to the elevated intrinsic cell motility, however the behavior of motile cells may be different from that of FGD1(SA)-expressing cells, in which the direction of cell migration is determined by external guiding factors.

It is widely accepted that active JNK enters the nucleus, where it phosphorylates several transcription factors, resulting in an increase in transcriptional activity.²⁹ However, active JNK is also known to be localized in other intracellular compartments including cytoplasm and focal adhesions.¹⁵ Huang *et al.* reported that JNK phosphorylation of paxillin on the serine residue at 178 is critical for EGF-stimulated cell migration.³⁰ Paxillin is a multidomain adaptor protein that recruits a number of signal transducers to focal adhesions, where it performs a critical role in transducing signals from integrins and growth factors to downstream regulation of cell migration and gene expression.³¹ Although we have not examined the effect of FGD1 expression on the phosphorylation status of paxillin yet, JNK-mediated phosphorylation of paxillin may be elevated downstream of external guiding factors-dependent translocation of FGD1 to the leading edge membrane (Fig. 5).

Many GEFs contain a regulatory domain that blocks activity through an intramolecular interaction.⁴ It is assumed that activation of full-length GEF is through the relief of autoinhibition by phosphorylation or by binding to other proteins, although the mechanism is still obscure in most cases.⁴ In the case of FGD1, S²⁰⁵ phosphorylation may trigger the interaction of FGD1 with adaptor proteins, such as cortactin, thereby enabling FGD1 to translocate to the membrane and form the leading edge where downstream signaling pathways are activated (Fig. 5). The PH or FYVE domains may also contribute to the stabilization of FGD1 at the leading edge

membrane through the binding with polyphosphoinositides. On the other hand, FGD1 that is not stabilized at this intracellular membrane compartment may be constitutively degraded through the destruction pathway composed of GSK-3 β /SCF^{FWD1}/the proteasome (Fig. 5).⁹

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