The Faciogenital Dysplasia Gene Product FGD1 Functions as a Cdc42Hs-specific Guanine-Nucleotide Exchange Factor

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The Rho family of small GTP-binding proteins plays important roles in the regulation of actin cytoskeleton organization and cell growth. Activation of these GTPases involves the replacement of bound GDP with GTP, a process catalyzed by the Dbl-like guanine-nucleotide exchange factors, all of which seem to share a putative catalytic motif termed the Dbl homology (DH) domain, followed by a pleckstrin homology (PH) domain. Here we have examined the role of a Dbl-like molecule, the faciogenital dysplasia gene product (FGD1), which when mutated in its DH homology domain, cosegregates with the developmental disease Aarskog-Scott syndrome. We report that a polypeptide of FGD1 encompassing the DH and PH domains can bind specifically to the Rho family GTPase Cdc42Hs and stimulates the GDP-GTP exchange of the isoprenylated form of Cdc42Hs. Microinjection of this FGD1 polypeptide into Swiss 3T3 fibroblast cells induces the formation of peripheral actin microspikes when microinjected into Swiss 3T3 cells, an effect reversible by coinjection of dominant-negative mutant of Cdc42Hs. Examination of NIH 3T3 cells expressing the FGD1 fragment revealed that similar to cells expressing Dbl, two independent elements downstream of Cdc42Hs, the Jun NH2-terminal kinase and the p70 S6 kinase, become activated. Hence, our results indicate that FGD1, through its DH and PH domains, acts as a Cdc42Hs-specific guanine-nucleotide exchange factor and suggest that the Cdc42Hs GTPase may have a role in mammalian development.

The Rho family of small GTP-binding proteins have been implicated in multiple signaling pathways leading to cytoskeleton reorganization (1) and cell growth (2). RhoA has been shown to be involved in actin stress fiber and focal adhesion formation (3); Rac1 is required for membrane ruffling and lamellipodia formation induced by growth factors (4); and Cdc42Hs has been demonstrated to mediate the induction of peripheral actin microspikes (PAM) and filopodia by bradykinin (5). In fibroblast cells, Cdc42Hs/Rac1/RhoA have been suggested to work in a hierarchical cascade mediating these changes in the actin cytoskeleton (6). In addition, Rho, Rac, and Cdc42Hs all seem to be involved in the transcriptional regulation by serum response factor (7) and in the regulation of cell cycle progression (8). Particularly, Cdc42Hs and Rac participate in the p21-activated kinase-mediated Jun N-terminal kinase (JNK) activation (9, 10) and, through an independent pathway, in the regulation of p70 S6 kinase (S6K) (11).

The activation of Rho proteins requires the exchange of bound GDP for GTP (12), a process catalyzed by a growing family of guanine-nucleotide exchange factors (GEFs) for which the Dbl oncoprotein is a prototype (13). These putative GEFs share the structural arrangement of a 200-amino acid motif, the Dbl homology (DH) domain, followed by a second putative signaling motif, the pleckstrin homology (PH) domain (13). The DH domain of oncogenic Dbl is essential both for the transforming activity and for its GEF catalytic activity of Cdc42Hs (14), whereas the PH domain seems to mediate the transforming activity of Dbl by targeting it to specific cytoskeletal locations (15). Studies of other members of the GEF family have also provided evidence consistent with the idea that the DH domain is responsible for the GEF catalytic activity and that the PH domain in these molecules may be involved in determining intracellular localization (16–18).

In the present study, we report that a polypeptide composed of the DH and PH domains of the Dbl-like molecule, the faciogenital dysplasia gene product FGD1 (19), functions as a Cdc42Hs-specific GEF both in vitro and in fibroblast cells. In particular, we show that this polypeptide derived from FGD1 can specifically bind to and catalyze the GDP-GTP exchange of Cdc42Hs GTPase. It potently stimulates the formation of peripheral actin microspikes when microinjected into Swiss 3T3 cells, an effect reversible by coinjection of dominant-negative Cdc42Hs protein. Furthermore, we found that two independent downstream elements of Cdc42Hs, the JNK and the S6K, have become constitutively activated in FGD1-expressing cells. These results implicate Cdc42Hs as a substrate of FGD1 and, together with the previous evidence that mutation of FGD1 in the DH domain cosegregates with the human developmental disease faciogenital dysplasia (Aarskog-Scott syndrome) (19), suggest that the FGD1-Cdc42Hs pathway may be required for normal mammalian development.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH 3T3 and Swiss 3T3 mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum. The Dbl- and Lbc-expressing NIH 3T3 cell clones were gifts from Drs. A. E. Guda (Istituto Gaslini, Italy) and D. Toksoz (Tufts University). Transfections were carried out using the calcium phosphate transfection kit (Invitrogen). To establish cell lines that stably
express the FGD1 polypeptide (amino acid sequences 330–710), transfected cells were selected in growth medium that was supplemented with 400 μg/ml G418, and the drug-resistant colonies were selected 21 days after transfection.

Expression and Purification of Recombinant Proteins—The glutathione S-transferase (GST)-fusion products of small GTP-binding proteins and their mutants were expressed in Escherichia coli using the pGEX-2T plasmid and were purified as described (14, 20). The GST-Cdc24 protein was synthesized in the baculovirus-infected Sf9 insect cells and purified by glutathione-agarose affinity chromatography (21). Cdc42Hs from Sf9 insect cells was produced similarly as described (22) from the membrane fraction of insect cells infected with recombinant Cdc42Hs virus, except that a His6 tag was added to the amino terminus of Cdc42Hs to allow its purification using the nickel-agarose affinity column (Qiagen).

The cDNA fragment of FGD1 encoding amino acids encompassing the DH and PH domains (amino acid residues 330–710) was amplified by polymerase chain reaction using the Pfu polymerase (Stratagene) from the full-length FGD1 cDNA after introducing a BamHI site on both ends. This polymerase chain reaction-generated cDNA fragment of FGD1 was cloned into the pBluescript vector and was sequence-confirmed by automated sequencing from both ends. The BamHI insert of FGD1 was then cloned into the mammalian expression vector pFlag (15) and separately was inserted into the baculovirus transfer vector pVL1393 (Invitrogen) together with a cDNA encoding GST. Recombinant baculovirus of RhoA and Rac2 were produced by cloning the BamHI-EcoRI fragments of cDNA coding from (pGEX-2T) vector into the pVL1393 vector by transformation into Sf9 insect cells.

Complex Formation Assay between Small GTP-binding Proteins and FGD1—Complex formation of FGD1 polypeptide with GST-fused small GTP-binding proteins was carried out essentially as described for the Dbl-G-protein interactions (14), except that cell lysates containing Flag-FGD1 polypeptide were used in place of the Dbl-expressing Sf9 cell lysates. Western blot was probed with the M5 anti-Flag monoclonal antibody (IBI, Inc.). Immunocomplexes were visualized by ECL reagents (Amersham Corp.).

GDP Dissociation and GTP·S Binding Assays—The time courses for [3H]GDP dissociation and [35S]GTP·S binding of Cdc42Hs under the catalysis of GST-Cdc24, GST-FGD1, or GST were determined exactly as described using the nitrocellulose filtration method (23).

Microinjection of Recombinant Proteins—Swiss 3T3 cells were plated onto Locator glass coverslips (Eppendorf) and cultured overnight in DMEM supplemented with 1% calf serum. Subconfluent cells were placed in serum-free medium 3 h before microinjection. Proteins were injected in a buffer containing 20 mM Tris·HCl (pH 7.6), 100 mM NaCl, and 0.5% (w/v) of Texas-red-dextran (Molecular Probes) using an Eppendorf micromanipulator mounted on an Olympus IMT2 microscope. Successful injections were determined by visual inspection of the cells for fluorescence. Typically all cells in the view of a grid would be injected to facilitate later identification. After microinjection cells were incubated at 37°C for 20 min, inspected under a phase contrast microscope for morphological changes, and fixed and stained with rhodamine-conjugated phalloidin (Sigma) for filamentous actin as described (24).

Immunoprecipitation and Kinase Assays—The rabbit polyclonal anti-JNK1 and anti-p70 S6K antibodies and kinase substrates (GST-c-Jun(79) and p70S6K substrate peptide) were used in incubation with GST-Cdc42Hs expressed in Sf9 cells by GST-FGD1 was observed (25). Although incubation of Cdc42Hs expressed in E. coli with GST-FGD1 had no detectable effect on the release of Cdc42Hs-bound [3H]GDP, the rate of [3H]GDP dissociation from the Cdc42Hs isolated from S9 cell membrane was increased drastically (Fig. 2B). In parallel, stimulation of the rate of [35S]GTP·S binding to Cdc42Hs expressed in S9 cells by GST-FGD1 was observed (Fig. 2C). Again, Cdc42Hs produced in E. coli appeared to be insensitive to FGD1 stimulation in the GTP binding assay (Fig. 2C). On the other hand, the Cdc42-specific GEF, Cdc24 from O. newyorica expressed in E. coli, together with potential Cdc24 binding partners from E. coli was demonstrated to stimulate the GDP dissociation and [35S]GTP·S binding of both the isoprenylated and nonisoprenylated forms of Cdc42Hs (Fig. 2B and C). None of the other small GTPases expressed in E. coli that tested
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Fig. 2. The insect cell expressed FGD1 polypeptide stimulates the guanine-nucleotide exchange activity of the post-translationally modified form of Cdc42Hs. A, 10% SDS-PAGE of the purified GST-Cdc24 and GST-FGD1 prepared from Sf9 insect cells. These proteins were used in the GEF assays and/or microinjections. Lane 1, 2 μg of GST-Cdc24; lane 2, 2 μg of GST-FGD1. B, time courses of the \(^{3}H\)GDP dissociation from the E. coli-(open symbols) or Sf9 cell-expressed Cdc42Hs (~0.5 μg) (closed symbols) in the presence of 1 μg of GST-Cdc24 (squares), 1 μg of GST-FGD1 (triangles), or 2 μg of GST (diamonds). C, measurement of the \(^{35}S\)GTP\(\gamma\)S binding to the E. coli-(open symbols) or the Sf9 cell-expressed Cdc42Hs (~0.5 μg) (closed symbols) under catalysis of 1 μg of GST-Cdc24 (squares), 1 μg of GST-FGD1 (triangles), or 2 μg of GST (diamonds). Data are the means of two assays.

negative in the FGD1 binding assay (Fig. 1) showed any detectable sensitivity to the GST-FGD1 polypeptide; neither did the membrane fractions of RhoA and Rac2 prepared from insect cells infected with recombinant RhoA or Rac2 virus (data not shown). These experiments indicate that FGD1 acts as a Cdc42Hs-specific GEF in a reconstituted system and that effective catalysis of guanine-nucleotide exchange by FGD1 requires the post-translational lipid modification of Cdc42Hs, a property similar to that reported for the Rom2 protein in the activation of the Rho1 GTPase in yeast (28).

To see whether the FGD1 polypeptide could act as a GEF in vivo, serum-starved Swiss 3T3 cells were microinjected with the recombinant GST-FGD1 polypeptide. Cell morphology was observed under a phase contrast microscope, and 20 min after injection the cells were fixed, and filamentous actin was localized by fluorescence microscopy. Under phase contrast microscope within 10 min of injection of the FGD1 protein, long membrane protrusions could be seen extending from the cells (data not shown). Staining of the cells with rhodamine-conjugated phalloidin revealed that these dynamic membrane protrusions were mostly due to the formation of PAM (Fig. 3B), similar to the effect observed when a constitutively active form of Cdc42Hs was injected (5, 6). In addition, cell retraction and rounding occurred 20 min after injection of the FGD1 polypeptide, also similar to that reported for cells injected with Cdc42Hs (5). In contrast, little change could be detected in the control cells when the GST protein was injected alone (Fig. 3A).

The induction of PAM by FGD1 and cell retraction were reversed when a dominant-negative mutant of Cdc42Hs (Cdc42HsT17N) was coinjected into cells (Fig. 3C). Together with the previous observation that the formation of filopodia and PAM is through a Cdc42-mediated pathway (5, 6), therefore, these results indicate that FGD1 is capable of activating endogenous Cdc42Hs in fibroblasts to effect the actin organization.

To look further for downstream events that may be coupled to the activation of Cdc42Hs by FGD1, we examined FGD1-expressing cells for the activities of JNK and S6K, both of which have been implicated in the Cdc42Hs-mediated pathway (11, 25, 26). Endogenous JNK1 and S6K were immunoprecipitated from serum-starved NIH 3T3 cells expressing the FGD1 polypeptide (F1 and F2, two independent clones), and the kinase activities were compared with that of wild-type cells and also with that of cells expressing the Rho family GEF Dbl or Lbc coproteins (Fig. 4). Both clones of FGD1-expressing cells (F1 and F2) showed pronounced increases in JNK1 and S6K enzymatic activities when compared with that of wild-type NIH 3T3 cells. This is similar to that detected in Dbl-expressing cells, in which both of these kinases were activated (Fig. 4) (11, 25). Consistent with the previous observations that a constitutively activated mutant of RhoA failed to stimulate the JNK and S6K activities in transiently transfected Cos and NIH 3T3 cells (11, 25, 26), the JNK and S6K activities in cells expressing the Rho-specific activator Lbc (33) were found to be at the basal levels as in wild-type cells (Fig. 4). From these results we conclude that the DH and PH domains of FGD1 can effectively induce pathways leading to the activation of JNK and S6K,
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Fig. 4. FGD1 stimulates the Cdc42Hs-sensitive JNK1 and S6K activities in vivo. A, expression of the Flag-tagged Lbc and FGD1 proteins in NIH 3T3 cells. Two independent FGD1 transfectants were included (F1 and F2). Lysates containing 20 μg of proteins from wild-type NIH 3T3 cells and the Dbl, Flag-Lbc, and Flag-FGD1 (amino acids 330–710) transfected cells were subjected to Western blot analysis with the M5 anti-Flag antibody. B, JNK1 activities of the serum-starved wild-type NIH 3T3 cells and the Dbl, Lbc, and FGD1 transfectants (F1 and F2). JNK1 was immunoprecipitated from various cell lysates by the anti-JNK1 Western blot. C, S6K activities immunoprecipitated from various cell lysates by the anti-S6K Western blot. Data are representative of three independent experiments.

as in the case with constitutively activated Cdc42Hs (8, 11, 25, 26).

Cdc42 is known to play a central role in signaling pathways of cell polarization (29, 30) and cytokinesis (27). In S. cerevisiae, the physiological activator of Cdc42 was identified as the Dbl family member Cdc24, a cell division cycle protein that is a Cdc42-specific GEF (21) and is required for both budding and pheromone-induced mating (31, 32). In the present study we provide evidence that the product of a gene responsible for a skeletal dysplasia, FGD1, functions as a specific GEF of Cdc42His in a mammalian system. The polypeptide of FGD1 containing the DH and PH domains, similar to Cdc24 (21), specifically binds to and stimulates the GDP-GTP exchange of Cdc42His. Introduction of this fragment of FGD1 into fibroblast cells causes the Cdc42His-mediated actin-cytoskeleton reorganization and the activation of the signaling pathways leading to JNK and S6K. Together with the developmentally regulated pattern of expression of FGD1 and the previous evidence that a mutation of FGD1 in the catalytic DH domain co segregates with the human developmental disease FGDY (19), our results suggest that Cdc42His may have a role in mammalian development and that aberrations of the FGD1-Cdc42His pathway could lead to developmental disorders.

Based primarily on the structural and functional studies of the Dbl oncoprotein (14, 15) and supported by studies of a few other Dbl-like GEFs (16–18), it is proposed that the DH domain together with the PH domain of the GEFs constitutes the minimum structural unit conferring biological activity and that the DH domain is responsible for the GEF catalytic activity whereas the PH domain is essential for the proper cellular localization (13). The results of the present study utilizing the FGD1 polypeptide encompassing only the DH and PH domains provide further evidence that the DH domain together with the PH domain is sufficient for the GEF catalytic activity and further downstream signaling events involving its substrate, Cdc42His. The additional putative domains in FGD1, a proline-rich SH3 domain-binding motif and a cysteine-rich zinc finger-like motif located at the amino and carboxyl termini of the molecule, respectively, have been found in many signaling proteins, including other Dbl family GEFs (13), Rho GTPase-activating proteins (34), and Rho GTPase targets (35). It remains to be seen whether these additional structural units of FGD1 contribute to the direct regulation of the GEF activity of the DH domain and if they are involved in the formation of larger signaling complexes of FGD1, coupling it to other signaling machineries required for Cdc42His function.

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