Frabin, a Novel FGD1-related Actin Filament-binding Protein Capable of Changing Cell Shape and Activating c-Jun N-terminal Kinase*

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We purified from rat brain a novel F-actin-binding protein with a M\textsubscript{r} of about 105,000 (p105), which was estimated by SDS-polyacrylamide gel electrophoresis. We cloned its cDNA from a rat brain cDNA library and characterized it. p105 was a protein of 766 amino acids and showed a calculated M\textsubscript{r} of 86,449. p105 consisted of one F-actin-binding domain at the N-terminal region, one Dbl homology domain and one pleckstrin homology domain at the middle region, and one cysteine-rich domain at the C-terminal region. This domain organization of p105 was similar to that of FGD1, which has been determined to be the genetic locus responsible for facio-genital dysplasia or Aarskog-Scott syndrome. We therefore named p105 frabin (FGD1-related F-actin-binding protein). Frabin bound along the sides of F-actin and showed F-actin-cross-linking activity. Overexpression of frabin in Swiss 3T3 cells and COS7 cells induced cell shape change and c-Jun N-terminal kinase activation, respectively, as described for FGD1. Because FGD1 has been shown to serve as a GDP/GTP exchange protein for Cdc42 small G protein, it is likely that frabin is a direct linker between Cdc42 and the actin cytoskeleton.

Dynamic reorganization of the actin cytoskeleton is essential for many cell functions, including cell shape change, adhesion, and motility (for reviews see Refs. 1–3). Evidence is accumulating that the Rho family small G proteins are important regulators of these actin-dependent cell functions (for reviews see Refs. 4 and 5). The Rho family is divided into three subfamilies: the Cdc42, Rac, and Rho subfamilies (Cdc42, Rac, and Rho, respectively). In fibroblasts, Cdc42 induces filopodium and membrane ruffle formation, and Rac induces lamellipodium and membrane ruffle formation; and Rho regulates assembly of stress fibers and focal adhesions. In addition to these functions, the Rho family members are involved in the regulation of cell growth: Cdc42 and Rac, but not Rho, induce mitogen-activated protein kinase-mediated JNK\textsuperscript{2} activation (6, 7); and Cdc42, Rac, and Rho all induce transcriptional activation of the genes containing the serum response element (8).

The Rho family members cycle between the GDP-bound inactive and GTP-bound active forms (4, 5). The conversion from the GDP-bound form to the GTP-bound form is stimulated by a GEP. Many GEPs for the Rho family members have thus far been identified and shown to share two conserved domains: a DH domain of about 250 aa and a PH domain of about 100 aa. The majority of the GEPs have been isolated as oncogenes (4, 5), but FGD1, a GEP specific for Cdc42, has been determined to be a positional cloning to be the genetic locus responsible for facio-genital dysplasia or Aarskog-Scott syndrome (9, 10).

In preceding papers, we purified from rat brain novel F-actin-binding proteins, neura-in (11) and (II-12) and lafadin (13). During the purification of lafadin, we identified another F-actin-binding protein with a M\textsubscript{r} of about 105,000. Molecular cloning of this protein revealed that it showed a significant homology to FGD1. We named this protein frabin (FGD1-related F-actin-binding protein). Frabin bound to F-actin in vivo and in vitro and showed activities similar to those of FGD1.

EXPERIMENTAL PROCEDURES

Materials—Rabbit skeletal actin monomer (14), F-actin (11), and 125\textsuperscript{I}-labeled F-actin (11) were prepared as described. Various GST fusion proteins of full-length and truncated forms of frabin were expressed in Escherichia coli and purified (11). Various Myc-tagged proteins of full-length and truncated forms of frabin were expressed in COS7 cells as described (11). 125\textsuperscript{I}-Labeled F-actin blot overlay, immunofluorescence microscopy, gel filtration, cosedimentation of various GST fusion proteins of full-length and truncated forms of frabin with F-actin, and transmission electron microscopy of negatively stained specimens were done as described (11). Protein concentrations were determined with bovine serum albumin as a reference protein (15).

Purification and Molecular Cloning of Frabin—Fetal brains from 420 mother rats (17-day gestation) were homogenized with a solution containing 10 mM Tris/Cl at pH 8.0, 2 mM EDTA, 5 mM EGTA, and a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 20 \mu M of leupeptin, and 1 \mu M of pepstatin A). The homogenate was mildly stirred for 1 h and centrifuged at 200,000 \times g for 1 h. The supernatant was stored at \(-80^\circ\)C until use. One-seventh of the supernatant (515 ml, 1.9 g of protein) was applied on a Q-Sepharose FF column (2.6 \times 34 cm; Amersham Pharmacia Biotech) equilibrated with Buffer A (20 mM Tris/Cl at pH 8.0, 0.5 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol). Elution was performed with a 800-ml linear gradient of NaCl (0–0.5 M) in Buffer A. Fractions of 10 ml each were collected. Frabin appeared in Fractions 35–55. These fractions (210 ml, 300 mg of protein) were collected. Half of the sample was diluted with a 2-fold volume of Buffer A and applied on a Mono Q HR 10/10 (Amersham Pharmacia Biotech) equilibrated with Buffer A. Elution was performed with a 48-ml linear gradient of NaCl (0–0.3 M) in Buffer A, followed by 34 ml of Buffer A containing 1 M NaCl. Fractions of 2 ml each were collected. Frabin appeared in Fractions 20–22. These fractions (6 ml, 19 mg of protein) were collected. The other half of the sample of the Q-Sepharose FF column chromatography was also subjected to the Mono Q column chromatography in the same manner as described above. The active fractions of the two Mono Q column chromatographies were combined,

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‡ The abbreviations used are: JNK, c-Jun N-terminal kinase; GEP, GDP/GTP exchange protein; DH, Dbl homology; aa, amino acid(s); PH, pleckstrin homology; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; S1, subfragment 1.
cDNA library in protein band were determined with a peptide sequencer. A rat brain described (11). The aa sequences of the peptides derived from the cells were subjected to immunofluorescence microscopy as described each) was then microinjected into the nuclei of the cells, and 18 h later pCMV-Myc-full-length frabin or pEF-BOS-Myc-V12Cdc42 (0.05 mg/ml subconfluent cells were serum-starved for 24 h before microinjection. The active fractions of the seven phenyl-5PW RP column chromatographies were combined, lyophilized, and stored at −80 °C. The purified phenyl-5PW RP sample (300 μg of protein) was subjected to SDS-PAGE (10% polyacrylamide gel). The protein band corresponding to a protein with a Mr of about 105,000 was processed as described (11). The aa sequences of the peptides derived from the protein band were determined with a peptide sequencer. A rat brain cDNA library in ZAP II (Stratagene) was screened using the oligonucleotide probes designed from the partial aa sequences. Assay for JNK Activity—Assay for JNK activity was done as described (16). Briefly, pRo-HA-JNKα was transfected with pCMV-Myc-full-length frabin, pEF-BOS-Myc-V12Cdc42 (17), or pEF-BOS-Myc-Dbl (18) in COS7 cells. After incubation for 16 h in serum-starved Dulbecco’s modified Eagle’s medium, the cell lysates were subjected to immunoprecipitation with an anti-HA antibody. Each immunoprecipitate was then washed and used for assay with GST-c-Jun as a substrate. During the purification of lafadin from rat brain using a blot overlay method with 125I-labeled F-actin (13), we detected another protein band of 125I-labeled F-actin binding activity with other protein band of 125I-labeled F-actin binding activity with a Mr of about 105,000. We purified it by chromatographies of Q-Sepharose, Mono Q, phenyl-5PW, and phenyl-5PW RP columns. On the final phenyl-5PW RP column chromatography, the 125I-labeled F-actin-binding protein band coincided well with a protein with a Mr of about 105,000 (p105), which was identified by protein staining (Fig. 1). We determined the partial aa sequences of p105. The aa sequences of p105 were not found in current protein data base. On the basis of this information, we isolated a p105 cDNA from a rat brain cDNA library and determined its nucleotide sequence (GenBank™ accession number AF038388) (Fig. 2A). This Mr value was less than that estimated by SDS-PAGE. To confirm whether this clone contained a full-length cDNA of p105, we constructed the eukaryotic expression vector with this cDNA and expressed the protein in other protein band of 125I-labeled F-actin binding activity with a Mr of about 105,000. We purified it by chromatographies of Q-Sepharose, Mono Q, phenyl-5PW, and phenyl-5PW RP columns. On the final phenyl-5PW RP column chromatography, the 125I-labeled F-actin-binding protein band coincided well with a protein with a Mr of about 105,000 (p105), which was identified by protein staining (Fig. 1). We determined the partial aa sequences of p105. The aa sequences of p105 were not found in current protein data base. On the basis of this information, we isolated a p105 cDNA from a rat brain cDNA library and determined its nucleotide sequence (GenBank™ accession number AF038388) (Fig. 2A). This Mr value was less than that estimated by SDS-PAGE. To confirm whether this clone contained a full-length cDNA of p105, we constructed the eukaryotic expression vector with this cDNA and expressed the protein in other protein band of 125I-labeled F-actin binding activity with a Mr of about 105,000. We purified it by chromatographies of Q-Sepharose, Mono Q, phenyl-5PW, and phenyl-5PW RP columns. On the final phenyl-5PW RP column chromatography, the 125I-labeled F-actin-binding protein band coincided well with a protein with a Mr of about 105,000 (p105), which was identified by protein staining (Fig. 1). We determined the partial aa sequences of p105. The aa sequences of p105 were not found in current protein data base. On the basis of this information, we isolated a p105 cDNA from a rat brain cDNA library and determined its nucleotide sequence (GenBank™ accession number AF038388) (Fig. 2A). This Mr value was less than that estimated by SDS-PAGE. To confirm whether this clone contained a full-length cDNA of p105, we constructed the eukaryotic expression vector with this cDNA and expressed the protein in
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Frabin was purified from COS7 cells. The expressed protein showed the mobility similar to that of native p105 on SDS-PAGE and had the $^{125}$I-labeled F-actin binding activity (Fig. 2B). Therefore, we concluded that this clone encoded the full-length cDNA of p105.

By use of fusion proteins of several truncated forms of p105 with GST, we determined the minimum $^{125}$I-labeled F-actin-binding domain to be aa 1–150 (Fig. 2C). Computer homology search revealed that the aa sequence of this domain showed no significant homology to any protein in current protein data base. However, the aa sequence following the F-actin-binding domain showed a significant homology to that of FGD1 (Fig. 2D). p105 had one DH domain and one PH domain the aa sequences of which showed 71% and 57% identity to those of FGD1, respectively. p105 also had one cysteine-rich domain the aa sequence of which showed 65% identity to that of FGD1. We therefore named p105 frabin.

When GST-full-length frabin was incubated with F-actin followed by ultracentrifugation, the fusion protein was recovered with F-actin in the pellet (Fig. 3A). The stoichiometry of the binding of GST-frabin to actin was 1 GST-frabin molecule/14 actin molecules. The $K_d$ was about $9 \times 10^{-7}$ M. Similar results were obtained with GST-frabin-1 (aa 1–150) (data not shown). The binding of native frabin to $^{125}$I-labeled F-actin was inhibited by an excessive amount of myosin S1, a protein that binds along the sides of F-actin (19, 20) (Fig. 3C). The inhibition was reversed by the addition of MgATP because MgATP dissociates the actin-myosin complex (21), indicating that frabin bound along the sides of F-actin. The F-actin-cross-linking activity of GST-full-length frabin caused F-actin to associate into bundles, whereas GST-frabin-1 or GST alone did not (Fig. 3C).

The $\alpha$-actinin/spectrin family members have most extensively been studied as F-actin-binding proteins having F-actin-cross-linking activity (22, 23). They usually form oligomers and thereby show the F-actin-cross-linking activity. When frabin was subjected to gel filtration, it appeared at a position corresponding to a $M_r$ of about 250,000 (data not shown). Because the $M_r$ values of frabin estimated by SDS-PAGE and calculated from its predicted aa sequence were about 105,000 and 86,000, respectively, these results suggest that frabin forms an oligomer with multiple F-actin-binding heads and thereby shows the F-actin-cross-linking activity.

To further obtain the evidence that frabin is an F-actin-binding protein, the binding of GST-full-length frabin to F-actin was determined by cosedimentation of GST-full-length frabin in the presence of F-actin (Fig. 3B). Therefore, we concluded that frabin bound along the sides of F-actin with an inhibitory capacity of the F-actin-cross-linking activity. When frabin was incubated with F-actin in the absence of ATP, the F-actin-cross-linking activity of GST-full-length frabin was almost completely inhibited (Fig. 3B). This inhibition was reversed by the addition of MgATP because MgATP dissociates the actin-myosin complex (21), indicating that frabin bound along the sides of F-actin. The F-actin-cross-linking activity of GST-full-length frabin and GST-frabin-1 (aa 1–150) was examined by transmission electron microscopy of negatively stained specimens. GST-full-length frabin caused F-actin to associate into bundles, whereas GST-frabin-1 or GST alone did not (Fig. 3C).

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binding protein, we expressed Myc-tagged full-length frabin or a Myc-tagged mutant, which lacked the F-actin-binding domain, in COS7 cells and compared the localization of each expressed protein with that of endogenous F-actin. Frabin having the F-actin-binding domain was colocalized with F-actin, but frabin lacking the domain was not (Fig. 3D). On the basis of these observations, we have concluded that frabin serves as an F-actin-binding protein in intact cells and that the F-actin-binding domain is located at the N-terminal region (aa 1–150).

Of the activities of FGD1 thus far reported (10), we examined whether frabin is also capable of changing cell shape and activating JNK. When frabin or a dominant active mutant of Cdc42 (V12Cdc42) was expressed in Swiss 3T3 cells, they similarly induced cell shape change characterized by microspike formation (Fig. 4). Although we did not determine whether the microspikes were filopodia or retraction fibers, the former is likely because Cdc42 has been shown to induce filopodium formation (24). Frabin or V12Cdc42 was expressed in COS7 cells together with HA-tagged JNK. The JNK was immunoprecipitated, and its kinase activity toward GST-c-Jun was determined. Frabin as well as V12Cdc42 similarly induced JNK activation (Fig. 5). These results indicate that frabin shows at least the two activities similar to those of FGD1. We have not determined here whether frabin shows GEP activity to Cdc42. However, because the DH and PH domains of frabin are highly homologous to those of FGD1, it is likely that frabin serves as a GEP for Cdc42 and induces cell shape change and JNK activation through Cdc42 activation as described for FGD1 (10).

We have isolated here a novel F-actin-binding protein named frabin that has a structure homologous to FGD1 and shows activities similar to those of FGD1. The aa sequences of the DH, PH, and cysteine-rich domains are well conserved between frabin and FGD1, but the nucleotide sequence differences were not clustered but distributed throughout the sequences. Therefore, although we have not determined the genetic locus of frabin, it is likely that frabin is not a splicing variant but an isoform of FGD1 derived from a different gene. The aa sequence of the N-terminal region of frabin, which is responsible for the F-actin binding activity, is not conserved in FGD1, and it remains unknown whether the N-terminal proline-rich domain of FGD1 binds to F-actin.

The physiological significance of frabin binding to F-actin and showing activities similar to those of FGD1 has not been clarified here, but there are two possible roles of the F-actin binding activity of frabin: one is to be involved in Cdc42 activation, and the other is to be involved in Cdc42 action. It has been shown that a GEP specific for Rac, Tiam1, is localized at the membrane through its N-terminal PH domain, but not its PH domain adjacent to the DH domain, and that this domain is required for the Rac-induced membrane ruffling and JNK activation (25). On the basis of these observations, it has been suggested that the localization of Tiam1 at the membrane is an important factor for Rac activation (25). By analogy, it is possible that the localization of frabin at the actin cytoskeleton through its F-actin-binding domain is important for Cdc42 activation.

Mutations to the FGD1 gene, either by interruption of the gene by chromosomal translocation or by insertion of a single base pair causing premature translational termination within the DH domain, have been detected in faciogenital dysplasia or Aarskog-Scott syndrome (9). Mutations to the frabin gene may also cause multisystemic developmental diseases including faciogenital dysplasia. Further studies are necessary to understand the physiological and pathological roles of frabin.

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