Association of Frabin with the Actin Cytoskeleton Is Essential for Microspike Formation through Activation of Cdc42 Small G Protein*

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We have recently isolated a novel actin filament-binding protein, named frabin. Frabin has one actin filament-binding domain (ABD), one Dbl homology domain (DHD), first pleckstrin homology domains (PHD) adjacent to DHD, one cysteine-rich domain (CRD), and second PHD from the N terminus to the C terminus in this order. Full-length frabin induces microspike formation and c-Jun N-terminal kinase (JNK) activation. We found here that the fragment of frabin containing DHD and first PHD stimulated guanine nucleotide exchange of Cdc42Hs small G protein, but not that of RhoA or Rac1 small G protein. However, this fragment of frabin did not induce microspike formation, and ABD was additionally necessary for microspike formation. Frabin having ABD was associated with the actin cytoskeleton, whereas frabin lacking ABD was diffusely distributed in the cytoplasm. In contrast, ABD was not necessary for JNK activation but CRD and second PHD were additionally necessary for this activation. These results indicate that the association of frabin with the actin cytoskeleton is essential for microspike formation but not for JNK activation and that different domains of frabin are involved in microspike formation and JNK activation through Cdc42 activation.

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Dynamic reorganization of the actin cytoskeleton is implicated in many cell functions, including cell shape change, adhesion, and motility (for reviews, see Refs. 1–3). Evidence is accumulating that the Rho family small GTP-binding proteins (G proteins) are important regulators of these actin-dependent cell functions (for reviews, see Refs. 4–6). The Rho family consists of three major subfamilies: the Cdc42, Rac, and Rho subfamilies (Cdc42, Rac, and Rho, respectively). In fibroblasts, Cdc42 induces filopodium formation; 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 gene expression, cell growth, cell-cell adhesion, and cell motility (4–6).

The Rho family members cycle between the GDP-bound inactive and GTP-bound active forms (4–6). The conversion from the GDP-bound form to the GTP-bound form is stimulated by a GDP/GTP exchange factor (GEF). Many GEFs for the Rho family members have thus far been identified and shown to share two conserved domains: a Dbl homology domain (DHD) of about 250 amino acids (aa) and a pleckstrin homology domain (PHD) of about 100 aa adjacent to DHD. We have recently isolated a novel actin filament (F-actin)-binding protein, named frabin (7). Frabin has one F-actin-binding domain (ABD), one DHD, first PHD adjacent to DHD, one cysteine-rich domain (CRD), and second PHD from the N terminus to the C terminus in this order. This domain structure of frabin is similar to that of a GEF specific for Cdc42, FGD1, determined by positional cloning to be the genetic locus responsible for facioscapulohumeral dystrophy or Aarskog-Scott syndrome (8, 9), except that FGD1 lacks ABD but has a proline-rich domain. Overexpression of frabin in Swiss 3T3 cells and COS7 cells induces microspike formation and c-Jun N-terminal kinase (JNK) activation, respectively, as described for Cdc42 and FGD1 (9). However, we had not examined whether frabin shows GEF activity on Cdc42. In this study, we first examined this activity of frabin and then the role of each domain of frabin in microspike formation and JNK activation.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Lipid-modified RhoA, Rac1, and Cdc42Hs were purified from the membrane fraction of Spodoptera frugiperda cells transfected with baculovirus carrying the respective cDNAs (10). Glutathione S-transferase (GST)-DbI and GST-Rho GDP dissociation inhibitor (GDI) were prepared as described (10). The GST carrier of GST-Rho GDI was cleaved off from Rho GDI by digestion with thrombin. Primary cultured rat hippocampal neurons were prepared as described previously (11). A rabbit antiserum against frabin was raised against GST-frabin-h (aa 1–208) described below. This antiserum was affinity-purified with GST-frabin-h covalently coupled to N-hydroxysuccinimide-activated Sepharose (Amersham Pharmacia Biotech, Ltd.) and used as an anti-frabin antibody. A monoclonal anti-Myc antibody was from American Type Culture Collection (Manassas, VA). An anti-hermagglutinin (HA) antibody was prepared as described previously (12). pSRα-HA-JNKα and GST-c-Jun (13) were kindly supplied by Dr. E. Nishida (Kyoto University, Kyoto, Japan). pEF-BOS-Myc-
V12Cdc42Hs was prepared as described previously (14). Phosphatidylinositol 4,5-diphosphate (PIP₂) was purchased from Sigma.

Construction of Expression Vectors—Eukaryotic and prokaryotic expression vectors of frabin were constructed in pCMV-Myc (11), pCMV-green fluorescent protein (GFP), and pGEX-KG (15) using standard molecular biology methods (16). Various pCMV-Myc constructs of frabin shown in Fig. 2A contained the following aa residues: pCMV-Myc-frabin-a, aa 1–766 (full length); pCMV-Myc-frabin-b, aa 1–150; pCMV-Myc-frabin-c, aa 151–766; pCMV-Myc-frabin-d, aa 169–539; and pCMV-Myc-frabin-e, aa 1–539. GST fusion constructs of frabin contained the following aa residues: GST-frabin-h, aa 1–208; and GST-frabin-c, aa 1–539. GST fusion constructs of frabin were transfected into COS7 cells using the DEAE-dextran method (17). After incubation for 16 h in serum-starved Dulbecco’s modified Eagle’s medium, the cell lysates were subjected to immunoprecipitation with the anti-HA antibody. Each immunoprecipitate was then washed and used to assay the phosphorylation of GST-c-Jun after incubation in the presence of [32P]ATP. The phosphorylation was detected by autoradiography and quantified using an image analyzer (Fujix BAS-2000II).

Other Procedures—Immunofluorescence microscopy of cultured COS7 cells and hippocampal neurons were done as described (7, 11). Protein concentrations were determined with bovine serum albumin as a reference protein (18). SDS-polyacrylamide gel electrophoresis was done as described previously (19).

RESULTS

GEF Activity of Frabin on Cdc42 and Its Responsible Domain—FGD1 has been described to show GEF activity on Cdc42Hs, but not on Rac1 or RhoA (9). The domains responsible for this activity of FGD1 are DHD and first PHD (9). We first examined whether a GST fusion protein of frabin containing only these domains (GST-frabin DH/PH) shows GEF activity on Cdc42Hs. GST-frabin DH/PH showed GEF activity on Cdc42Hs in a dose-dependent manner (Fig. 1A). GST-Dbl also showed this activity as described (10), but the efficiency of GST-frabin DH/PH on Cdc42Hs activation was about 1.6% that of GST-Dbl (data not shown). GST-frabin DH/PH was inactive on RhoA and Rac1 under the conditions where GST-Dbl was active on all the three Rho family members (Fig. 1B). This result is consistent with the properties of FGD1 described previously (9). It has been shown that PHD binds acidic phospholipids, such as PIP₂ (20). We examined the effect of PIP₂ on GEF activity of frabin on Cdc42Hs. However, addition of various doses of PIP₂ (0–20 μM) did not affect the activity of
of c-Jun. COS7 cells were transfected with pSRα along with pEF-BOS-Myc-V12Cdc42Hs or various pCMV-Myc constructs of frabin. pCMV-Myc vector was used as a control. HA-tagged JNK was then immunoprecipitated with the anti-HA antibody. A comparable amount of each immunoprecipitate was subjected to the assay for JNK activity using GST-c-Jun as a substrate. The phosphorylation of GST-c-Jun was detected by autoradiography.

We have previously shown that Rho GDI inhibits GEF activity of frabin (4). We have also shown that Rho GDI inhibits GEF activity of frabin (4). We have previously shown that Rho GDI inhibits GEF activity of frabin (4). We have previously shown that Rho GDI inhibits GEF activity of frabin (4). We have previously shown that Rho GDI inhibits GEF activity of frabin (4). We have previously shown that Rho GDI inhibits GEF activity of frabin (4). We have previously shown that Rho GDI inhibits GEF activity of frabin (4).

The phosphorylation of GST-c-Jun was expressed as fold activation relative to the level of the phosphorylation with the pCMV-Myc vector control. Data shown represent means ± S.D. of three independent experiments.

GST-frabin DH/PH (data not shown). It is not known at present why GEF activity of frabin is very low, but an unidentified factor(s) may enhance the activity.

Rho GDI is a general regulator of all the Rho family members (4). We have previously shown that Rho GDI inhibits GEF activity of GST-Dbl on RhoA, Rac1, and Cdc42Hs (10). Similarly, Rho GDI inhibited GEF activity of GST-frabin DH/PH on Cdc42Hs in a dose-dependent manner (Fig. 1C).

Domains of Frabin for Microspike Formation—We then determined the domains of frabin responsible for microspike formation in COS7 cells. We constructed the Myc-tagged, full-length and various fragments of frabin which contained various combinations of each domain (Fig. 2A). Each Myc-tagged protein was transiently expressed in COS7 cells and cell shape was analyzed by F-actin staining using fluorescent phallolidin. Consistent with our previous observation in Swiss 3T3 cells (7), full-length frabin (Myc-frabin-a) induced the formation of F-actin-containing microspikes at the periphery of cultured COS7 cells (Fig. 2, B and C). The microspikes were apparently similar to those induced by a dominant active mutant of Cdc42Hs (V12Cdc42Hs). To determine whether the frabin-induced microspikes were filopodia or retraction fibers, GFP-tagged, full-length frabin was transiently expressed in COS7 cells. Time-lapse phase-contrast microscopy of the expressing cells revealed that most microspikes were filopodia, but some of them were retraction fibers, consistent with an earlier observation with Cdc42Hs-induced microspikes (21) (data not shown). The fragment lacking ABD (Myc-frabin-c) induced the accumulation of F-actin at the cell periphery, but it did not induce microspike formation. Neither the fragment containing ABD alone (Myc-frabin-b) nor the fragment containing DHD and first PHD (Myc-frabin-d) induced microspike formation. The fragment containing ABD, DHD, and first PHD (Myc-frabin-e) induced microspike formation. Consistent with our previous observation (7), full-length frabin (Myc-frabin-a) and the fragments having ABD (Myc-frabin-b and -e) were colocalized with F-actin whereas the fragments lacking ABD (Myc-frabin-c and -d) showed diffuse distribution throughout the cytoplasm. These results indicate that ABD in addition to DHD and first PHD is necessary for microspike formation.

Domains of Frabin for JNK Activation—We have shown previously that full-length frabin induces JNK activation to an extent about 40% that induced by V12Cdc42Hs (7). We next analyzed the domains responsible for this activity. We coexpressed a fragment of frabin containing various combinations of each domain with HA-tagged JNK in COS7 cells. The expressed JNK was immunoprecipitated and its kinase activity toward GST-c-Jun was assayed. The fragment of frabin lacking ABD (Myc-frabin-c) induced JNK activation to the extent similar to that induced by full-length frabin (Myc-frabin-a) (Fig. 3, A and B). Neither the fragment containing ABD alone (Myc-frabin-b), the fragment containing DHD and first PHD (Myc-frabin-d), nor the fragment containing ABD, DHD, and first PHD (Myc-frabin-e) was active in this activity. These results indicate that DHD and first PHD are necessary not only for microspike formation but also for JNK activation, but that CRD and second PHD are additionally necessary for JNK activation.

Subcellular Localization and Tissue Distribution of Frabin—We have shown previously that full-length frabin induces JNK activation to an extent about 40% that induced by V12Cdc42Hs (7). We next analyzed the domains responsible for this activity. We coexpressed a fragment of frabin containing various combinations of each domain with HA-tagged JNK in COS7 cells. The expressed JNK was immunoprecipitated and its kinase activity toward GST-c-Jun was assayed. The fragment of frabin lacking ABD (Myc-frabin-c) induced JNK activation to the extent similar to that induced by full-length frabin (Myc-frabin-a) (Fig. 3, A and B). Neither the fragment containing ABD alone (Myc-frabin-b), the fragment containing DHD and first PHD (Myc-frabin-d), nor the fragment containing ABD, DHD, and first PHD (Myc-frabin-e) was active in this activity. These results indicate that DHD and first PHD are necessary not only for microspike formation but also for JNK activation, but that CRD and second PHD are additionally necessary for JNK activation.

Subcellular Localization and Tissue Distribution of Frabin.
Frabin—We analyzed the localization of frabin in growth cones of cultured rat hippocampal neurons in which filopodia are markedly formed. Immunofluorescence microscopy showed that frabin was highly concentrated at filopodia and poorly detected at lamellipodia (Fig. 4A). We then examined tissue distribution of frabin in various rat adult tissues. Northern and Western blot analyses showed that frabin was expressed in all the tissues examined (Fig. 4, B and C). Lung and kidney showed strong signals on Northern blot analysis whereas brain and liver showed strong signals on Western blot analysis. The reason for this discrepancy is not known, but it may be due to the difference of the stability of frabin protein in various tissues. Two bands were detected on Western blot analysis. The exact relationship between these two bands remains to be clarified, but they may be splicing variants or posttranslationally modified forms, such as the phosphorylated form.

**DISCUSSION**

We have shown here that frabin shows GEF activity specific for Cdc42 as described for FGDI (9). Like other GEFs for the Rho family members, frabin as well as FGDI has both DHD and its adjacent PHD. The aa sequences of these domains of many GEFs for the Rho family members thus far identified are highly homologous. The aa sequences of DHD and its adjacent PHD of frabin show 26% and 23% identity to those of DbI, respectively. However, those between frabin and FGDI are more highly homologous than those among other GEFs: the aa sequences of DHD and its adjacent PHD of frabin show 71% and 57% identity to those of FGDI, respectively. Of the many GEFs for the Rho family members, only frabin and FGDI are specific for Cdc42. It is likely that some specific region in these domains of frabin and FGDI determines the specificity for Cdc42, but it remains unknown how each GEF determines their substrate specificity.

We have then analyzed here the role of ABD of frabin in microspike formation and JNK activation and shown that ABD is additionally necessary for microspike formation but not for JNK activation. In our previous (7) and present report, we have furthermore shown that full-length frabin (Myc-frabin-a) is associated with the actin cytoskeleton in intact cells and that the fragment of frabin lacking ABD (Myc-frabin-c) is diffusely distributed throughout the cytoplasm. Myc-frabin-c contains DHD and first PHD which are capable of activating Cdc42 in a cell-free system. This fragment of frabin is indeed active in intact cells, because it induces JNK activation. However, Myc-frabin-c is unable to induce microspike formation. It is likely that Cdc42 activation in the vicinity of the actin cytoskeleton is essential for reorganization of the actin cytoskeleton followed by microspike formation. It has been shown that a fragment of FGDI containing only DHD and first PHD induces microspike formation through Cdc42 activation when microinjected into Swiss 3T3 cells (9, 22). It is not known why there is the difference in the ability of microspike formation between the similar fragments of FGDI and frabin, but this difference may be due to experimental conditions: In the FGDI experiments, Swiss 3T3 cells were used and the protein or cDNA samples were microinjected, whereas in our experiments, COS7 cells were used and the cDNA samples were transfected. However, it could be concluded, at least from these two experiments, that ABD is necessary for more efficient microspike formation.

In contrast, the association of frabin with the actin cytoskeleton is not essential for JNK activation. Of course, in intact cells, full-length frabin is associated with the actin cytoskeleton and activates Cdc42 around there, which then induces both microspike formation and JNK activation. It may be noted that the fragment of frabin lacking ABD (Myc-frabin-c) is active but the fragment of frabin containing only DHD and first PHD (Myc-frabin-d) is inactive for JNK activation. It is not known whether this fragment is capable of activating Cdc42 in intact cells, but these results suggest that ABD, CRD, and second PHD intramolecularly or intermolecularly affect the conformation of DHD and first PHD of frabin which determines GEF activity.

We have shown here that frabin is expressed in all the tissues thus far examined, but the protein is most abundant in brain and liver. The tissue distribution of frabin on Northern blot analysis is different from that of FGDI which is expressed in heart, brain, lung, and skeletal muscle (8). We have moreover shown that frabin is localized at filopodia at least in growth cones of cultured neurons. It is not known how frabin is highly concentrated at filopodia, but this localization is consistent with its ability to induce microspike formation through Cdc42 activation. Frabin which is associated with the cortical actin cytoskeleton activates Cdc42, which then reorganizes the actin cytoskeleton to induce filopodium formation. Repetition of this process may lengthen the microspike. In other words, the frabin-Cdc42 system reorganizes the pre-existing actin cytoskeleton to a new structure. Further study is necessary to clarify how frabin is localized at filopodia and how it is activated there for our understanding of the mechanisms of microspike formation.

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