Fhos, a mammalian formin, directly binds to F-actin via a region N-terminal to the FH1 domain and forms a homotypic complex via the FH2 domain to promote actin fiber formation

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Summary

Formins constitute a family of eukaryotic proteins that are considered to function as a cytoskeleton organizer to regulate morphogenesis, cell polarity and cytokinesis. Fhos is a recently identified mammalian formin, which contains the conserved domains FH (formin homology) 1 and FH2 in the middle region and the Dia-autoregulatory domain (DAD) in the C-terminus. The role of Fhos in the regulation of cytoskeleton, however, has remained unknown. Here we show that Fhos, in an active form, induces the formation of actin stress fibers and localizes to the actin-based structure. Fhos appears to normally exist in a closed inactive form via an intramolecular interaction between the N-terminal region and the C-terminal DAD. Both FH1 and FH2 domains are required for the induction of the stress fiber formation. However, the N-terminal region of Fhos is required for the targeting of this protein to stress fibers, which is probably mediated via its F-actin-binding activity. We also show that Fhos occurs as a homotypic complex in cells. The self-association of Fhos seems to be mediated via the FH2 domain: the domains bind to each other in a direct manner. Thus, the mammalian formin Fhos, which directly binds to F-actin via the N-terminal region, forms a homotypic complex via the FH2 domain to organize actin cytoskeleton.

Key words: Formin proteins, Fhos, Diaphanous proteins, Actin, Stress fiber, Rac

Introduction

The actin cytoskeleton functions in various cellular events, including cell motility, morphogenesis, cytokinesis, and establishment and maintenance of cell polarity. To perform these multiple tasks, actin cytoskeleton dynamics must be controlled by a variety of proteins that regulate processes such as the polymerization of actin monomers into filaments and the bundling of the filaments into a network (Amann and Pollard, 2000; Ayscough, 1998; Chen et al., 2000). Among the regulatory proteins is the family of formins that are structurally characterized by the presence of two conserved regions, the FH (formin homology) 1 and FH2 domains. In the fission yeast *Schizosaccharomyces pombe*, there exists three members of the family, i.e. fus1, for3 and cdc12, each participating in the construction of distinct actin-based structures (Chang et al., 1997; Feierbach and Chang, 2001; Pelham and Chang, 2002; Petersen et al., 1998; Sawin, 2002). In mammals, more than five formins have thus far been identified, including mDia, Fhos and FRL (formin-related gene in leukocytes) (Watanabe et al., 1997; Westendorf et al., 1999; Yayoshi-Yamamoto et al., 2000). It is known that mDia in an active state induces the formation of actin stress fibers (Ishizaki et al., 2001; Watanabe et al., 1999), whereas roles of other mammalian formins in the regulation of actin cytoskeleton have remained largely elusive.

In resting cells, mDia is probably folded in an inactive form, which is maintained by an intramolecular interaction between the N- and C-terminal regions (Alberts, 2001; Ishizaki et al., 2001; Watanabe et al., 1999). Disruption of the intramolecular interaction by deleting the N- or C-terminal region leads to the activation of mDia, thereby inducing the formation of stress fibers. On cell activation, binding of the small GTPase Rho to the N-terminal region of mDia is considered to induce a conformational change to render the protein in an open active state (Watanabe et al., 1999). The activated mDia seems to function via the core modules FH1 and FH2 (Nakano et al., 1999; Watanabe et al., 1999). The FH1 region of formins is rich in proline residues, which appears to serve as a target of the actin monomer-binding protein profilin and/or proteins containing an SH3 or WW domain (Bedford et al., 1997; Chan et al., 1996; Evangelista et al., 1997; Imamura et al., 1997; Kamei et al., 1998; Watanabe et al., 1997). The FH2 domain, which locates C-terminal to the FH1 domain, is the most conserved region among the formin family proteins. Recent studies have shown that the FH2 domain of Bni1p, a formin required for actin cable formation in the budding yeast (Evangelista et al., 2002; Sagot et al., 2002a), stimulates de novo actin polymerization in vitro (Pruyne et al., 2002; Sagot et al., 2002b). The molecular function of FH2 domains of most formin proteins is, however, under investigation.

We had fortuitously cloned cDNA encoding a novel formin

homologue, which was also identified as Fhos (formin homologue overexpressed in spleen) by other investigators during the course of the present study (Westendorf et al., 1999). Although this formin might be involved in transcription from the serum response element (Westendorf, 2001), its role in the regulation of actin cytoskeleton has remained unknown.

Here we show that Fhos in an active form induces thick actin stress fibers, which seems to require both the FH1 and FH2 domains. Fhos localizes to the actin-based structure: the N-terminal region of Fhos probably mediates the targeting of this protein to actin stress fibers, probably via its F-actin-binding activity. We also show that the Fhos forms a homotypic complex in cells. The self-association of Fhos seems to be mediated via the FH2 domain: the domains bind to each other in a direct manner. The novel function of the FH2 domain may participate in processes that involve F-actin organization, such as promotion of actin filament assembly.

Materials and Methods

Cloning of cDNA for human Fhos and plasmid construction

In the process of a yeast two-hybrid screening (Takeya et al., 2000), we obtained by chance a human cDNA clone partially encoding a novel formin homologue. On the basis of the sequence, we prepared the PCR product of 522 nucleotides (corresponding to amino acids 880-1053) and obtained, using the product as a probe, a full-length cDNA clone for human p127/Fhos (GenBank accession #AB041046) from a human T-cell cDNA library (Stratagene, La Jolla, CA). The cDNA fragments encoding Fhos-F (amino acids 1-1164), ΔC (1-1053), ΔNΔC (415-1053), N (1-569), FH1 (451-619), FH2 (613-1053), FH1FH2 (533-1053), ΔC2 (1-1071), ΔC3 (1-1120), ΔC4 (1-886) and ΔC5 (1-721) were amplified from the human Fhos cDNA by PCR using specific primers. The cDNA encoding an FH1-truncated protein, ΔFH1ΔC (1-566 plus 639-1053), was obtained by PCR-mediated site-directed mutagenesis. The PCR products were ligated to pGEX-6P (Amersham Biosciences, Tokyo, Japan), pMALc2 (New England Biolabs, Beverly, MA), pProEX-HTb (Invitrogen, Carlsbad, CA), pEGFP-C1 (Clontech, Palo Alto, CA) or pEF-BOS (Noda et al., 2001). All the constructs were sequenced to confirm their identities. The construct pEF-BOS-Myc–RhoA-G14V was a generous gift from Yoshimi Takai (Osaka University).

Cells and fluorescence microscopy

COS-7 and HeLa cells were cultured in Dulebecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS. Cells were transfected with plasmids using Lipofectamine (Invitrogen), and cultured for 3 hours. After the addition of DMEM containing 10% fetal calf serum (FCS), cells were cultured for another 13 hours. Following three washes with PBS, cells were fixed for 15 minutes in 3.7% formaldehyde. Alternatively, cells were treated simultaneously with 1.9% formaldehyde and 0.1% Triton X-100 in PBS for the first 2 minutes, and further fixed with 3.7% formaldehyde in PBS for 15 minutes (in the case of Fig. 4D). In either case, cells were subsequently permeabilized for 4 minutes with 0.1% Triton X-100 in PBS. After being washed three times, the permeabilized cells were blocked with PBS containing 3% bovine serum albumin (BSA) for 60 minutes (Ishizaki et al., 1997; Stokoe et al., 1994). Indirect immunofluorescence analysis was performed using an anti-Myc primary antibody (9E10, Roche, Tokyo, Japan) and AMCA (aminomethylcoumarin)-conjugated donkey anti-mouse secondary antibody (CHEMICON, Temecula, CA) or Alexa Fluor 488-labeled goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR). For F-actin staining, Texas Red-X phalloidin (Molecular Probes) was used. Images were visualized with a Nikon Eclipse TE300 microscope and captured on an ORCA digital camera (Hamamatsu Photonics, Hamamatsu, Japan), with the exception of those shown in Fig. 3A and Fig. 5C, which were acquired by laser confocal microscopes LSM5 PASCAL (Zeiss, Tokyo, Japan) and Radiance 2100 (Bio-Rad), respectively.

An in vitro pull-down binding assay

GST (glutathione *S*-transferase)-, MBP (maltose-binding protein)-, or His-tagged proteins were expressed in *Escherichia coli* strain BL21 and purified by glutathione–Sepharose-4B (Amersham Pharmacia Biotech), amylose resin (New England Biolabs), or His-bind resin (Novagen, Madison, WI), respectively, according to the manufacturer's protocol. Pull-down binding assays were performed as previously described (Ago et al., 1999). Briefly, a pair of a GST-fusion (10 μg) and an MBP- or His-tagged protein (10 μg) were mixed in 1 ml of phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4, pH 7.4) containing 1% Triton X-100. A slurry of glutathione-Sepharose-4B or amylose resin was added to the mixture, followed by incubation for 30 minutes at 4°C. After washing three times with PBS, proteins were eluted with 10 mM glutathione or with 10 mM amylose. The eluates from the resin were subjected to SDS-PAGE and stained with CBB (Coomassie Brilliant Blue).

F-actin co-sedimentation assay

Rabbit skeletal muscle actin was purchased from Cytoskeleton (Denver, CO). The depolymerized G-actin in a G-buffer (5 mM Tris, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 0.5 mM dithiothreitol) was polymerized by the addition of KCl, $MgCl_2$, and ATP at the final concentrations of 50 mM, 2 mM, and 1mM, respectively, and incubated for 60 minutes at 25°C. His-tagged Fhos-N was diluted into a F-buffer (5 mM Tris, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.2 mM $CaCl_2$, and 0.5 mM DTT) and clarified by centrifugation for 1 hour at 4°C at 100,000 ***g***. Polymerized actin and His-Fhos-N at the indicated concentrations were mixed in F-buffer and incubated for 60 minutes at 25°C. The mixture was then centrifuged for 60 minutes at 100,000 ***g***. Both supernatants and pellets were subjected to SDS-PAGE, followed by staining with CBB. In the case of the quantification of the free and bound His–Fhos-N, the amounts of the protein on the gel were estimated by the image analyzer LAS1000 (Fuji Photo Film, Tokyo, Japan).

F-actin blot overlay

F-actin blot overlays were performed by the method of Luna (Luna, 1998) with minor modifications. Briefly, lysates of *E. coli* expressing GST-fusion proteins or purified His-tagged proteins were subjected to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated for 60 minutes at 25°C with 50 μg/ml of F-actin in the presence of 40 μM phalloidin in TBS (20 mM Tris, pH 7.4, and 150 mM NaCl) containing 0.1% Tween-20 and 3% BSA. The blots were washed three times for 10 minutes in the same buffer and probed with an anti-actin monoclonal antibody (Roche, Tokyo, Japan). Proteins were also analyzed by immunoblot with anti-GST polyclonal antibodies (Amersham Pharmacia Biotech) or an anti-His monoclonal antibody (Qiagen, Tokyo, Japan).

Immunoprecipitation

HeLa cells (4×10^6 cells) were transfected with indicated plasmids using Lipofectamine (Invitrogen) and cultured for 36 hours in DMEM supplemented with 10% FCS. The cells were broken with a lysis buffer (135 mM NaCl, 5 mM EDTA, 10% glycerol, and 20 mM

HEPES, pH 7.4) containing 1% NP-40. The lysate was precipitated with an anti-Myc antibody (9E10, Roche) or anti-Flag antibody (M2, Sigma-Aldrich) in the presence of protein G-Sepharose. After washing three times with the lysis buffer, the precipitants were applied to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was probed with the anti-Flag monoclonal antibody, anti-Myc polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GFP rabbit polyclonal antibodies (Clontech) or anti-Fhos rabbit polyclonal antibodies. The anti-Fhos antibodies were raised against the C-terminal peptide (1125-1144) of human Fhos.

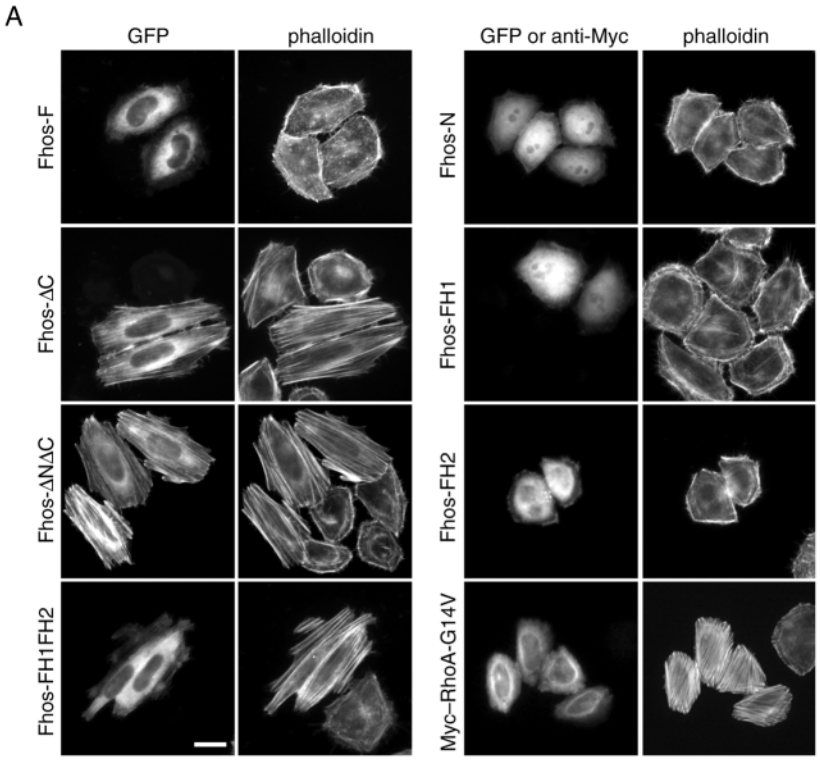


Fig. 1. Stress fiber formation induced by Fhos mutant proteins. (A) HeLa cells were transfected with vectors encoding the indicated mutant proteins of Fhos or RhoA-G14V. Cells were fixed 16 hours after the addition of DNA-lipofectamine, and expressed proteins were detected by GFP fluorescence or immunostaining for the Myc epitope (left panel). F-actin was detected by phalloidin staining (right panel). Bar, 20 μm. (B) The structure of Fhos and its truncation mutants. Numbers denote amino acid positions in Fhos. Although the FH2 domain is originally defined as a conserved sequence of approximately 100 residues, the analysis of additional formins revealed that the similarity extends over about 500 amino acids (Frazier and Field, 1997; Zeller et al., 1999). The FH2 domain of Fhos (amino acids 613-1053) is also delineated by the extended definition. All constructs were tagged with GFP at the N-termini. The effect of each mutant on the induction of actin stress fibers is summarized on the right.

Results

Stress fiber formation induced by mutant Fhos proteins

To study the role of Fhos in regulation of the actin cytoskeleton, we transiently expressed the protein in HeLa cells. As shown in Fig. 1A, expression of the full-length protein (Fhos-F) hardly affected a pattern of F-actin staining or cell shape. It is known that the best characterized mammalian formin mDia appears to be folded in an inactive form via an intramolecular interaction between the N- and C-terminal regions: a mutant protein deleting either termini are considered to lack the interaction, and thus acts as an open active form accordingly (Alberts, 2001; Ishizaki et al., 2001; Watanabe et al., 1999). It has been also suggested that Fhos normally exists as a closed inactive form via such a head-to-tail interaction (Westendorf, 2001). We therefore transiently expressed in HeLa cells a C-terminally truncated mutant of Fhos (Fhos-ΔC), which is expected to function as an active form. As shown in Fig. 1A, expression of Fhos-ΔC led to the elongation of cells and the formation of actin stress fibers aligned with the long axis of the cells. The finding is consistent with the current idea that formin family proteins regulate dynamic cytoskeletal networks during a variety of biological processes (Afshar et al., 2000; Emmons et al., 1995; Habas et al., 2001; Woychik et al., 1990). The Fhos-induced phenotypes, i.e. cell elongation and stress fiber formation, are similar to those elicited by active forms of mDia (Watanabe et al., 1999), but different from those by an active form of the small GTPase RhoA (G14V), in that Rho did not induce cell elongation (Fig. 1A). The mutant protein Fhos-FH1FH2, which retains the entire FH1 and FH2 domains (amino acids 533-1053) (Fig. 1B), was also capable of fully inducing the stress fiber formation. However, a mutant protein solely comprising the N-terminal, FH1 or FH2 domain failed to form the stress fiber (Fig. 1A).

The DAD of Fhos in the C-terminus appears to regulate the Fhos activity probably by interacting with the N-terminus

Deletion of the C-terminus of Fhos seems to render the protein in an active form, as described above (Fig. 1). Alberts has recently reported that the diaphanous-related formin homology proteins, such as mDia1-3 and Bni1p, contain a conserved module in the C-termini, designated the Dia-autoregulatory domain (DAD), in which several residues situated towards the C-termini are basic ones (Fig. 2C): the DAD seems to interact intramolecularly with the N-terminus, thereby regulating the protein activity (Alberts, 2001). Alignment of the sequence of Fhos revealed that the C-terminal region of this protein (amino acids 1101-1132) exhibits a modest homology with

the DAD sequences of the Diaphanous-related proteins, as shown in Fig. 2C; this was also raised by Westendorf (Westendorf, 2001). To study the role of the DAD in the regulation of Fhos, we prepared some C-terminally truncated mutants and expressed them in HeLa cells (Fig. 2A,B). Fhos-ΔC3 (1-1120), which lacks the polybasic region of the DAD, could promote actin fiber formation, suggesting that the DAD plays an important role.

We next purified GST–Fhos-DAD (1081-1145) and performed an in vitro pull-down binding assay to investigate whether the DAD of Fhos is responsible for the head-to-tail interaction. As shown in Fig. 2D, GST–Fhos-DAD directly interacted with His–Fhos-N, whereas it was incapable of binding to His–Fhos-FH1FH2. In addition, GST-Fhos (1081-1120), which lacks the polybasic region in the DAD, failed to interact with His–Fhos-N (Fig. 2E), suggesting that the polybasic region is essential for the binding to the N-terminus of Fhos. On the basis of these findings, we propose that Fhos probably occurs in a closed inactive form via the DAD-mediated intramolecular interaction, and that disruption of the interaction appears to lead to activation of Fhos, thereby promoting the formation of actin stress fibers.

Fhos in an active form localizes to actin stress fibers

Active forms of Fhos, such as Fhos-ΔC and Fhos-ΔNΔC, not only induce stress fiber formation but also appear to be recruited to the actin-based structure (Fig. 1). To verify the localization of activated Fhos, we expressed GFP–Fhos-ΔNΔC in HeLa cells and performed optical sectioning by confocal microscopy. The analysis revealed that Fhos-ΔNΔC exactly localized to these thick actin fibers that mainly aligned with the long axis of the spindle-shaped cells, in sections throughout from the bottom to the top of the cells (Fig. 3A). The localization of this protein provides a remarkable contrast to a cytoplasmic distribution pattern of an active mDia, which can also induce the formation of actin stress fibers (Watanabe et al., 1999). In some cells, thick actin fibers were observed to wind round and Fhos was still targeted to such unusual fibers (Fig. 3B). Furthermore, also in COS-7 cells, the active mutant protein Fhos-ΔNΔC induced the formation of thick actin fibers and localized to the induced actin-based structure (Fig. 3C).

Localization of active Fhos to actin stress fibers is probably mediated via its N-terminal F-actin-binding region

Although a mutant protein comprising only the entire FH1 and FH2 domains (amino acids 533-1053) could induce the formation of actin stress fibers, it failed to localize to the fibers in HeLa cells (Fig. 1) and COS-7 cells (data not shown), in contrast with Fhos-ΔC and Fhos-ΔNΔC. Because the protein Fhos-FH1FH2 lacks a fragment N-terminal to the FH1 domain (amino acids

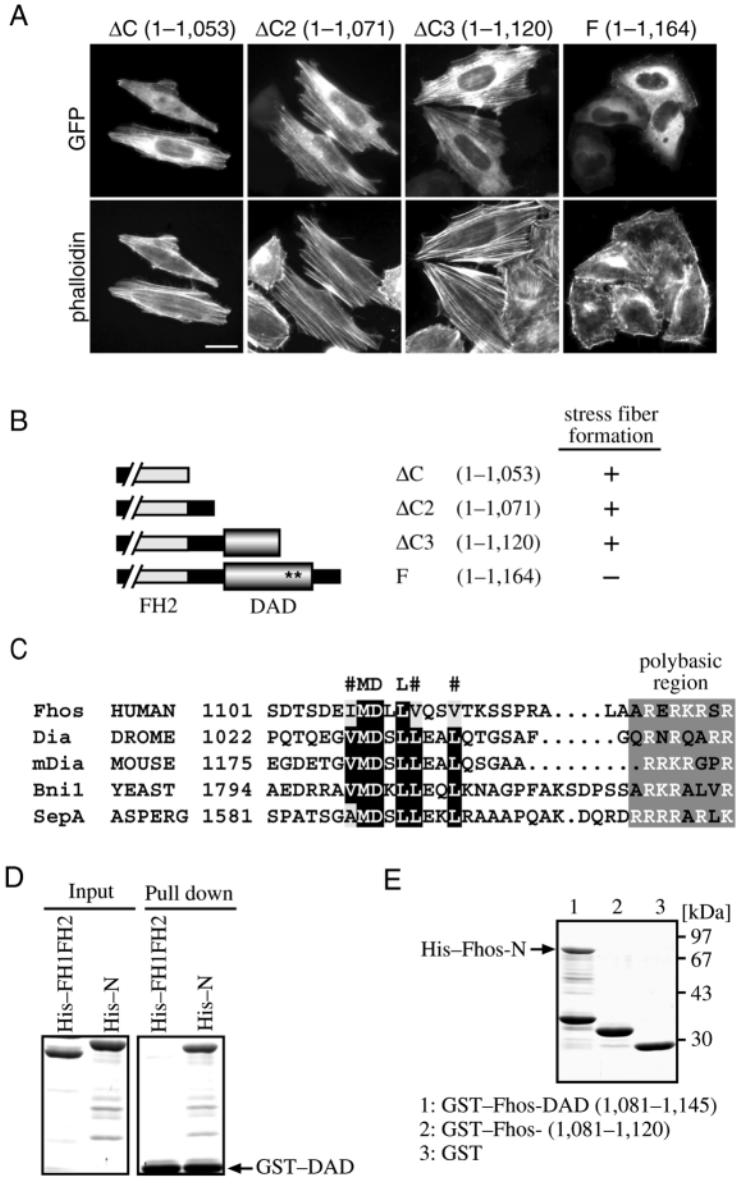


Fig. 2. The DAD of Fhos in the C-terminus appears to regulate the Fhos activity by interacting with the N-terminus. (A) HeLa cells were transfected with vectors encoding the indicated GFP-fused C-terminally truncation mutants of Fhos. Cells were fixed and then detected by GFP fluorescence (upper panels) or phalloidin staining (lower panels). Bar, 20 μm. (B) The C-terminally truncated mutant proteins are illustrated on the left, and the effects of each mutant on the induction of actin stress fibers (shown in Fig. 2A) are summarized on the right (indicated by plus and minus signs). Asterisks in the DAD indicate the polybasic region (amino acid sequences are shown in Fig. 2C). (C) Sequence alignment of the DAD and the polybasic region of Fhos, Diaphanous, p140mDia, Bni1 and SepA. Identical residues are shown on a black background and similar residues are shown on a gray background. The polybasic region is shaded in dark gray and basic residues are indicated by white letters. (D) The direct interaction between the N-terminus and DAD of Fhos. His–Fhos-N (1-569) or His–Fhos-FH1FH2 (533-1053) was incubated with GST–Fhos-DAD (1081-1145). Proteins were pulled down with glutathione-Sepharose-4B, subjected to SDS-PAGE, and stained with CBB. (E) Role for the polybasic region of the DAD in the intramolecular interaction. His–Fhos-N (1-569) binds directly to GST–Fhos-DAD (1081-1145), whereas it failed to bind to GST-Fhos (1081-1120), which lacks the polybasic region, or GST alone. An in vitro pull-down binding assay was performed as in Fig. 2D.

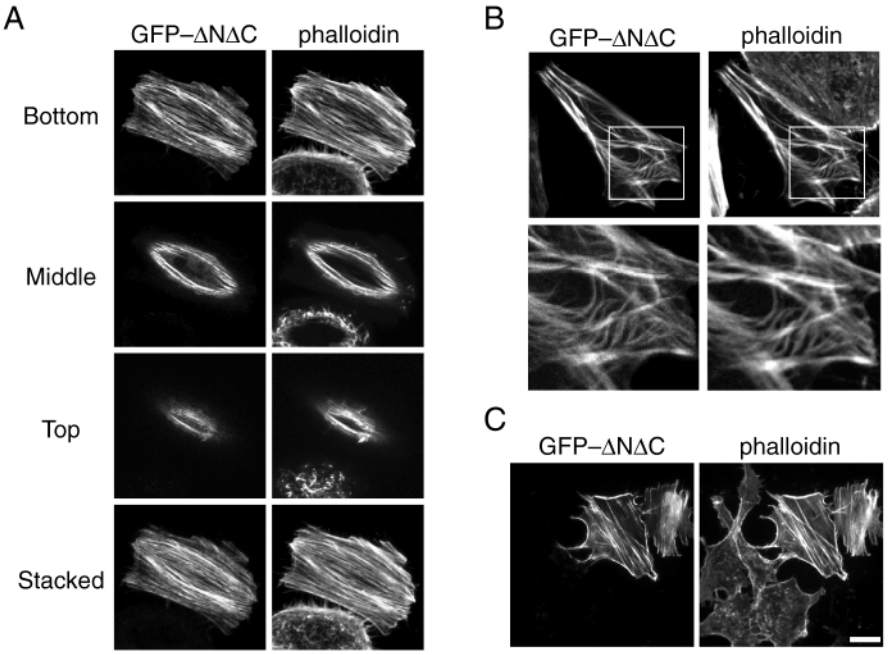


Fig. 3. Fhos in an active form localizes to actin stress fibers. (A) Confocal images of HeLa cells expressing the GFP–Fhos-ΔNΔC (415-1053), a constitutively active mutant protein. Confocal images near the bottom, middle and top of the cells, as well as stacked images, are shown. For each pair of images, GFP fluorescence (left panel) and phalloidin staining (right panel) are shown. (B) Actin fibers in HeLa cells expressing the GFP-ΔNΔC mutant. GFP fluorescence (left panels) or phalloidin staining (right panels) are shown. A magnified view of the insert is also shown in the lower panels. (C) COS-7 cells expressing the GFP-ΔNΔC mutant were fixed and detected by GFP fluorescence (left panel) or phalloidin staining (right panel). Bar, 20 μm.

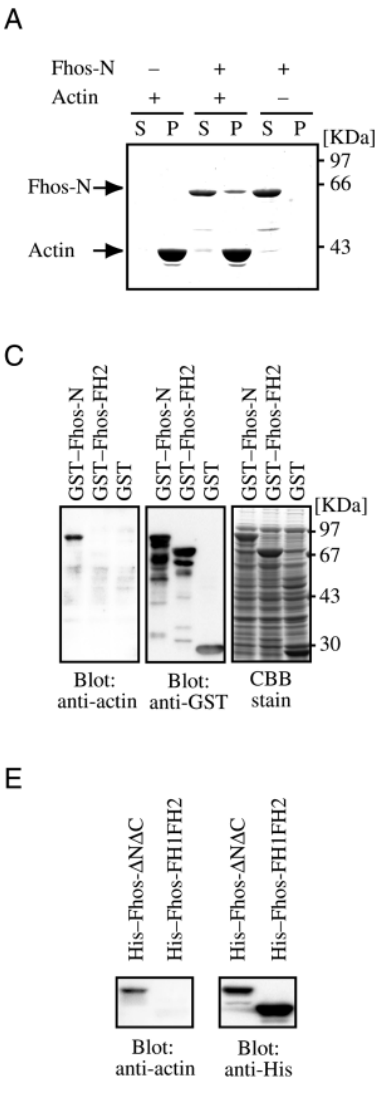
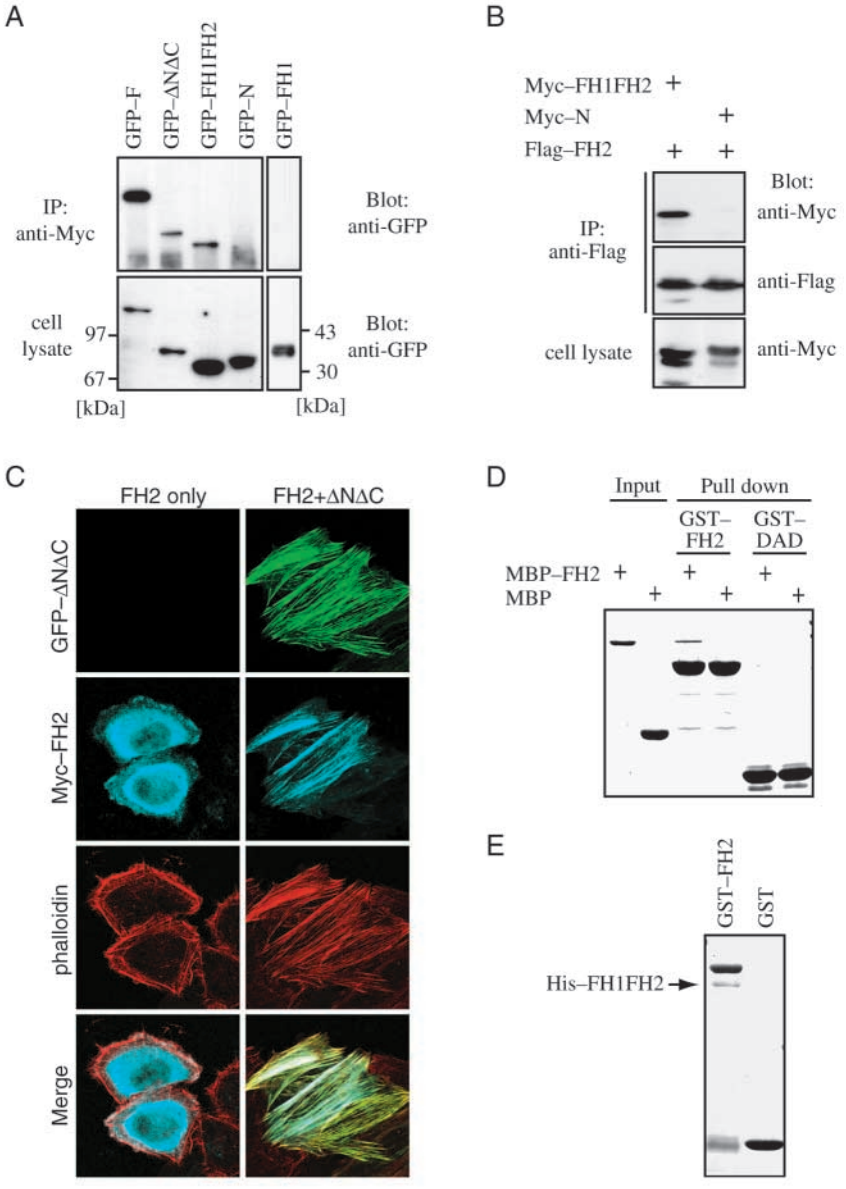


Fig. 4. Localization of active Fhos to actin stress fibers is mediated via its N-terminal F-actin-binding region. (A) Binding of Fhos to F-actin in a co-sedimentation assay. Polymerized F-actin and His-tagged Fhos-N (1-569) were mixed in F-buffer to a final concentration of 6.6 μM and 1.3 μM, respectively, and incubated for 60 minutes at 25°C. The mixture was centrifuged for 60 minutes at 100,000 ***g***, and both supernatants (S) and pellets (P) were analyzed by SDS-PAGE, followed by staining with CBB. (B) Quantitative analysis for binding of Fhos-N to F-actin. Various amounts of His-tagged Fhos-N (1-20 μg) were incubated with 1 μg of polymerized actin in a total volume of 50 μl. After ultracentrifugation, the free and bound His–Fhos-N were subjected to SDS-PAGE followed by staining with CBB. The amounts of the protein on the gel were estimated by an image analyzer. (C) Direct binding of F-actin to GST–Fhos-N in an F-actin overlay assay. Lysates of *E. coli* expressing GST–Fhos-N, GST–Fhos-FH2 and GST alone were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was probed with F-actin, and bound F-actins were detected using an anti-actin antibody (left panel). Proteins in bacterial lysates were also analyzed by immunoblot with the anti-GST antibodies (middle panel) or CBB staining (right panel). (D) Localization of Myc–Fhos-N to actin stress fibers in HeLa cells. Myc–Fhos-N and Flag–Fhos-FH1FH2 were coexpressed in HeLa cells. The cells were fixed and stained with the anti-Myc antibody (left panel) and phalloidin (right panel). Bars, 20 μm. (E) Direct binding of F-actin to His–Fhos-ΔNΔC in an F-actin overlay assay. His–Fhos-ΔNΔC and His–Fhos-FH1FH2 proteins were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was probed with F-actin, and bound F-actins were detected using an anti-actin antibody (left panel). Proteins were also analyzed by immunoblot with the anti-His antibody (right panel).

415-532) compared with Fhos-ΔNΔC, it seems possible that the N-terminal region is responsible for the targeting to actin filaments. To test this possibility, we prepared the N-terminal region (amino acids 1-569), Fhos-N, as a His-tagged protein, and carried out an F-actin co-sedimentation assay. As shown in Fig. 4A, Fhos-N was pelleted together with F-actin but not precipitated in the absence of F-actin, indicative of its direct interaction with F-actin. We next titrated in different amounts of the formin to estimate a dissociation constant, and found that Fhos-N bound to actin in a dose-dependent manner with an apparent K_d of about 2 μM (Fig. 4B). The K_d value is similar to those of actin-binding proteins such as α-actinin and talin (Bennett et al., 1984; McCann and Craig, 1997). The specific interaction of Fhos-N with F-actin was confirmed by an F-actin overlay assay: after the lysate of *E. coli* expressing GST–Fhos-N, GST–Fhos-FH2 or GST alone was subjected to SDS-PAGE, proteins were transferred to a membrane and probed with F-actin. As shown in Fig. 4C, F-actin bound solely to Fhos-N on the filter membrane in a direct manner; F-actin did not interact with GST–Fhos-FH2, GST alone or bacterial proteins.

We also tested whether the N-terminal region of Fhos associates with F-actin in vivo. When expressed in HeLa cells, Fhos-N was targeted to the stress fibers elicited by Fhos-FH1FH2 (Fig. 4D), although a part of Fhos-N was distributed in the cytoplasm. Thus, Fhos-N appears to interact with F-actin in vivo as well as in vitro.

To verify the role of the F-actin-binding activity in the localization of Fhos, we performed the F-actin overlay assay using His–Fhos-ΔNΔC (533-1053) and His–Fhos-FH1FH2 (415-1053); the former localized to stress fibers, but the latter did not (Fig. 1). As shown in Fig. 4E, F-actin bound directly to His–Fhos-ΔNΔC, whereas it failed to interact with His–Fhos-FH1FH2, which lacks the N-terminal region. Taken together, the present findings indicate that the N-terminal region of Fhos mediates the targeting of this protein to actin stress fibers via its F-actin-binding activity.



Fhos forms a homotypic complex via the FH2 domain

It is known that the organization of individual actin filaments into higher ordered structures is controlled by bivalent actin-crosslinking proteins that contain two discrete F-actin-binding sites or by noncovalently dimerized F-actin-binding proteins (Ayscough, 1998; Janmey, 2001; Puius et al., 1998). To test the possibility that the F-actin-binding protein Fhos functions as a dimer, we expressed both GFP- and Myc-tagged Fhos proteins in HeLa

Fig. 5. Fhos forms a homotypic complex via the FH2 domain. (A) HeLa cells co-expressing Myc-tagged Fhos-F and GFP-fused Fhos mutants were lysed, and proteins were immunoprecipitated with the anti-Myc antibody. The precipitants were analyzed by immunoblot with the anti-GFP antibodies. Proteins in cell lysates were also analyzed directly by immunoblot (lower panel). (B) HeLa cells co-expressing Flag-tagged Fhos-FH2 and Myc-tagged Fhos mutants (FH1FH2 or N) were lysed and immunoprecipitated with the anti-Flag antibody. The immunoprecipitates were analyzed by immunoblot with the anti-Myc or anti-Flag antibodies. Proteins in cell lysates were also analyzed directly by immunoblot. (C) HeLa cells co-expressing Myc-tagged Fhos-FH2 and GFP-fused Fhos-ΔNΔC. Cells were fixed and detected by triple-fluorescence microscopy for GFP fluorescence, Myc immunostaining and phalloidin staining. Merged images are shown in the lowest panel. (D) The direct interaction of MBP-FH2 with GST-FH2. MBP-FH2 or MBP alone was incubated with GST-FH2 or GST-DAD. Proteins were pulled down with glutathione-Sepharose-4B and subjected to SDS-PAGE, and stained with CBB. (E) The direct interaction between His-tagged FH1FH2 and GST-FH2. His-FH1FH2 was incubated with GST-FH2 or GST alone. An in vitro pull-down binding assay was performed as in Fig. 5D.

cells and assessed the ability of the proteins to interact with each other. As shown in Fig. 5A, GFP–Fhos-F was co-immunoprecipitated with Myc–Fhos-F, indicating that the full-length Fhos formed a homotypic complex in cells.

We next examined which region mediates the homotypic interaction using truncation mutants of Fhos. The truncated proteins Fhos-ΔNΔC and Fhos-FH1FH2 were co-precipitated with Fhos-F, whereas neither Fhos-N nor Fhos-FH1 interacted with Fhos-F (Fig. 5A). In addition, Myc-tagged Fhos-FH1FH2, but not Fhos-N, was coprecipitated with Flag-tagged FH2 (Fig. 5B). These findings indicate that the FH2 domain is required for formation of the homotypic complex of Fhos. The role of the FH2 domain in the intermolecular interaction was corroborated by the analysis for subcellular colocalization of truncated proteins. The protein Fhos-FH2 distributed in a cytoplasmic pattern when it is solely expressed in HeLa cells (Fig. 1A, Fig. 5C). However, when Fhos-FH2 was co-expressed with Fhos-ΔNΔC, Fhos-FH2 became colocalized with Fhos-ΔNΔC at thick actin fibers (Fig. 5C), supporting the idea that the FH2 domain plays a crucial role in the self-association of Fhos.

To determine whether the FH2 domains interact with each other in a direct manner, we performed an in vitro pull-down binding assay using purified proteins. As shown in Fig. 5D, MBP–Fhos-FH2 on the one hand was capable of directly binding to GST–Fhos-FH2. On the other hand, MBP–Fhos-FH2 failed to interact with GST-DAD, and MBP alone did not associate with GST-FH2 (Fig. 5D). Furthermore, we prepared another FH2-containing protein with a distinct tag, His–Fhos-FH1FH2, and confirmed that the FH2 domains directly bind to each other (Fig. 5E). It is thus likely that the FH2 domains homotypically interact in a direct manner, thereby mediating the self-association of Fhos.

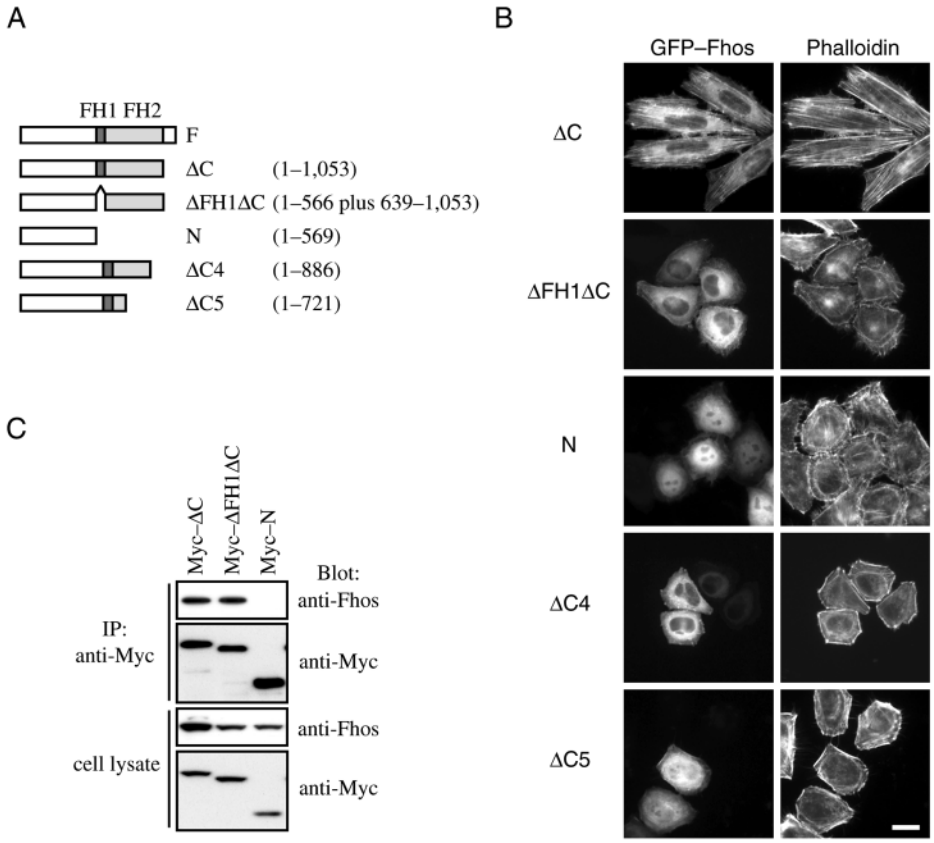


Fig. 6. Both FH1 and FH2 domains of Fhos are required for promoting actin fiber formation, but the FH1 domain is dispensable for homotypic complex formation. (A) The structure of Fhos and its FH1- or FH2-truncated mutant proteins. (B) HeLa cells were transfected with vectors encoding the indicated Fhos truncation mutants. Cells were fixed and then detected by GFP fluorescence (left panels) or phalloidin staining (right panels). Bar, 20 μm. (C) HeLa cells expressing Myc-tagged Fhos mutants were lysed and immunoprecipitated with the anti-Myc antibody. The immunoprecipitates were blotted with anti-Fhos or anti-Myc antibodies. Proteins in cell lysates were also analyzed directly by immunoblot.

Both FH1 and FH2 domains of Fhos are required for promoting actin fiber formation

The present study has shown that the N-terminal region of Fhos interacts with F-actin and that the FH2 domain mediates the homotypic interaction of this protein. However, the role for the FH1 domain of Fhos in vivo has remained uncertain. As shown in Fig. 1A, the FH1 domain alone was incapable of promoting the stress fiber formation. To address the question of whether this domain is necessary for actin reorganization in vivo, we expressed the FH1-truncated protein ΔFH1ΔC (amino acids 1-566 plus 639-1053) (Fig. 6A). This mutant protein failed to induce the formation of actin stress fibers (Fig. 6B), albeit it retained the activity to form a homotypic complex (Fig. 6C). Thus, the FH1 domain of Fhos appears to be required for the actin reorganization, but not for the homotypic interaction.

It seems likely that the FH2 domain of Fhos is also indispensable to actin stress fiber formation, given that the formation was elicited by Fhos-FH1FH2 but not by Fhos-FH1 (Fig. 1). To further study the function of the FH2 domain, we prepared the FH2-truncated proteins Fhos-ΔC4 (1-886) and Fhos-ΔC5 (1-721) (Fig. 6A). As shown in Fig. 6B, the two proteins failed to induce the formation of actin stress fibers, supporting the idea that the FH2 domain is crucial for stress fiber formation.

Discussion

Fhos is a mammalian protein that belongs to the formin family, and contains not only the FH1 and FH2 domains but also the conserved module DAD in the C-terminus. In the present study, we show that Fhos, in an active form, induces the formation of actin stress fibers and localizes to the actin-based structure.

Fhos appears to normally occur in a closed inactive form via an intramolecular interaction between the N-terminal region and the C-terminal DAD. The activity of Fhos to induce the stress fiber formation requires not only the FH1 but also the FH2 domain (Figs 1, 6).

In the case of mDia, binding of GTP-bound RhoA to the N-terminus is considered to induce the disruption of the DAD-mediated intramolecular interaction, i.e. activation of mDia (Alberts, 2001; Watanabe et al., 1999). However, the N-terminus of Fhos can interact with the small GTPase Rac1 but in a guanine nucleotide-independent manner (Westendorf, 2001). However, the interaction with Rac1 does not appear to lead to activation of Fhos, because the wild-type full-length Fhos was incapable of inducing the formation of actin stress fibers, even when co-expressed with a constitutively active or a dominant negative form of Rac (data not shown). It also seems unlikely that RhoA directly activates Fhos; GTP-RhoA was incapable of interacting with Fhos under the conditions where it bound to mDia (data not shown), which is consistent with a previous observation (Westendorf, 2001). In addition, the possibility that Fhos acts upstream of RhoA may be also excluded, as neither expression of a dominant negative form of Rho (T19N) nor inactivation of Rho with C3 exotoxin affected the stress fiber formation induced by Fhos (data not shown). Further studies are necessary to know how Fhos is converted into the active state.

We show here that Fhos in the active state localizes to actin stress fibers (Fig. 1); by contrast, active mDia is not recruited to the actin-based structure (Alberts, 2001; Watanabe et al., 1999). Furthermore, the present study shows that the targeting of Fhos to the stress fibers is mediated by the N-terminal region (Figs 1, 4), which can directly bind to F-actin (Fig. 4). The N-terminal regions of other formin proteins also seem to be involved in their subcellular localization: for instance, the N-terminus of the yeast formin fus1 is responsible for its recruitment to the projection tip that contains F-actin, whereas the N-terminal region of mDia is required for its localization to mitotic spindles, which are composed of microtubules in dividing cells (Kato et al., 2001; Petersen et al., 1998). It is, however, unknown at present whether the N-termini of these proteins directly interact with the cytoskeletal components, except that the N-terminal region of Fhos exhibits an F-actin-binding activity (Fig. 4).

As shown in this study, the FH2 domain is capable of directly mediating a homotypic interaction of Fhos (Fig. 5D,E), which is probably responsible for the present finding that Fhos exists as a homotypic complex in cells (Fig. 5A-C). Although the physiological role of the homotypic complex formation is currently obscure, the function of the FH2 domain as a mediator of homotypic interaction might underlie the actin-nucleating activity of this modular domain. The self-association of Fhos may also participate in processes involving F-actin organization, such as promotion of actin filament bundling. The formation of linear actin structures is considered to be divided into two molecular processes, i.e. actin polymerization and bundling of the formed actin filaments. It has recently been shown that yeast formins Bni1p and Cdc12p are required for the actin-polymerizing step in the assembly of actin cables or contractile actin rings, respectively (Evangelista et al., 2002; Pelham and Chang, 2002; Sagot et al., 2002a). However, it is currently unclear whether formins regulate the bundling of the actin filaments directly or indirectly. The bundling of actin filaments can be caused by dimerization of F-actin-binding proteins, such as α-actinin (Ayscough, 1998; Janmey, 2001; Puius et al., 1998). It seems thus possible that, in addition to the process of actin polymerization, Fhos may be also involved in the process of F-actin bundling via the formation of a homotypic complex.

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