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Identification of FHOD1-Binding Proteins and Mechanisms of FHOD1-Regulated Actin Dynamics

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Abstract Formin homology-2-domain containing protein 1 (FHOD1) regulates gene transcription, actin-cytoskeleton structure, and cell migration. To gain insight into the mechanisms by which FHOD1 mediates these diverse activities, a yeast-two-hybrid screen was performed to identify FHOD1-binding proteins. Three proteins specifically interacted with the carboxy-terminal two-thirds of FHOD1, which includes the FH1, FH2, and diaphanous activating domains (DAD). The newly identified FHOD1-binding proteins are protein kinase C binding protein 1 (PRKCBP1), cyclophilin B, and an isoform of WASP-interacting SH3-domain protein/diaphanous-interacting protein 1 (WISH/DIP1), named WISH-B. The proline-rich FH1 domain of FHOD1 was sufficient to interact with the central portion of PRKCP1 and full-length cyclophilin B. The FH1 domain also interacted with full-length WISH-B, but the extreme amino-terminus was sufficient to associate with WISH-B as well. WISH-B altered the solubility of FHOD1 in vitro and a truncation mutant containing the amino-terminal 227 residues of WISH-B disrupted FHOD1-induced stress fibers. WISH-B did not affect FHOD1-induced gene transcription through the serum response factor (SRF) recognition site on the skeletal α actin promoter (SkA). However, stabilization of F-actin prevented FHOD1 dependent activation of this promoter in presence of high, but not low serum concentrations. Thus, the identification of a new FHOD1-binding protein provides insight into the mechanisms by which FHOD1 regulates actin polymerization and transcription. J. Cell. Biochem. 92: 29–41, 2004.

Key words: diaphanous; FHOD1; FHOS; formin; stress fibers; WISH; DIP1; cyclophilin B; PRKCP1; RACK7

Formins are evolutionarily conserved proteins that act as scaffolds and regulators of actin cytoskeleton remodeling [Evangelista et al., 2003; Wallar and Alberts, 2003]. Formins activate signaling pathways and nucleate actin filaments in an Arp2/3-independent manner to mediate cytokinesis and cell polarity [Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002; Li and Higgs, 2003]. In yeast and *Drosophila*, formins organize cortical actin filaments and the contractile ring [Castrillon and Wasserman, 1994; Emmons et al., 1995; Chang et al., 1997; Evangelista et al., 1997, 2002, 2003; Imamura et al., 1997]. In mammalian cells, formins participate in stress fiber formation, motility, phagocytosis, signaling, gene transcription, and embryonic development [Nakano et al., 1999; Sotiropoulos et al., 1999; Tominaga et al., 2000; Yayoshi-Yamamoto et al., 2000; Habas et al., 2001; Westendorf, 2001; Koka et al., 2003]. Increasing evidence indicates that alterations in formin levels affect cell growth, differentiation, and migration. Naturally occurring formin mutations are associated with deafness and premature ovarian failure in humans [Lynch et al., 1997; Bione et al., 1998] and they affect neural function, oogenesis, and limb and kidney morphogenesis in mice [Maas et al., 1990; Woychik et al., 1990; Leader and Leder, 2000; Leader et al., 2002]. Overexpression of diaphanous proteins was observed in highly metastatic rat osteosarcomas and

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leiomyosarcomas [Fukuda et al., 1999; Skubitz and Skubitz, 2003].

Several structural and functional domains characterize formins. Two structural elements called formin homology (FH) domains are present in all formins [Castrillon and Wasserman, 1994; Wasserman, 1998]. The FH1 domain is proline rich and mediates interactions with a variety of proteins, including profilins, src family kinases, and SH3-domain containing factors [Chang et al., 1997; Evangelista et al., 1997; Imamura et al., 1997; Watanabe et al., 1997; Tominaga et al., 2000; Satoh and Tominaga, 2001]. The FH2 domain is necessary for actin nucleation, microtubule stabilization, and serum response factor (SRF) activation [Sotiropoulos et al., 1999; Tominaga et al., 2000; Ishizaki et al., 2001; Westendorf, 2001; Pruyne et al., 2002]. A subset of formins contains a diaphanous activation domain (DAD) that consists of a leucine-rich region accompanied by a basic region [Alberts, 2000]. These proteins bind Rho-family GTPases and are referred to as diaphanous related formins (Drfs). In some Drfs, binding of activated Rho relieves intramolecular interactions between the DAD and amino-terminal sequences [Watanabe et al., 1997, 1999]. This induces the activation of Drfs and allows the FH1 and FH2 domains to bind to various effector proteins which subsequently change gene transcription or actin organization [Watanabe et al., 1999]. In vitro, constitutively activated formins are created by deletion of either the carboxy-terminus and DAD or the amino-terminus and intramolecular DAD-binding region.

Formin homology-2-domain containing protein 1 (FHOD1; also known as FHOS) is a Drf that is expressed at high levels in the spleen and skeletal muscle [Westendorf et al., 1999; Tojo et al., 2003]. Ectopic expression of FHOD1 enhances cell migration and insulin-stimulated glucose uptake [Koka et al., 2003; Tojo et al., 2003]. Activated forms of FHOD1 induce gene transcription from the serum response element [Westendorf, 2001] and actin stress fiber formation [Gasteier et al., 2003; Koka et al., 2003; Takeya and Sumimoto, 2003]. FHOD1 interacts with Rac1 in a guanine-nucleotide independent manner; however, dominant negative Rac1 inhibits FHOD1-induced gene transcription and stress fiber formation [Westendorf, 2001; Koka et al., 2003]. Moreover, activated Rac1 recruits FHOD1 to filamentous actin and lamellipodia [Gasteier et al., 2003]. Although FHOD1-dependent stress fiber formation is sensitive to RhoA and Rho kinase inhibitors, FHOD1 does not interact with these proteins [Gasteier et al., 2003]. The only other proteins that are known to interact with FHOD1 are actin and profilin IIa [Koka et al., 2003; Takeya and Sumimoto, 2003; Tojo et al., 2003]. In this report, we describe the identification of three FHOD1-interacting proteins and demonstrate how one of these proteins, WISH-B, influences FHOD1-dependent activities.

MATERIALS AND METHODS

Plasmids

Mammalian (pCMV5) and yeast (pAS-2-1)-FHOD1 expression plasmids were previously described [Westendorf et al., 1999; Westendorf, 2001]. Skeletal α-actin-luciferase (SkA-Luc) reporter plasmids were kindly provided by Dr. Michael D. Schneider (Baylor College of Medicine) [Paradis et al., 1996]. pGEX-4T-2-Cyclophilin B was generated by subcloning the EcoRI/XhoI fragment from the yeast-two-hybrid library vector, pACT-II. WISH-B cDNA expression plasmids were created by removing the cDNA from the pACT2 yeast-two-hybrid vector with EcoRI and XhoI and subcloning into pENTR-1A (Invitrogen, Carlsbad, CA). WISH-B truncation mutants were generated by PCR with gene-specific oligonucleotides (sequences available upon request) and subcloned into pENTR-1A with EcoRI and XhoI. The cDNAs were then transferred with the Gateway LR Clonase Enzyme mix (Invitrogen) into pDEST-FLAG-CMV2, which was created by adding the Gateway vector conversion cassette A (Invitrogen) into pFLAG-CMV-2.

Yeast-Two Hybrid Assays

The yeast-two-hybrid "bait", FHOD1 residues 491–1165, was generated by subcloning a PstI fragment from pCMV5-HA-FHOD1 into pAS2-1 (Clontech, Palo Alto, CA). pAS2-1-FHOD1 (491–1165) was transformed into *S. cerevisiae* strain Y190 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, −112 +URA3::GAL → lacZ, LYS2::GAL → HIS3). Transformants were selected by growth on SD-agar lacking tryptophan (W; SD-W), grown in SD-W medium and then transformed with an oligo-dT primed human bone marrow cDNA

library in pACT2 (Clontech). Double transformants were selected by growth on SD-agar lacking histidine (H), W, and leucine (L) (SD-HWL) and containing 45 mM 3-aminotriazole (3-AT). After 3 days, 54 colonies greater than 2 mm in size were selected for further analysis. Clones capable of producing Lac-Z in β-galactosidase assays were cultured in SD-L medium to segregate the pACT2 plasmids from pAS2-1. Resulting clones were tested for Lac-Z production. Only four clones did not make Lac-Z in the absence of the bait. The specificity of the "prey" cDNAs for FHOD1 (491–1165) was determined by transforming Y190 cells expressing prey plasmid with the original bait, pAS2-1 FHOD1 (491–1165), pAS2-1 or pLAMC-5 (GAL_{DBD}-Lamin C; Clontech) and selecting for growth on SD-HWL containing 45 mM 3-AT. Yeast-two hybrid assays to map FHOD1 interaction sites were performed as described above and in a previous report [Westendorf, 2001].

Immunoprecipitations and Cellular Fractionation Experiments

For co-immunoprecipitation experiments with HA-FHOD1 and FLAG-WISH-B proteins, HEK293T cells were co-transfected with 6 μg of the indicated mammalian plasmid(s) and 10 μg pKS-Bluescript by calcium phosphate precipitation. Two days later, the cells were washed twice with cold phosphate buffered saline (PBS) and lysed with 1 ml PBS containing 1% Triton X-100, 0.1% EMPIGEN BB (Calbiochem, San Diego, CA), 3 U/ml aprotinin, and 5 μg/ml leupeptin. Lysates were incubated for 5 min on ice and then precleared with 20 μl 50% slurry of protein A-sepharose beads for 30 min at 4°C. Whole cell lysates (WCL) were cleared by centrifugation and 30 μl was removed for immunoblot analysis. The remaining portions of the lysates were incubated for 2.5 h with anti-FLAG M2-Agarose beads (Sigma, St. Louis, MO; 15 μl 50% slurry) that were pre-blocked with 3 mg/ml bovine serum albumin (BSA), activated with 0.1 M glycine, pH 3.5, and resuspended in lysis buffer. Beads were washed three times with 1 ml of lysis buffer. Proteins were eluted from the beads with SDS sample buffer, resolved by SDS-10% PAGE and transferred to Immobilon P (Millipore, Bedford, MA).

For cell fractionation experiments, COS cells were transfected with the indicated HA-FHOD1 or FLAG-WISH-B expression plasmids with DEAE-Dextran. Cells were lysed on ice for 10 min in modified radioimmunoprecipitation buffer (1% NP-40, 0.25% sodium deoxycholate (DOC), 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 3 U/ml aprotinin, and 5 μg/ml leupeptin). Lysates were fractionated by microcentrifugation at maximum speed for 20 min at 4°C. Soluble proteins in the supernatants were removed and the pellets containing insoluble proteins were suspended in SDS-sample buffer and sonicated. Proteins were resolved by SDS-8% PAGE and transferred to Immobilon P. HA-FHOD1 and FLAG-WISH-B proteins were detected by immunoblotting with mouse anti-HA (clone 12CA5, Sigma) or mouse anti-FLAG (M2, Sigma) monoclonal antibodies, respectively, followed by anti-mouse-IgG-Fc secondary antibodies conjugated to horseradish peroxidase (Sigma) and enhanced chemiluminescence.

GST-Pulldown Assays

GST proteins were produced in *E.coli*, DH5α, during a 3 h induction with 0.2 mM IPTG. Bacteria were lysed by resuspension in PBS containing 0.1% NP-40, 5 μg/ml leupeptin, aprotinin, and pepstatin A. Following sonication, insoluble material was separated by centrifugation. GST fusion proteins were purified from the lysates with glutathione-sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). ^{35}S-labeled FHOD1 proteins were in vitro transcribed and translated with the TNT T3-Coupled Reticulocyte Lysate Systems (Promega, Madison, WI). One-tenth of the lysate was incubated with equal amounts of purified GST or GST-CYPB proteins for 2 h at 4°C in 200 μl pulldown buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP40, 1 mM EDTA, 0.1% BSA, aprotinin, leupeptin, pepstatin A, and PMSF). Beads were washed twice with pulldown buffer and once with a 10 mM Tris-HCl, pH 7.5, 0.1% NP-40 buffer. Proteins were resolved by SDS-7.5% PAGE. Gels were fixed in 40% methanol and 10% acetic acid, incubated with Amplify (Amersham Pharmacia Biotech), dried, and exposed to film.

In Situ Immunofluorescence

NIH-3T3 cells were grown on coverslips and transfected with expression plasmids encoding HA-FHOD1 (1–1010) and the indicated FLAG-WISH-B protein. Cells were washed with cytoskeletal buffer and fixed in 3% paraformaldehyde as previously described [Koka et al.,

2003]. F-actin was detected with phalloidin-rhodamine. HA-FHOD1 was detected with a rabbit-anti-HA Ab and FITC-conjugated donkey anti-rabbit secondary Ab. FLAG-WISH-B proteins were detected with mouse anti-FLAG Ab and Cy5-conjugated goat anti-mouse Ab. Cells were visualized with a Nikon confocal microscope as previously reported [Koka et al., 2003].

Transcription Assays

C2C12 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Biowhittaker), 200 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were transfected with SkA-Luc (0.2 μg), pCMV-secreted alkaline phosphatase (SEAP; 0.2 μg), and the indicated FHOD1 and/or WISH-B expression pCMV plasmid (0.4 μg) or pCMV5 (control) with 6–8 μg lipofectamine (Invitrogen) in 12-well plates. Jasplankinolide (JSP; Molecular Probes, Eugene OR) or latrunculin B (LatB; Sigma) was added to a final concentration of 1 μM after removing the transfection medium. Supernatants and cell lysates were collected 40–48 h after the start of the transfection. Luc activity in cell lysates was measured with a Luciferase Assay System (Promega). SEAP activity in culture supernatants was measured as previously described [Westendorf, 2001]. Luc activities were normalized with SEAP values for the same sample. Values represent the means of triplicate samples.

RESULTS

Identification of FHOD1 Interacting Proteins

Activated forms of FHOD1 lacking either the amino- or carboxy-terminus (FHOD1 (469–1165) and (1–1010), respectively) stimulate transcription of the SRE [Westendorf, 2001]. In an effort towards determining the mechanism of FHOD1-induced SRE activation, a yeast-two-hybrid screen was performed with a carboxy-terminal portion of FHOD1 including residues 491–1165 (Fig. 1A). Four FHOD1-interacting clones representing three distinct genes were identified in a human bone marrow cDNA library that interacted with the original FHOD1 bait upon retransformation, but did not interact with the GAL-DNA binding domain, p53, and/or Lamin C (Fig. 1B–D). The first two clones were identified as a central portion of the protein kinase C binding protein (PRKCBP) 1 [Fossey et al., 2000] and cyclophilin B (CYPB). The other two clones were identical and highly similar in sequence to AF3p21, SH3 protein interacting with Nck (SPIN90), diaphanous interacting protein (DIP)1, vimentin/VacA-interacting protein (VIP54), and WASP-interacting SH3 protein (WISH) [de Bernard et al., 2000; Sano et al., 2000; Fukuoka et al., 2001; Lim et al., 2001; Satoh and Tominaga, 2001]. The major distinction is that they lack the variable carboxy-terminal region present in the other proteins, perhaps due to an alternative splicing event. This FHOD1-interacting protein will be referred to as WISH-B (Fig. 1D, GenBank Accession number AY453794).

FH1 Domain Interacts With the Identified FHOD1-Binding Proteins

The FHOD1 protein used as bait in the yeast-two-hybrid system contains a proline-rich FH1 domain, a FH2 domain, coiled-coil, and a dia-like autoregulatory domain (DAD), but lacks the amino terminus (Fig. 1A) [Westendorf et al., 1999; Westendorf, 2001]. The interactions between full-length FHOD1 and WISH-B or CYPB were verified in biochemical assays. The PRKCPB1 interaction was not verified because a full-length clone has not yet been obtained. To confirm the interaction between WISH-B and FHOD1, HEK293T cells were transiently transfected with expression plasmids for FLAG-WISH-B and HA-FHOD1 proteins. FHOD1 was specifically co-immunoprecipitated with FLAG-WISH-B (Fig. 2B). The interaction between FHOD1 and CYPB could not be verified in cells because overexpression of CYPB inhibited FHOD1 expression (data not shown). However, GST-CYPB interacted with full-length FHOD1 in vitro (Fig. 2C). CYBP also interacted with several FHOD1 proteins (1–1010, 1–717, and 1–421), but it did not bind to FHOD1 (1–328).

To determine which FHOD1 domains are required for the interactions with all the proteins, truncated FHOD1 cDNAs were fused to the GAL-DBD (Fig. 2A) and transformed into yeast expressing the GAL-activation domain fused to PRKCBP1, CYPB, or WISH-B. As expected, the original bait for the 2-hybrid screen, FHOD1 (491–1165), interacted with all three proteins (Fig. 2D). Truncation mutants lacking the FH1 domain, FHOD1 (668–1165) and (854–1165), did not interact with any of the proteins. Two carboxy-terminally truncated

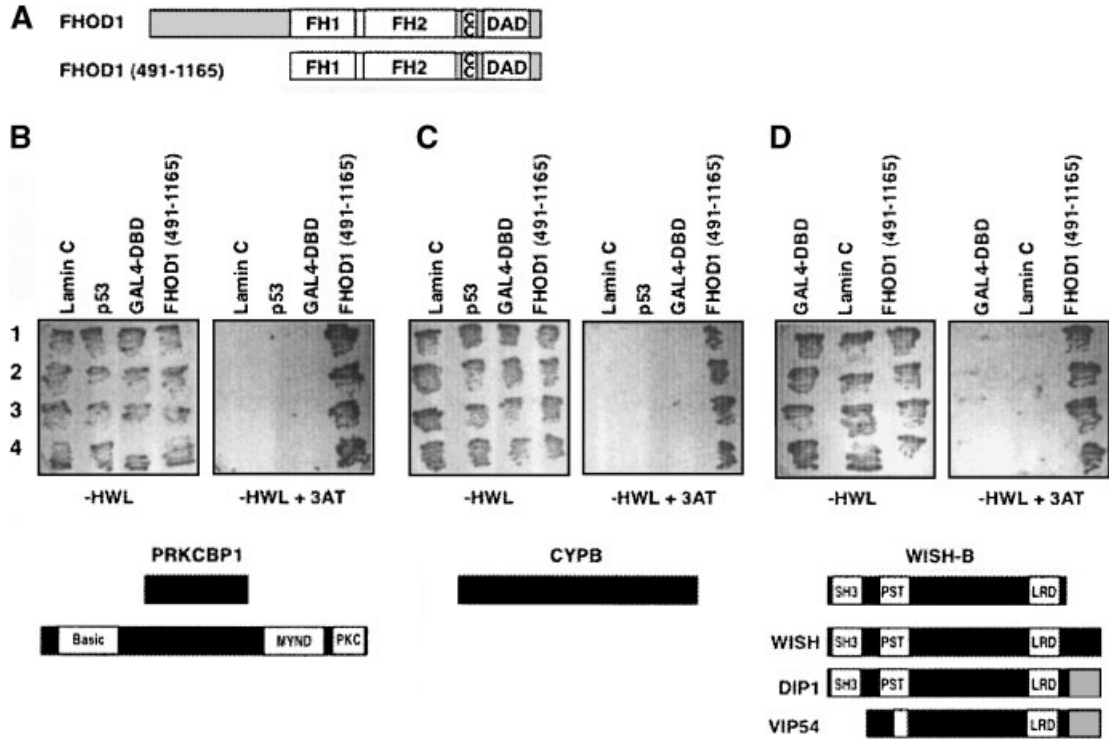

Fig. 1. The identification of three FHOD1-binding proteins. **A**: Schematic representation of FHOD1 and the truncated FHOD1 protein (residues 491–1165) that was used to identify interacting proteins in a yeast-two-hybrid assay. FH denotes the formin homology (FH) domains. CC is the coiled-coil region and DAD is the diaphanous activating domain. **B–D**: The interactions between FHOD1 and the interacting proteins are specific. Yeast-two hybrid assays were performed by co-transfecting Y190 cells with expression plasmids for GAL_{DBD} or GAL_{DBD} fusion proteins (lamin C, p53 or FHOD (491–1165)) and GAL_{AD}-PRKCBP1 (B), -CYPB (C) or -WISH-B (D). Four colonies from each co-transfection were picked from plates containing growth medium lacking HWL and streaked on plates containing the same medium in the presence or absence of 3-AT. Growth on medium lacking HWL indicates that the yeast contain both expression plasmids. Growth on medium lacking HWL and containing 3-AT indicates a protein interaction. Diagrams of the FHOD1-binding proteins are aligned with highly homologous proteins at the bottom of the panels.

FHOD1 proteins, (1–421) and (1–322), that contain residues not present in the FHOD1 bait did not interact with PRKCBP1. Although FHOD1 (1–421) bound to CYBP in vitro (Fig. 2C), it did not interact with it in vivo (Fig. 2D). FHOD1 (1–322) also did not interact with CYBP. In contrast, both interacted with WISH-B in the yeast-two hybrid binding assay but the interaction between WISH-B and FHOD1 (1–421) was much weaker than with FHOD1 (1–322). Thus, the FH1 domain of FHOD1 is necessary for the interactions with these three proteins. An additional region in the amino terminus may also interact with WISH-B.

FHOD1 Interacts With an Amino-Terminal Portion of WISH-B

The SH3 domain is the only known structural domain in WISH-B; however, WISH-B also contains the proline-serine-threonine (PST) and leucine-rich domains (LRD) identified in other isoforms (Fig. 1E). SH3 domains interact with proline-rich regions, thus it was hypothesized to mediate an interaction with the FHOD1 FH1 domain. To determine which regions of WISH-B were necessary to interact with FHOD1 in vivo, various portions of WISH-B were fused to the FLAG-epitope tag in a mammalian expression vector (Fig. 3A). Initial experiments to detect the expression of the FLAG-WISH-B proteins in vivo were largely unsuccessful because the majority of WISH-B was not soluble in a buffer containing NP-40 and DOC (Fig. 4A). However, addition of the zwitterionic detergent, EMPIGEN BB, to a Triton X-100-containing lysis buffer solubilized the majority of the FLAG-WISH-B proteins. This allowed us to test for interactions between FLAG-WISH-B proteins and HA-FHOD1 in vivo. HEK293T cells were transiently transfected with pCMV5-HA-FHOD1 in the presence or absence of a

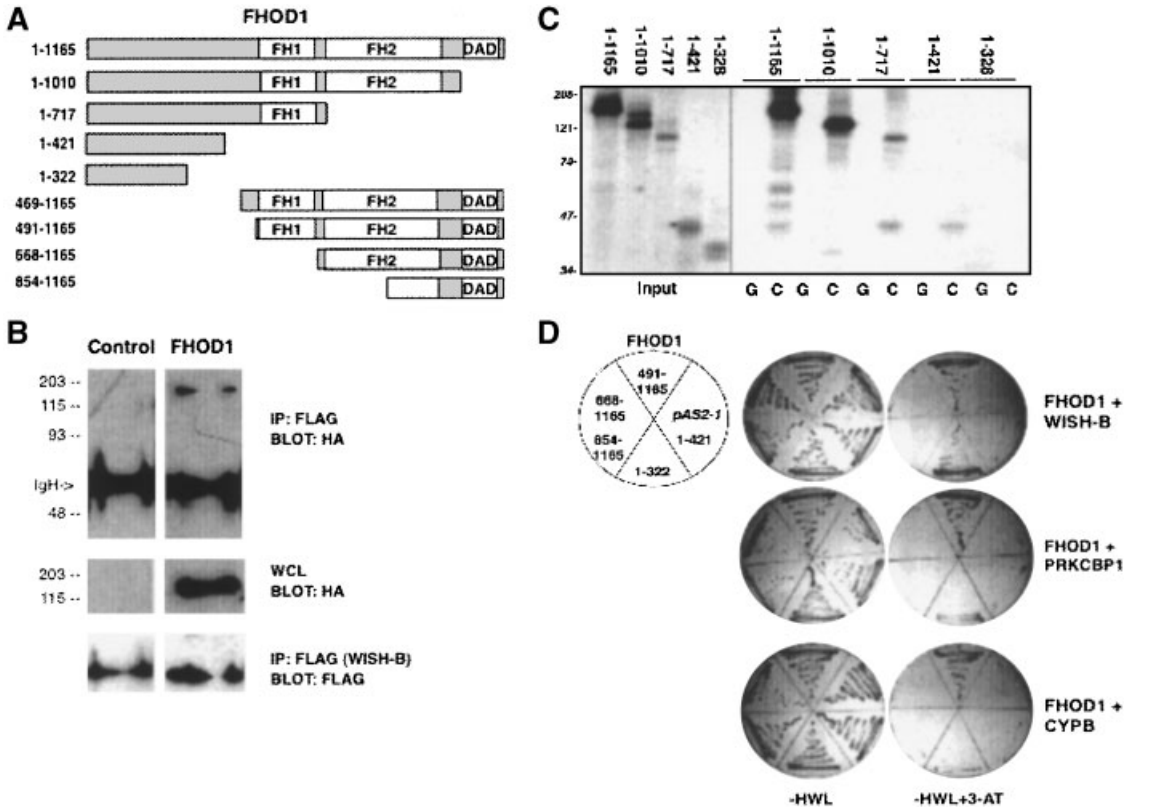

Fig. 2. The FHOD1-binding proteins interact with the FHOD1 FH1 domain. **A**: Schematic of the truncated FHOD1 proteins used in these experiments. **B**: WISH-B interacts with full-length FHOD1. COS cells were co-transfected with FLAG-WISH-B and HA-FHOD1 or an empty expression plasmid, pCMV5 (control). Cell extracts were immunoprecipitated with FLAG Abs. Proteins were detected in the immunoprecipitates or whole cell lysates (WCL) by immunoblotting with FLAG or HA antibodies. **C**: FHOD1 proteins containing the FH1 domain interact with CYPB. In vitro transcribed and translated ^{35}S-FHOD1 proteins were incubated with GST (G) or GST-CYPB (C). Input represents 20% of the material added to the GST binding reactions. **D**: The FH1 domain of FHOD1 is necessary for interacting with PRKCBP1, CYPB, and WISH-B. Y190 cells were co-transfected with expression plasmids encoding GAL_{AD}-WISH-B, -PRKCBP1 or -CYPB and the indicated GAL_{DBD}-FHOD1 protein. Cells were grown as described in Figure 1.

pCMV5-FLAG-WISH-B. WISH-B complexes were immunoprecipitated from cell lysates with anti-FLAG antibodies. The immunoprecipitates and WCL were then analyzed by immunoblotting with FLAG- or HA-specific antibodies. As shown in Figure 3B, FHOD1 co-immunoprecipitated with full-length WISH-B (1–683). FHOD1 also interacted with carboxy-terminally truncated-WISH-B proteins (1–431) and (1–247). FHOD1 was similarly co-immunoprecipitated with WISH-B mutants lacking the SH3 domain (61–683) and (61–431). However, FHOD1 did not interact as strongly with WISH-B (248–683) and (248–431), which lack the SH3 and PST domains. Together these data demonstrate that the WISH-B SH3 and PST domains provided strong interaction sites for FHOD1 but that the SH3 domain is not necessary for the interaction.

WISH-B Recruits FHOD1 to a Triton X-100-Insoluble Cell Fraction

As described above, WISH-B was insoluble in a lysis buffer containing NP-40 and DOC (Fig. 4A). In contrast, FHOD1 is completely soluble in these buffers. Given that FHOD1 interacts with WISH-B, it was hypothesized that WISH-B may alter the solubility of FHOD1. Indeed, when full-length FHOD1 (1–1165) was co-expressed with FLAG-WISH-B, it was detected in both the soluble and insoluble fractions (Fig. 4B). WISH-B was only detected in the insoluble fraction. Similarly, FHOD1 truncation mutants, FHOD1 (469–1165) and (1–1010), which contain the FH1 and FH2 domains, also were present in the both the soluble and insoluble fractions in the presence of WISH-B (Fig. 4B, lanes 5, 6, 11, and 12).

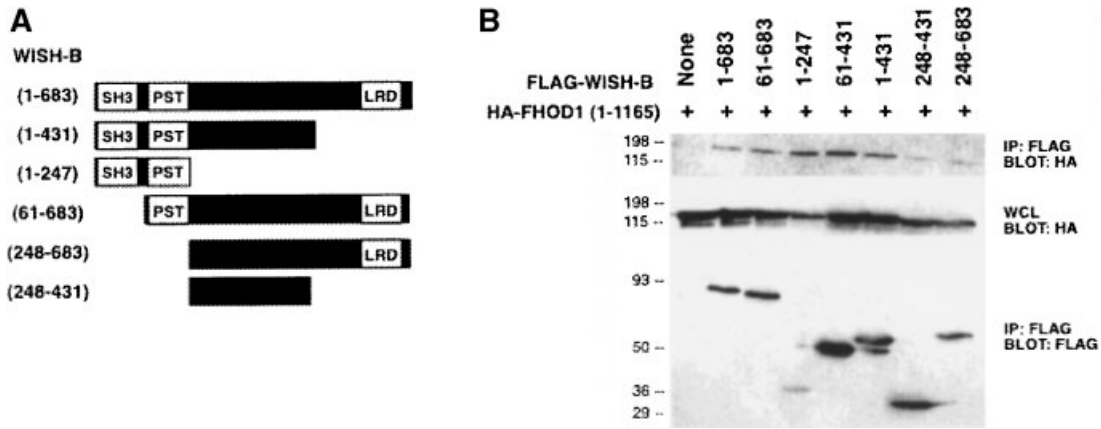

Fig. 3. The amino-terminus of WISH-B confers maximal binding to FHOD1. **A**: Schematic of the WISH-B proteins used in these studies. **B**: COS cells were co-transfected with expression plasmids for HA-FHOD1 (1–1165) and the indicated FLAG-WISH-B protein. Cell extracts were immunoprecipitated with FLAG Abs. Proteins were detected in the immunoprecipitates or WCL by immunoblotting with FLAG or HA antibodies.

These proteins were completely soluble in NP-40 and DOC in the absence of WISH-B (lanes 2, 3, 8, and 9). These data suggest that WISH-B recruits FHOD1 to cellular complexes that are insoluble in non-ionic detergents.

WISH-B Amino Terminus Prevents FHOD1-Induced Stress Fiber Formation

Ectopic expression of an activated form of FHOD1 (1–1010) in cells results in the appearance of prominent stress fibers [Gasteier et al., 2003; Koka et al., 2003; Takeya and Sumimoto, 2003]. Co-expression of full-length WISH-B (1–663) did not affect the appearance of FHOD1-induced stress fibers (Fig. 5A). However, in the presence of the WISH-B truncation mutant (1–247) that contains the SH3 and PST domains, FHOD1 was dissociated from stress fibers and it was partially relocalized to the nucleus. In addition, cells did not present with thick actin stress fibers nor did they exhibit the elongated shape typical of cells overexpressing FHOD1 (1–1010) and WISH-B (1–663). A WISH-B truncation mutant (248–663) that did not bind FHOD1 well in vitro did not affect FHOD1-induced stress fibers. These data provide further evidence that the SH3 and PST domains of WISH-B interact with FHOD1. WISH-B does not induce stress fibers on its own (Fig. 5B).

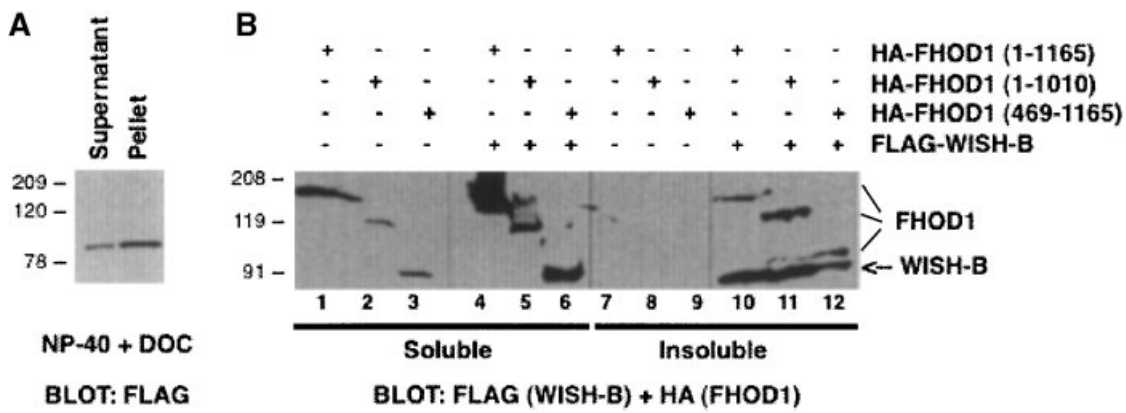

Fig. 4. WISH-B alters the solubility of FHOD1 Proteins. **A**: WISH-B is weakly soluble in non-ionic detergents. COS cells were transfected with FLAG-WISH-B. Cell extracts were prepared by lysing the cells in modified RIPA buffer containing NP-40 and DOC and high speed centrifugation. Equal volumes of the supernant (soluble) and pellet (insoluble) were resolved by SDS–PAGE. WISH-B was detected by immunoblotting with FLAG mAb. **B**: A fraction of the FHOD1 protein is retained in the insoluble fraction in the presence of WISH-B. COS cells were co-transfected with FLAG-WISH-B and either full-length HA-FHOD1 or a HA-FHOD truncation protein, (1–1010) or (469–1165). Cells were lysed as described in A. Proteins were detected by immunoblotting with HA or FLAG mAbs.

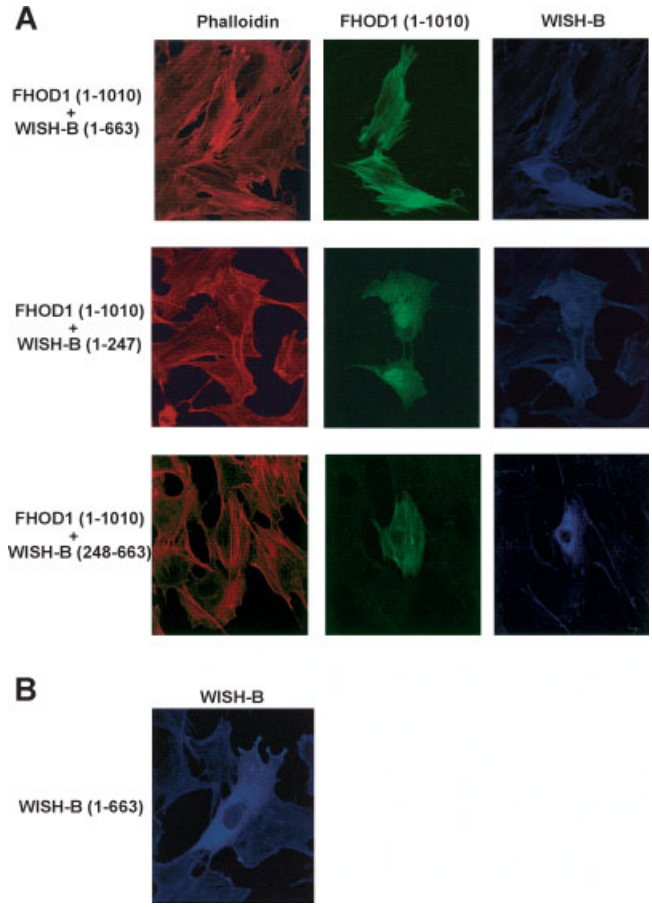

Fig. 5. FHOD1-induces stress fiber formation is blocked by interactions with the WISH-B amino-terminus. **A**: NIH3T3 cells were co-transfected with expression plasmids for HA-FHOD1 (1–1010) and the indicated FLAG-WISH-B protein. Cells expressing both proteins were identified by immunofluorescence (FHOD1 (1–1010), FITC; WISH-B, Cy5) and actin filaments were detected with rhodamine-conjugated phalloidin. **B**: WISH-B does not induce stress fibers. NIH-3T3 cells were transfected with WISH-B (1–663). WISH-B positive cells and actin filaments were detected as described above.

FHOD1-Induced Transcriptional Activation of the Skeletal Actin Promoter Requires the SRF Binding Element

Activated FHOD1 proteins induce transcription of a reporter gene though multimerized SRE elements from the c-fos promoter [Westendorf, 2001]. This SRE contains binding sites for SRF and its heterodimeric binding partners, the ternary complex factors (TCFs), but in muscle-specific promoters, the SRE is composed of SRF and E-box binding elements [Paradis et al., 1996]. FHOD1 mRNA is expressed at high levels in the spleen and skeletal muscle [Westendorf et al., 1999; Tojo et al., 2003]. To determine if FHOD1 acts through the SRF or its binding partners, FHOD1 was tested for its ability to activate the proximal skeletal α-actin promoter (SkA) and SkA mutants that lack binding sites for SRF and other known transcriptional regulators (Fig. 6A) [Paradis et al., 1996]. In agreement with previously published data with the multimerized SRE [Westendorf, 2001], full-length FHOD1 (1–1165) did not activate the SkA promoter, but truncated proteins lacking amino- and carboxy-terminal self-interaction domains, FHOD1 (469–1165) and (1–1010), markedly stimulated transcription of the wild-type SkA promoter (Fig. 6B). These truncated FHOD1 proteins however only poorly activated transcription of a mutant SkA promoter lacking the SRF binding element (Fig. 6C). Other SkA promoter mutants lacking binding sites for TEF, E-box proteins or Sp1 were activated to similar or greater levels by the truncated FHOD1 proteins as compared to the wild-type promoter (Fig. 6C,D). TATA box mutations decreased the transcriptional activity of the promoter, consistent with previous reports [Paradis et al., 1996]. These data

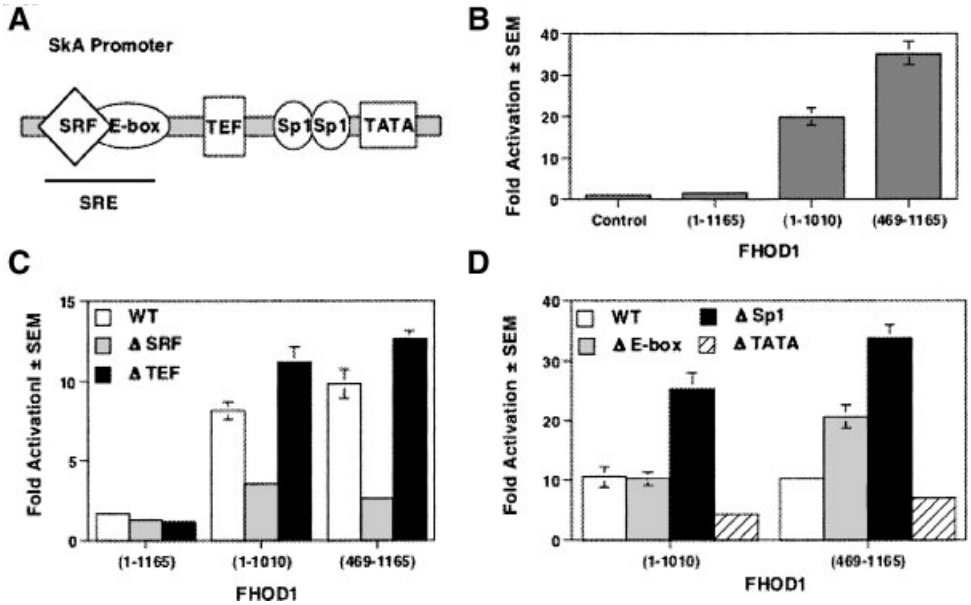

Fig. 6. FHOD1 induces transcription from the skeletal α-actin promoter (SkA). **A**: Model of the proximal SkA promoter and relative locations of transcription factor binding sites. **B**: FHOD1 activates the SkA promoter. C2C12 cells were co-transfected with SkA-Luc, CMV-SEAP, and the indicated FHOD1 protein. Luciferase activity was normalized to SEAP activity. Values represent the mean of triplicate samples ± the standard error of the mean (SEM). **C and D**: C2C12 cells were transfected as in B except mutant SkA promoter -Luc reporters were transfected as indicated. The activity of the wild-type promoter is shown with the white bars (C and D). The mutant SkA promoters lacked the binding sites for SRF (C, grey), TEF (C, black), E-box (D, grey) or Sp1 (D, black), or the TATA box (D, hatched).

demonstrate that active FHOD1 proteins stimulate SRF-dependent transcription of a muscle-specific promoter. Other FH proteins also affect SRF [Tominaga et al., 2000], thus SRF appears to be a common target of FH protein activation.

WISH-B Does not Affect FHOD1-Induced Transcription From the SkA Promoter

Because WISH-B interacts with FHOD1 and alters the solubility of transcriptionally active FHOD1 proteins, the effect of WISH-B on FHOD1-induced transcription of the SkA promoter was determined. WISH-B modestly inhibited the basal activity of the SkA promoter (Fig. 7). The WISH-B truncation mutant (1–247) that blocked FHOD1-induced stress fiber formation also suppressed the basal activity of this promoter. Although basal activity of the promoter was lower in the presence of either WISH-B protein, the FHOD1 proteins (469–1165) and (1–1010) retained their abilities to activate the SkA promoter 6–15 fold. These levels are similar to FHOD1-induced activation in the absence of WISH-B. Therefore, we conclude that WISH-B and WISH-B (1–247) do not prevent FHOD1 from activating the SkA promoter, even though they alter its subcellular location and the latter reduces its ability to induce stress fibers.

F-Actin Stabilization Inhibits FHOD1-Induced Transcription

The increasing evidence that SRF activity is dependent on actin dynamics [Sotiropoulos et al., 1999; Ellis et al., 2002; Schratt et al., 2002], led us to ask if FHOD1-dependent SRF activation was affected by F-actin stabilization. To test the role of actin dynamics, JSP or latrunculin B (LatB) were added to the cell culture medium prior to determining SkA activation in transcription assays. JSP binds to and stabilizes F-actin [Bubb et al., 1994], whereas LatB inhibits actin polymerization by sequestering G-actin monomers [Coue et al., 1987]. As expected [Sotiropoulos et al., 1999], JSP activated transcription of the SkA promoter in the presence of serum, while LatB inhibited it (Fig. 8A), indicating that serum-induced SRF activation is sensitive to high G-actin concentrations. Interestingly, FHOD1-dependent activation was sensitive to JSP, but not LatB (Fig. 8B) in the presence of 10% serum. In contrast, FHOD1-induced SRE activation was completely blocked by LatB in the presence of

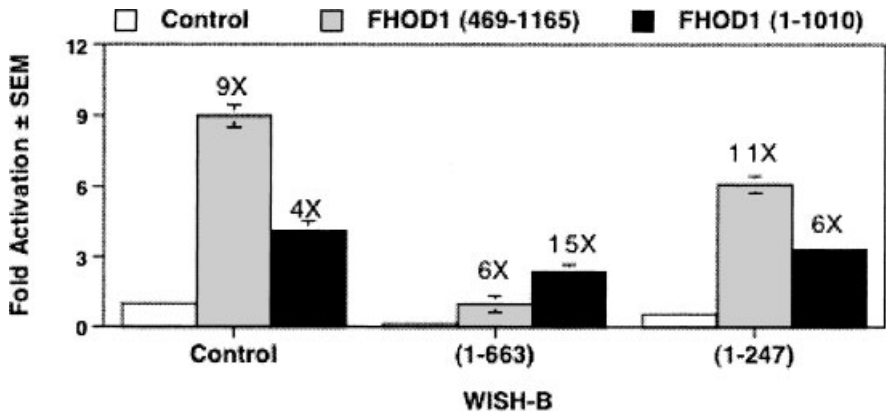

Fig. 7. WISH-B does not prevent FHOD1 from activating SkA. C2C12 cells were co-transfected with SkA-Luc, CMV-SEAP, and the indicated FHOD1 and WISH-B proteins. The numbers above the gray and black bars present fold activation by FHOD1 (469–1165) and (1–1010), respectively, over basal levels in the presence of the indicated WISH-B protein. The white bars in each group represent the effects of WISH-B and WISH-B (1–247) on the basal activity of the promoter. Luciferase activity was normalized to SEAP activity. Values represent the mean of triplicate samples ± SEM.

0.1% serum (Fig. 8C). Thus, FHOD1-dependent SRF activation is inhibited by F-actin stabilization in the presence of serum, and by G-actin sequestration in low serum. These results indicate that FHOD1 activity is differentially affected by actin dynamics depending on the culture conditions.

DISCUSSION

Formin proteins are often referred to as scaffolds for GTPases and other signaling molecules that coordinate the dynamics of the actin cytoskeleton [Evangelista et al., 2003; Wallar and Alberts, 2003]. FHOD1 is a diaphanous-related, FH domain-containing protein that regulates SRF transcriptional activity, actin organization, and cell migration [Westendorf et al., 1999; Westendorf, 2001; Gasteier et al., 2003; Koka et al., 2003; Takeya and Sumimoto, 2003]. Rac1 is the only known Rho GTPase family member that interacts with FHOD1. Rac1 activity is required for FHOD1-induced SRF activation, stress fiber formation, and its localization to actin filaments and lamellipodia [Westendorf, 2001; Gasteier et al., 2003; Koka et al., 2003]. To gain a better understanding of how FHOD1 organizes Rac1 and other cellular factors, we sought to identify FHOD1 interacting proteins. Three proteins were found to interact specifically with FHOD1. In this report, those interactions are defined and the effects of one of the proteins, WISH-B, on FHOD1 induced gene transcription and stress fiber formation are described.

WISH-B was identified in a yeast-two-hybrid screen as a FHOD1-interacting clone. It has high sequence similarity to AF3p21, SPIN90, DIP1, VIP54, and WISH [de Bernard et al., 2000; Sano et al., 2000; Fukuoka et al., 2001; Lim et al., 2001; Satoh and Tominaga, 2001]. These proteins share significant sequence identity but have divergent carboxy-termini. VIP54 also lacks the amino-terminal region common to the other proteins, but is otherwise identical to DIP1. These proteins thus appear to be isoforms of a single gene that may arise by alternative splicing. The purposes of these carboxy-terminal domains are not known. The FHOD1-interacting protein completely lacks the carboxy-terminal domain that is divergent in the other family members. Because it contains an SH3 domain and is similar to WISH, it was named WISH-B.

WISH-B contains a SH3 domain at its extreme amino-terminus. SH3 domains interact with proline-rich sequences. Because the cDNA clones identified in our yeast-two-hybrid screen contained the coding region for this domain, it was presumed to be important for interactions with the proline rich FH1 domain of FHOD1. Attempts to express the WISH-B SH3 domain in our biochemical assays were unsuccessful; therefore, we were unable to determine if this domain is sufficient to interact with FHOD1. The SH3 domain is not however necessary for the interaction. The PST-rich region following the SH3 domain was more important as it was required for interactions with FHOD1. WISH-B residues downstream of the

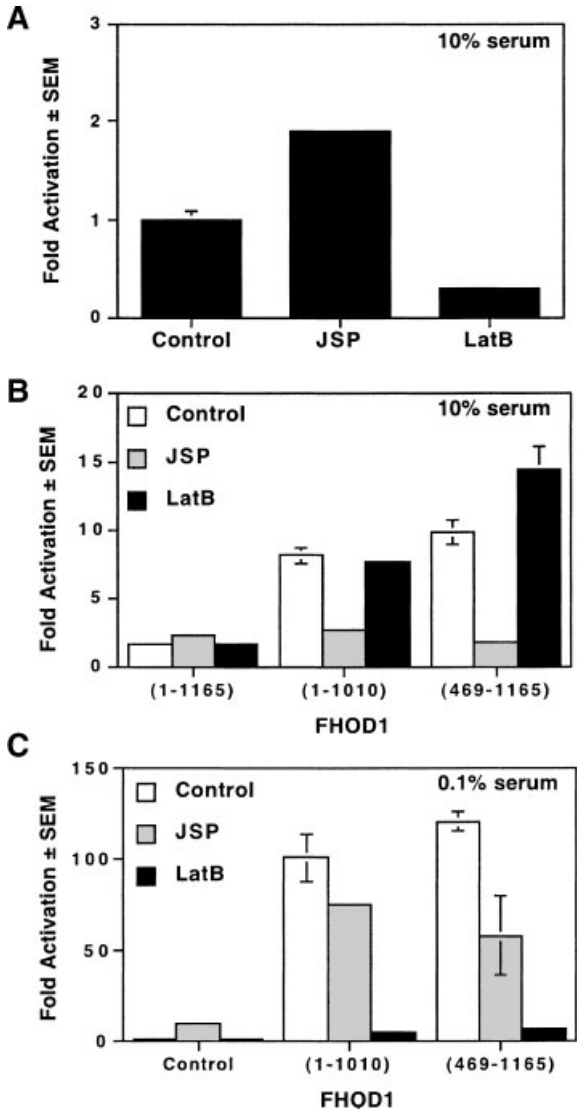

Fig. 8. FHOD1 activation of the SkA promoter is dependent on actin-organization and culture conditions. **A**: F-actin stabilization increases SkA promoter activity in presence of growth factors. COS cells were co-transfected with SkA-Luc and CMV-SEAP. JSP (1 μM) or LatB (1 μM) were added 18 h post-transfection. Cells were cultured for 40 h in medium containing 10% FBS. **B–C**: COS cells were treated as in A except they were also transfected with the indicated FHOD1 expression plasmid. Cell were cultured in either 10% FBS (B) or 0.1% FBS (C). Values represent the mean of triplicate samples ± SEM.

PST-domain were not sufficient to bind to FHOD1. These biochemical assays were supported in functional tests of stress fiber formation where the WISH-B amino-terminus altered FHOD1 localization and prevented stress fiber formation. Full-length WISH-B did not grossly affect FHOD1-induced stress fibers, but biochemical fractionation experiments demonstrate that WISH-B altered the solubility of FHOD1. Thus, WISH-B may influence the subcellular localization of FHOD1 or recruit it to larger protein complexes. While the WISH-B amino terminus interacts with FHOD1, the WISH-B carboxy-terminus may be responsible for tethering the FHOD1 to other proteins in the actin cytoskeleton. The redistribution of FHOD1 (1–1010) from stress fibers to the nucleus and diffuse cytoplasmic locations by the WISH-B protein lacking the carboxy-terminus (1–247) supports this model (Fig. 5).

FHOD1 is the second Drf to be described that interacts with a WISH-related protein. DIP1 interacted with mDia1 [Satoh and Tominaga, 2001]. Similar to our data with FHOD1 and WISH-B, mDia1 and DIP1 formed multiple contacts. The LRD of DIP1 interacted with the carboxy-terminus of mDia and prevented stress fiber formation. In contrast, only the amino-terminus of WISH-B interacted with FHOD1 and blocked FHOD1-induced stress fibers. Another notable difference is that the amino-terminus of mDia did not interact with DIP1, whereas the amino-terminus of FHOD1 interacts with WISH-B. It is possible that the extra residues in the DIP1 carboxy-terminus influence its interactions with Drfs. Alternatively, WISH-B/DIP1 may bridge interactions between FHOD1 and mDia1, and thereby integrate Rac1 and RhoA signaling. Further studies are necessary to finely elucidate the role of WISH/DIP1 in Drf signaling.

WISH interactions with Drfs are intriguing because both proteins regulate actin polymerization. WISH binds to N-WASP through its SH3 domain. WISH enhanced N-WASP -induced actin polymerization by activating the Arp2/3 complex independent of Cdc42 [Fukuoka et al., 2001]. The role of FHOD1 in actin polymerization remains to be determined; however, other formins promote actin nucleation in Arp2/3 independent manners [Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002]. It is possible that WISH isoforms are crucial crossroads for Arp2/3-dependent and -independent actin polymerization.

Activated forms of FHOD1 induce transcription of the SkA promoter and this activation is differentially sensitive to actin modifying drugs depending on culture conditions, but not to WISH-B expression (Figs. 7 and 8). Using the SkA promoter as a model, we show that FHOD1 induces SRE-dependent transcription via the

SRF and not through SRF-associated factors, TCF or E box proteins, as the SRF binding site was necessary for FHOD1-induced activation. Interestingly, in the presence of 10% serum, FHOD1-induced SRF activation is inhibited by the F-actin stabilizing drug, JSP, but not by the G-actin sequestering drug, Lat B. In contrast, when cells were cultured in low serum, FHOD1-dependent SRF activation was inhibited by G-actin sequestration, but not F-actin stabilization. Similarly, Lat B inhibited serum-induced SRF activation, indicating that this process is sensitive to high G-actin concentrations [Sotiropoulos et al., 1999]. Moreover, mDia1 controls SRF activity through its effects on actin polymerization [Copeland and Treisman, 2002]. Our results indicate that like other formin-related proteins, FHOD1-dependent SRF activation and gene transcription are intimately tied to actin polymerization events and are regulated by culture conditions. It is possible that activated FHOD1 fills an essential role in the absence of serum-derived growth factors, while FHOD1 has a more specialized function in the presence of serum. Additional studies are need however to understand these mechanisms.

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