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Activation of the Rac-binding Partner FHOD1 Induces Actin Stress Fibers via a ROCK-dependent Mechanism*

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Diaphanous related formins (DRFs) are part of the formin protein family that control morphogenesis, embryonic differentiation, cytokinesis, and cell polarity. DRFs organize the cytoskeleton in eukaryotic cells via the interaction with specific members of the Rho family of small GTPases including Rho, Rac, and Cdc42. This is best understood for Rho, which transmits signals to the actin cytoskeleton through the cooperation of its DRF effector mDia with ROCK (Rho-associated kinase). Here, we show that a constitutive active form of the Rac-interacting DRF FHOD1 (formin homology 2 domain containing 1) associates with F-actin in NIH3T3 cells, resulting in the formation of thick actin fibers. Cytoskeletal changes induced by FHOD1 correlated with the induction of serum response element transcription and were mediated by formin homology domains 1 and 2 of FHOD1. FHOD1-induced effects required the activity of the Rho-ROCK cascade that is targeted at a level downstream of Rho by the DRF. However, when the functional interaction of FHOD1 with individual GTPases was addressed, Rac but not Rho or Cdc42 bound to FHOD1 in cells and induced its recruitment to actin filaments and lamellipodia/membrane ruffles. Furthermore, activated FHOD1 interfered with lamellipodia formation. These results indicate that FHOD1 acts as an effector of Rac in actin rearrangements and transcriptional regulation and may provide a link for the Rac-dependent activation of the Rho cascade.

The dynamic regulation of the actin cytoskeleton is critical for central mechanisms of eukaryotic cells including polarization, division, motility, and adhesion. The overall polymerization status of cellular actin is regulated by multiple mechanisms including actin nucleation via Arp2/3 complex and inhibition of ADF-cofilin-induced depolymerization of actin filaments (1). Additionally, stabilization and/or bundling of actin filaments alter the turnover of actin, thereby affecting the balance between G- and F-actin pools (2). These events are primarily regulated by the small GTPases of the Rho family, including Rho, Rac, and Cdc42, which are key regulators of the transmission of exogenous stimulation to the inside of the cell. The activities of Rho, Rac, and Cdc42 GTPases are well understood because an array of distinct effector functions has been delineated, and specific reorganizations of the actin cytoskeleton upon individual activation of the GTPases have been established (3, 4). Whereas activation of Rho induces the formation of actin stress fibers, Rac and Cdc42 polymerize actin at the cell periphery, resulting in the formation of lamellipodia, membrane ruffles, and filopodia, respectively (5). It is generally assumed that actin reorganizations by Rac and Rho antagonize each other (6–9). In a model for cell motility, Rac-induced membrane ruffles create new contact sites that are subsequently matured by the action of Rho, thereby preventing the formation of new contacts by Rac. First evidence for this cross-talk between Rac and Rho was provided by the observation that the formation of lamellipodia upon activation of Rac is proceeded by the appearance of actin stress fibers (10). More recently, an opposing pathway leading to the activation of Rac by Rho was described (11). However, specific mediators for the control of Rho activity by Rac are missing, and the molecular mechanisms that allow transmission of GTPase signals to the actin cytoskeleton remain incompletely understood.

The diaphanous-related formins (DRFs)[1] have recently emerged as a group of proteins with the potential to bridge between G-protein signals and the cytoskeleton via their ability to bind activated small GTPases and to subsequently remodel the cytoskeleton (12, 13). DRFs are part of the formin protein family that control morphogenesis, embryonic differentiation, cytokinesis, and cell polarity (14). Formins are large modular molecules containing characteristic sequence motifs termed formin homology domains FH1, FH2, and FH3. Although the function of the FH2 and FH3 domains has remained elusive, the polyproline FH1 domain of some DRFs interacts with the actin-monomer-binding protein profilin and may thus provide a direct link to the actin cytoskeleton (15). Importantly, DRF-induced changes of actin polymerization correlate with transcriptional activation of certain cellular promotors such as the serum response element (SRE) (16, 17). As demonstrated for the interaction of activated mDia1 and mDia2 with Src, the FH1 domain also interacts with SH3 domains to regulate their effects on both the cytoskeleton and transcription (16). The activity of DRFs is regulated by an intramolecular interaction between the N-terminal GTPase-binding domain and the C-terminal Dia-autoregulatory domain that maintains the pro-

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[1] The abbreviations used are: DRF, diaphanous-related formin; FH, formin homology; SRE, serum response element; GST, glutathione *S*-transferase; GFP, green fluorescent protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; wt, wild type; GMP-PNP, guanylamide diphosphate; HA, hemagglutinin.

tein in an inactive form. Upon binding of an active GTPase to the GTPase-binding domain, autoinhibition is released (18).

Important insight into DRF function came from the characterization of the two yeast DRFs, Bni1p and Bnr1p, that synergize to assemble actin filaments resulting in cell polarization (19–23). Similar findings were made for the function of the mammalian DRFs mDia 1–3, because cooperative activation of the Rho effectors mDia1 and ROCK results in the formation of actin stress fibers (24, 25). Despite this overlap of functions of DRFs in yeast and mammals, recent work suggests that DRFs are not all functionally homologous. Individual specificity of DRFs for distinct GTPases likely regulates their activity. Originally, DRFs, such as mDia1, mDia2, and Bni1p, were defined as binding partners and downstream effectors of Rho, but mDia2 and Bni1p were found to interact also with Cdc42 to facilitate filopodia formation (15, 16, 26). It is becoming clear that specific GTPase-DRF pairs exist for distinct effects on actin remodeling, such as the regulation of endosome motility by RhoD via the Dia2C splice variant (27). The DRF Daam represents another variation of this theme by acting as upstream activator of Rho in the Wnt signaling cascade (28). For the Rac1 GTPase, the interaction with two formins, FRL and FHOD1 (previously known as FHOS (formin homologue overexpressed in spleen)) has been described, but their functional characterization awaits further characterization. FRL plays an as yet undefined role in regulating the actin cytoskeleton specifically in macrophages (29). FHOD1 was initially identified as an interaction partner of the AML-1B transcription factor (30). Subsequently, FHOD1 was shown to interact specifically with Rac1 *in vitro* and was implied in the activation of the SRE (31).

In this report, we demonstrate that FHOD1 is a specific interaction partner of Rac and mediates rearrangements of the actin cytoskeleton that correlate with activation of SRE transcription. A constitutive active form of FHOD1, lacking the C-terminal Dia-autoregulatory domain, associated with actin filaments to induce the formation of thick actin fibers in NIH3T3 fibroblasts and also interfered with lamellipodia formation by Rac. The association of FHOD1 with actin filaments correlated with the formation of actin fibers and an increase in cellular F-actin levels in an Arp2/3-independent manner. Because we found that FHOD1-actin fiber formation requires the activity of the Rho-ROCK cascade downstream of Rho, our data suggest FHOD1 as a cytoskeletal effector in the Rac-dependent activation of the Rho pathway that might play a role in lamellipodia formation and transcriptional regulation.

EXPERIMENTAL PROCEDURES

Cells and Reagents—NIH3T3 and HeLa cells were maintained in standard low or high glucose Dulbecco's modified Eagle's medium, respectively, complemented with 10% (v/v) fetal calf serum, glutamin, penicillin, and streptomycin. All medium components were purchased from Invitrogen. Mouse (F-7) or rabbit (Y-11) anti-HA antibodies were obtained from Santa Cruz Biotechnology. The anti-tubulin monoclonal antibody (B-5-1-2) as well as fluorescently labeled phalloidin were purchased from Sigma. Fluorescent secondary antibodies (Alexa488 or Alexa568) and Alexa660-conjugated phalloidin were obtained from Molecular Probes.

Expression Plasmids and GST Purification—Expression plasmids for GFP-tagged GTPases as well as the Arp2/3-interfering fragment of WASP were generous gifts of Michael Way (32). The expression plasmids for Arp3·GFP, C3 transferase, 5×SRE-Luc, ROCKΔ3, and RhoA-Myc were kindly provided by Dorothy Schafer (33), Art Alberts (16), Shuh Narumiya (24), and Stefan Offermans, respectively. pTk-*Renilla* was purchased from Clontech. The pCMV5-HA-FHOD1 full-length and HA-FHOD1(ΔC) expression plasmids were kindly provided by Jennifer Westendorf (30). The GST-RacL61 expression plasmid was a kind gift by Olivier Dorseuil, and the GST fusion constructs for the wild type GTPases were provided by Jean de Gunzberg. The respective fusion proteins were purified from BL21 *Escherichia coli* cells in the presence of 10 mM $MgCl_2$. All of the proteins prepared for binding analysis were subjected to SDS-PAGE and Coomassie Blue staining analysis. Deletions of the FH1 and FH2 domains in the full-length and ΔC constructs were introduced by ligating the *Afl*III/*Eco*RI-digested PCR amplicons into the *Nco*I/*Eco*RI sites of the EF-HA plink vector (34). All of the PCR products were generated using gene-specific oligonucleotides (MWG Biotech AG) and high fidelity polymerase (Roche Applied Science), and the correctness of the respective deletions was verified by sequencing in all clones.

Transfections and Immunofluorescence Microscopy—For transfection, NIH3T3 cells were kept in 10% FCS and plated onto glass coverslips overnight, subsequently transfected with a total of 1 μg of DNA using LipofectAMINE (Invitrogen) according to the manufacturer's instructions, and further processed 24–30 h post-transfection. For immunofluorescence, the cells were fixed with 3% paraformaldehyde (15 min at room temperature), except cells expressing GFP·GTPase fusion proteins, which were fixed with 3.7% formaldehyde. After permeabilization with PBS/0.1% Triton-X100 for 2 min, cells were blocked with PBS/1% BSA for 30min. For indirect immunofluorescence, primary antibodies were diluted 1:200 in PBS, 1% BSA and incubated for 45 min at room temperature. After four washing steps with PBS, the secondary antibodies were diluted 1: 1000 in PBS, 1% BSA and incubated for 45 min. Following extensive washing, the cells were mounted with Histogel (Linaris). For F-actin staining, paraformaldehyde fixed cells were treated with fluorescent phalloidin for 5 min and washed with PBS. The indirect fluorescence images were monitored with an Olympus 1 × 70 microscope and processed using Adobe Photoshop. Quantification of F-actin levels was achieved using Soft Imaging System Analysis (SIS) software. Pixel intensities of cells were determined from multiple representative cytoplasmic areas of FHOD1 expressing and directly neighboring control cells after subtraction of unspecific background. Average values from at least 75% of the entire cytoplasmic area of these cells were used to calculate the ratio of pixel intensity between transfected and control cells and used as measure for the relative F-actin levels of FHOD1-expressing cells. Statistical verification of the observed differences was ascertained with the Student's *t* test. For confocal analysis, the stained cells were examined using a confocal laser scanning microscope (Leica TCS-NT system, Leica) attached to a DM IRB inverted microscope with a PLAPO 63×1.32 oil immersion objective. Confocal images were collected as 512 × 512 pixel files and subsequently processed.

GTPase Binding Assay—24 h post-transfection, 4×10^6 wtFHOD1-expressing HeLa cells were lyzed in 50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 150 mM NaCl, and 1% Nonidet P-40. The cytoplasmic lysates were incubated overnight at 4 °C with 2 μg of GST or GST-RacL61 proteins immobilized on GSH-Sepharose beads (Amersham Biosciences) in lysis buffer supplemented with 2 mg/ml BSA (interaction buffer), respectively. For preloading of the GTPases, purified GST-Rac1, RhoA, and Cdc42 proteins (250 μM) were incubated with GMP-PNP (Roche Applied Science; 250 μM) in a buffer containing 50 mM Hepes, pH 7.6, 1 mM EDTA, and 0.2 mg/ml BSA for 30 min at 30 °C, before the addition of $MgCl_2$ to a final concentration of 10 mM. Binding assays using preloaded GMP-PNP GST-Rac1, -RhoA, and -Cdc42 proteins (10 μg) were carried out in the presence of 5 mM $MgCl_2$. The beads were washed three times in interaction buffer, and the bound cellular proteins were analyzed by Western blotting using the anti-HA antibody.

Drug Treatment—For the inhibition of ROCK, NIH3T3 cells were incubated for 30 min with 30 μM of the ROCK inhibitor Y-27632 (Calbiochem) 24 h post-transfection, fixed in paraformaldehyde, and subjected to immunohistochemical analysis. For exogenous Rho stimulation, the cells were incubated with lysophosphatidic acid (Sigma) (20 min, 5 μM).

Microinjection—Microinjection was performed with modifications as described (35). NIH3T3 cells grown on glass coverslips were cultivated in the presence of 0.1% FCS for 24 h prior to transfer to CO_2-independent medium (Invitrogen) and subsequent injection. Plasmids (10 μg/ml in 0.5× PBS) were injected with a microinjecton apparatus (AIS2 Microinjector, Cell Biology Trading) by using pulled borsolicate glass capillaries. At least 100 individual cells were injected per coverslip for each experiment. Unless specified otherwise, the cells were cultivated in the presence of 0.1% FCS for 6 h following injection prior to fixation and microscopic analysis.

Western Blotting—For Western blot analysis, transfected cells were lysed (50 mM Tris-HCl, pH 8, 1% Nonidet P-40, 150 mM NaCl for 1 h, 4 °C), and cleared lysates (10,000 × *g*, 10 min) corresponding to 5×10^5 cells/lane were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Protein detection was performed following

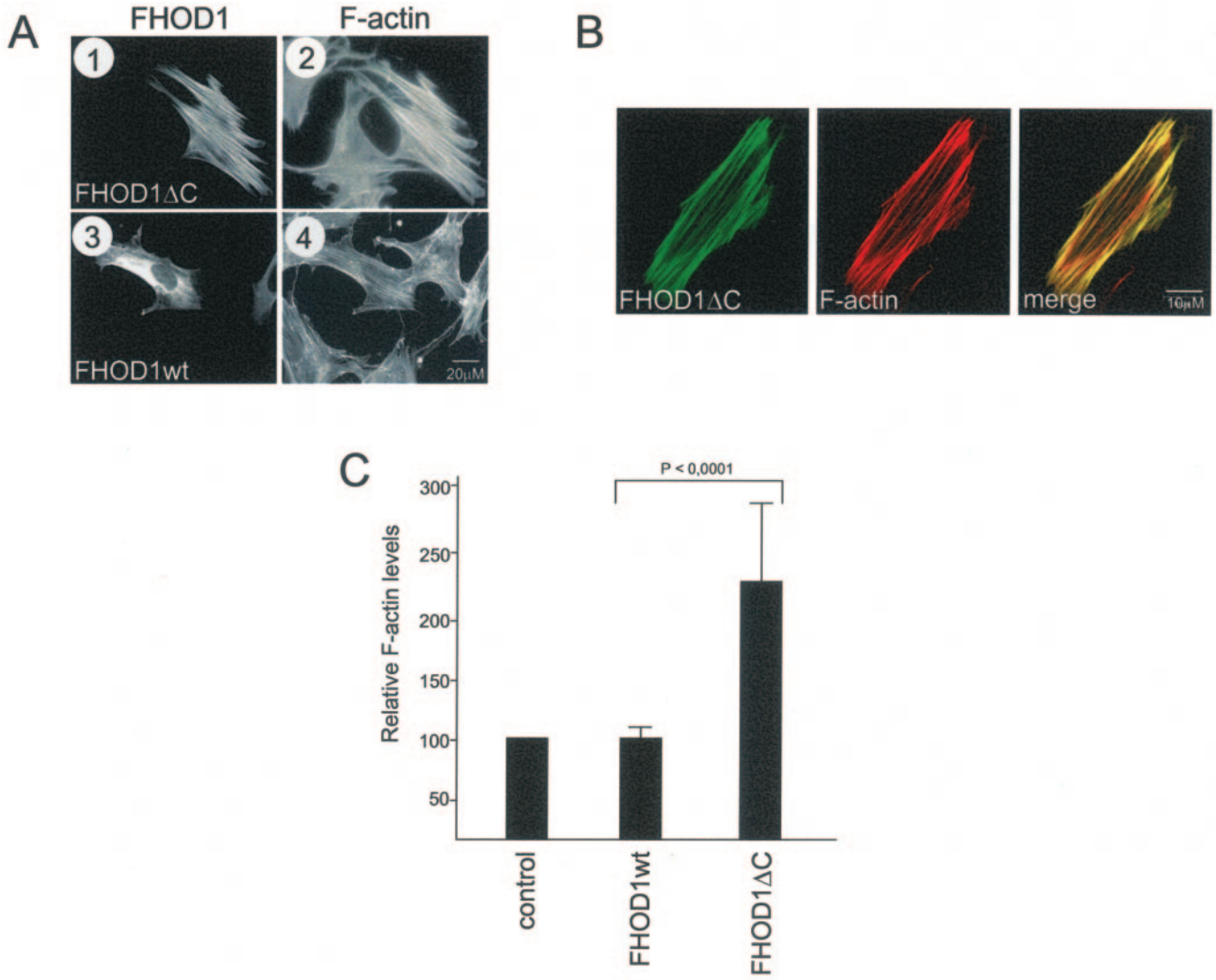

FIG. 1. **Activated FHOD1 causes the formation of thick actin-FHOD1 fibers.** *A*, subcellular localization of wild type (FHOD1wt) and active FHOD1 (FHOD1ΔC). NIH3T3 cells expressing FHOD1wt or FHOD1ΔC were stained for HA-FHOD1 and F-actin and analyzed by immunofluorescence. Cells with active FHOD1 form thick stress fibers associated with FHOD1 protein. *B*, confocal microscopy analysis of FHOD1-actin fibers in transfected NIH3T3 cells. Shown is one representative individual section. The *right panel* represents the merged picture. *C*, to quantify F-actin levels, pixel intensities of tetramethylrhodamine isothiocyanate-phalloidin-stained cells were measured using Soft Imaging System Analysis software, and the ratio of F-actin pixel intensities between FHOD1-expressing cells and untransfected neighboring cells was generated as measure for the relative F-actin levels of FHOD1-expressing cells. The average relative F-actin levels with the corresponding standard error of the mean for at least 30 cells are plotted with the *p* value from Student's *t* test analysis.

incubation with appropriate first and secondary antibodies using the Super Signal detection kit (Pierce) according to the manufacturer's instructions.

SRE Transcription Assay—To quantify activation of the SRE by FHOD1, 2×10^4 NIH3T3 cells were transfected with 1.3 μg of FHOD1 expression vector, 0.2 μg of pTK-*Renilla* encoding for *Renilla* luciferase to normalize for transfection efficiency, and 0.5 μg of the 5×SRE-Luc reporter plasmid. 24 h post-transfection, cell culture medium was changed to starve cells at 0.1% FCS, and the cells were harvested 48 h post-transfection. Luciferase activity was determined using the dual luciferase reporter assay system kit (Promega) with a Luminoskan Ascent luminometer (Thermo Labsystems). SRE firefly luciferase counts were normalized to the activity of the *Renilla* luciferase internal control and calculated as fold transactivation with the counts for FHOD1wt arbitrarily set to 1.

GTPase Activation Assay—Rho activity was quantified by measuring the amounts of Rho precipitated in a pull-down reaction from cell lysates with the GTPase-binding domain of Rhotekin as GST bait using the Rho activation assay biochemistry kit (Cytoskeleton) following the manufacturer's instructions. Briefly, 24 h post-transfection, 2×10^7 HeLa cells transiently co-expressing RhoA and FHOD1wt or FHOD1ΔC were serum-starved for 12 h, washed twice with ice-cold PBS, and collected. Following lysis, the cleared lysates were split into three equal aliquots. As negative and positive controls for the pull down, two aliquots were adjusted to 200 μM GDP or GTPγS, respectively, and incubated for 15 min at 30 °C to deplete or enrich Rho-GTP, whereas one aliquot remained untreated. Following the pull-down reaction and extensive washing, the precipitates and whole cell lysates were analyzed by Western blotting for the amounts of Rho.

RESULTS

Activated FHOD1 Induces the Formation of and Associates with Thick Actin Fibers—Given that formins are generally thought of as cytoskeletal organizers, we investigated whether the Rac-binding partner FHOD1 can affect cytoskeletal architecture. Like other formins, the C terminus of FHOD1 contains a Dia-autoregulatory domain autoinhibitory domain, and deletion of this domain results in a constitutive active molecule (31). NIH3T3 cells were therefore transfected with constructs directing the expression of the HA epitope-tagged wild type FHOD1 protein (FHOD1wt) or a FHOD1 mutant lacking the last 156 amino acids (FHOD1ΔC), and their subcellular localization and the F-actin organization were analyzed by fluorescence microscopy (Fig. 1*A*). Interestingly, the subcellular local-

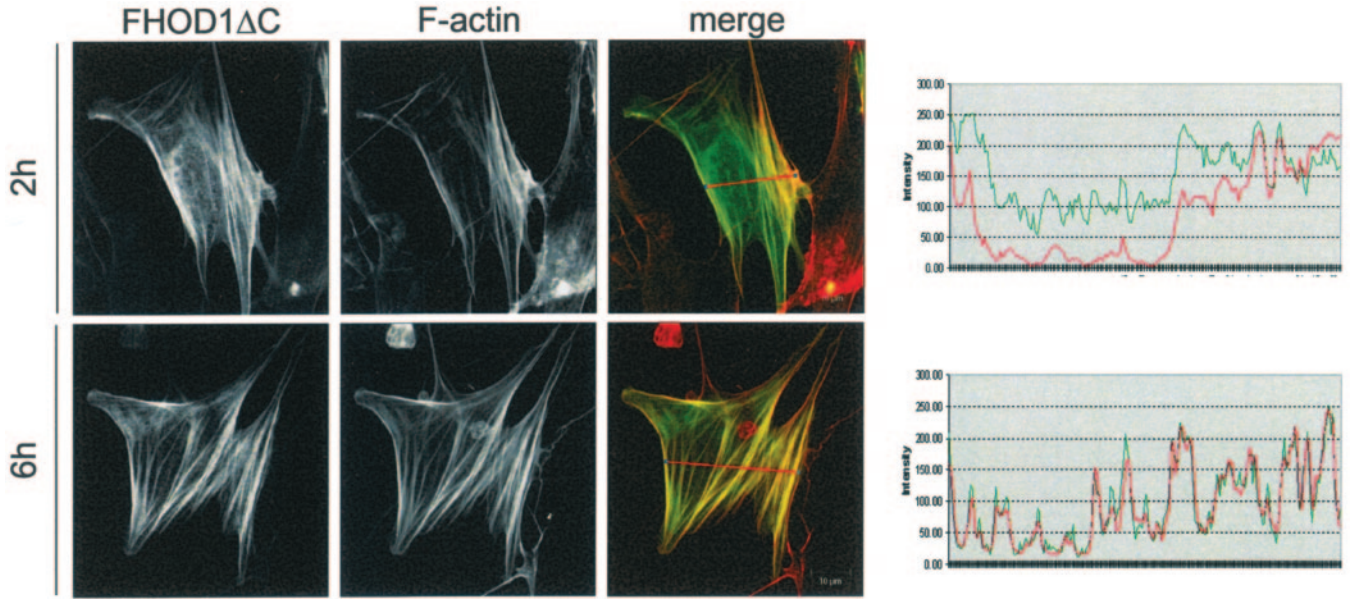

FIG. 2. **FHOD-actin fiber formation coincides with F-actin association of FHOD1.** NIH3T3 cells were microinjected with an expression plasmid for active FHOD1 (FHOD1ΔC), fixed after the indicated times, stained for FHOD1 and F-actin, and analyzed by confocal microscopy. Shown are immunomicrographs of individual sections representative for three independent experiments. On the *right*, fluorescence intensities measured according to pixel brightness were quantified along representative cell transects indicated by the *line* in the merge pictures and are depicted in an overlay of actin (*red curves*) with FHOD1ΔC (*green curves*).

ization and effects on the actin cytoskeleton of FHOD1wt and FHOD1ΔC proteins were found to be significantly different. FHOD1wt showed a diffuse cytoplasmic pattern (*panel 3*). In cells with pronounced membrane ruffles, FHODwt was also found at the cell periphery (data not shown). Similarly to nontransfected cells, the cells expressing FHOD1wt displayed only low amounts of thin actin filaments without particular orientation and co-localization with the DRF (*panel 4*). In contrast, FHOD1ΔC was exclusively localized to thick filamentous structures reminiscent of actin stress fibers that were aligned in parallel alongside the long axis of transfected cells (*panel 1*). In contrast to nontransfected control cells, prominent actin stress fibers were observed in the presence of FHOD1ΔC (*panel 2*). To verify that active FHOD1 associates with filamentous actin, confocal microscopy analysis was performed on FHOD1ΔC-expressing cells. As demonstrated in Fig. 1*B*, strong co-localization of FHOD1ΔC with actin fibers was observed. Together, these results indicate that activation of FHOD1 caused the formation of thick actin fibers. In agreement with these findings, FHOD1ΔC was mostly associated with the detergent-insoluble cell fraction, whereas FHOD1wt was strictly detergent-soluble (data not shown). The formation of stress fibers can result from bundling of pre-existing actin filament or from *de novo* formation of actin fibers. To assay for an involvement of actin polymerization in FHOD1-actin fiber formation, the fluorescence intensity of F-actin stains from FHOD1ΔC-expressing cells were compared with that of neighboring cells (*i.e.* as in Fig. 1*A*, *panels 2* and *4*), and the ratio of the two values was used to plot the changes in relative F-actin levels (Fig. 1*C*). Although expression of FHOD1wt did not markedly affect the amounts of F-actin levels as compared with control cells transfected with an empty plasmid (average, 100 ± 16.5%; $n = 30$), relative amounts of F-actin were significantly increased by the presence of FHOD1ΔC (average, 251 ± 64.1; $n = 30$). Thus, the formation of stress fibers by active FHOD1 involves the elevation of actin polymerization in cells.

FHOD1-Actin Fiber Formation Coincides with Actin Association of the DRF—To gain further insights into FHOD1-actin fiber formation, we compared the kinetics of fiber formation and actin association of FHOD1ΔC in cells. Serum-starved NIH3T3 cells were microinjected with the FHOD1ΔC expression plasmid, stained for F-actin and FHOD1, and analyzed by confocal microscopy (Fig. 2). When the distribution of FHOD1ΔC was monitored over time after microinjection, we found that in the early phase of expression (2 h post-injection), the majority of the protein was not associated with actin filaments but rather diffusely localized in the cytoplasm (*upper panels*). Importantly, only a few thickened actin fibers, already decorated by FHOD1ΔC, were detectable 2 h post-injection. Over time, the distribution changed toward the exclusive association of FHOD1ΔC with polymerized actin (*lower panels*). Formation of thick actin fibers occurred solely upon decoration of actin filaments by FHOD1ΔC. Quantification of the kinetics of the association of FHOD1 with F-actin and stress fiber formation in three independent experiments revealed that only about 5% of all cells displayed significant stress fiber formation and F-actin association of FHOD1ΔC 2 h post-injection. In contrast, at 6 h post-injection, FHOD1ΔC was exclusively found associated with thick actin fibers in more than 75% of all injected cells. These results indicate that the association of FHOD1 with F-actin occurs simultaneously with the formation of marked actin fibers, suggesting that FHOD1 actively causes the formation of these structures.

Arp2/3 Is Not Involved in the Formation of FHOD1-Actin Fibers—Yeast DRFs cause the formation of actin cables without *de novo* actin nucleation via Arp2/3 complex (22). Thus, we investigated whether the formation of FHOD1-actin fibers also occurred independently of Arp2/3. First, we used an Arp3·GFP fusion protein to test whether Arp2/3 complex is recruited to sites of FHOD1-actin fiber formation (Fig. 3*A*). No effect on the cellular distribution of Arp3·GFP by the expression of FHOD1ΔC could be detected (compare *panels 2* and *3*). Furthermore, Arp3·GFP did not localize to FHOD1-induced actin fibers (*panels 1* and *2*). Second, we sought to interfere with the activation of Arp2/3 by Wasp-like proteins. Arp2/3 activation can be inhibited by overexpression of the C-terminal effector domain (WA) of Wasp (32, 36, 37). When we co-expressed the WA·GFP fusion protein with active FHOD1, actin fiber forma-

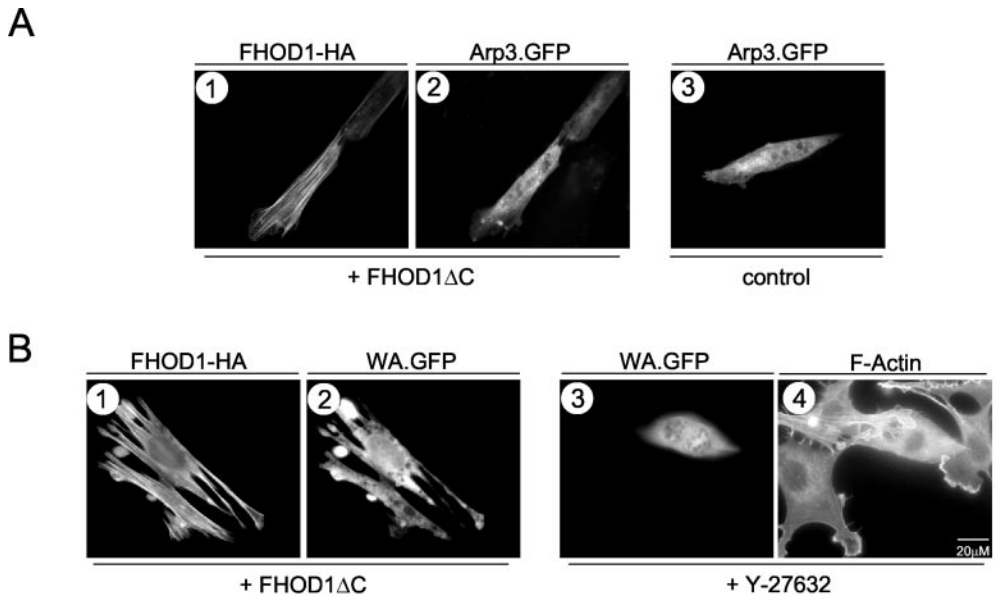

FIG. 3. **FHOD1-actin fibers are formed independently of Arp2/3 complex.** *A*, Arp3·GFP was expressed in NIH3T3 cells in combination with FHOD1ΔC (*panels 1* and *2*) or an empty control plasmid (*panel 3*). In *panel 1*, HA-FHOD1ΔC was analyzed by indirect immunofluorescence with anti-HA; *panels 2* and *3* show direct fluorescence of Arp3·GFP. *B*, the inhibitory domain of WASP (*WA.GFP*) was expressed in NIH3T3 cells in combination with FHOD1ΔC (*panels 1* and *2*) or an empty control plasmid (*panels 3* and *4*). In *panel 1*, HA-FHOD1ΔC was analyzed by indirect immunofluorescence with anti-HA; *panels 2* and *3* show direct fluorescence of WA·GFP; and *panel 4* represents an F-actin stain. The cells in *panels 3* and *4* were treated with the ROCK Inhibitor Y-27632 to induce lamellipodia and membrane ruffles.

tion and association of FHOD1ΔC were not affected (Fig. 3*B*, *panels 1* and *2*). In control cells, expression of WA·GFP alone did not cause the formation of stress fibers and significantly reduced lamellipodia formation induced by the ROCK inhibitor Y-27632 (11), indicating efficient inhibition of Arp2/3 (Fig. 3*B*, *panels 3* and *4*). Together, these results suggest that Arp2/3-mediated *de novo* polymerization of actin is not required for the cytoskeletal effects of FHOD1.

FH1 and FH2 Domains Are Required for the Effects of FHOD1 on the Actin Cytoskeleton and SRE Transcription—To characterize the molecular determinants of FHOD1 involved in actin rearrangements, the requirement for the FH1 and FH2 signature domains was investigated. As schematically outlined in Fig. 4*A*, deletions of these domains were introduced in the wild type and active FHOD1, and these mutated proteins were expressed in NIH3T3 cells to comparable levels (Fig. 4*B*). When tested for the effects on F-actin morphology in NIH3T3 cells, deletion of the FH1 and FH2 domains neither altered the distribution of FHOD1wt within the cytosol nor affected the actin cytoskeleton (Fig. 5*A*, *panels 1–6*). However, when the FH2 deletion was introduced in the context of activated FHOD1 (ΔCΔFH2), both the formation of actin fibers and the association of FHOD1 with polymerized actin were prevented (*panels 11* and *12*). In contrast, deletion of the FH1 domain from activated FHOD1 (ΔCΔFH1) still allowed the association of the DRF with actin filaments, but the formation of thick actin fibers was abrogated (*panels 9* and *10*). Thus, the FH2 domain is required for the association of FHOD1 with actin, whereas the FH1 domain is essential for the formation of FHOD1-actin fibers. Because DRF-induced actin rearrangements are thought to trigger transcriptional activation of the SRE element (16, 17), we next tested whether cytoskeletal rearrangements by FHOD1 correlate with its ability to induce SRE transcription (Fig. 5*B*). Although expression of FHOD1ΔC resulted in a 15-fold increase in SRE activity, all FHOD1wt variants and FHOD1ΔC lacking the FH1 or FH2 domains, respectively, failed to cause SRE activation. We conclude that intact FH1 and FH2 domains are simultaneously required for the cytoskeletal effects of FHOD1 and that both domains exert separable activities in the process. Furthermore, induction of FHOD1-actin fibers correlates with transcriptional activation of the SRE element by the DRF.

FHOD1 Is Responsive to and Associates with Rac—DRFs are regulated via their interaction with specific GTPases of the Rho family. To delineate the pathway that governs the activity of FHOD1, we tested which small GTPase would affect the subcellular localization and activity of FHOD1wt in NIH3T3 cells (Fig. 6*A*). Constitutive active Rho, Rac, or Cdc42 GTPases fused to GFP were co-expressed with FHOD1wt. Surprisingly, FHOD1 did not localize to actin stress fibers induced by activated Rho (*panels 3* and *7*), and identical results were obtained upon induction of stress fibers with the Rho activator lysophosphatidic acid (*panels 4* and *8*). In some cells, a faint recruitment of FHOD1 to actin fibers was observed at the cell periphery. Expression of activated Cdc42 (*panels 2* and *6*) or treatment with bradykinin (data not shown) led to the induction of microspikes but had also no effect on the subcellular localization of FHOD1. In sharp contrast, activation of Rac either by expression of RacL61 (*panels 1* and *5*) or treatment with PDGF (data not shown) recruited FHOD1 to filamentous actin. In particular, the DRF was found to be recruited into membrane ruffles and lamellipodia but also associated with actin fibers. Thus, in these experiments, FHOD1wt mirrors endogenous FHOD1 that is also recruited by activated Rac (Ref. 31 and data not shown). These filaments were clearly distinct from the thick actin fibers induced by the active FHOD1ΔC mutant, and a significant portion of FHOD1wt was still found diffusely in the cytoplasm. To correlate these observations with the association of FHOD1 with a specific GTPase, pull-down experiments from FHOD1-expressing cells were performed using recombinant GST-GTPase fusion proteins loaded with the nonhydrolyzable GMP-PNP GTP analogue (Fig. 6*B*). Identical results were obtained without prior GTP loading (data not shown). Consistent with our recruitment results and previous *in vitro* binding data (31), FHOD1wt efficiently interacted with Rac but not Rho and Cdc42. Interestingly, FHOD1ΔC associated neither with Rac nor with RhoA and Cdc42, indicating that, like other DRFs, upon removal of their GTPase binding

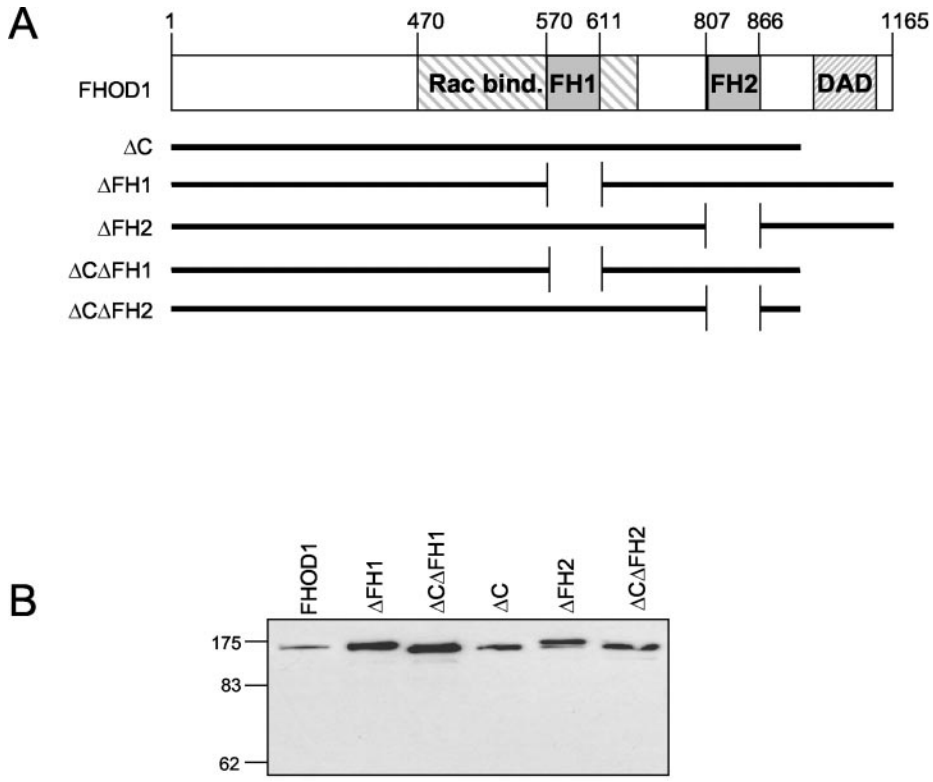

FIG. 4. **FHOD1 constructs used.** *A*, schematic representation of the FHOD1 mutants used. *B*, Western blot analysis of the expression levels of the respective proteins in transfected NIH3T3 cells.

domain, deletion of the C terminus of FHOD1 induces a deregulated conformation in which the DRF no longer interacts with the GTPase (16, 21, 24). At the same time, these results exclude that stress fiber formation by FHOD1ΔC results from the titration of endogenous Rac protein. Control experiments demonstrating the specificity of these pull-down experiments are shown in Fig. 6*C*. Rac and Cdc42 interacted with its effector Pak2 (*left panel*), whereas Rho specifically associated with its effector ROCK (*right panel*). Thus, FHOD1 specifically associates with and is recruited by Rac in cells.

A Role for FHOD1 in Rac-mediated Lamellipodia Formation—Next, we analyzed the requirements in FHOD1 for Rac-mediated recruitment into membrane ruffles by co-expression of various FHOD1 mutants in combination with RacL61 (Fig. 7*A*). Wild type and activated FHOD1 proteins were similarly recruited into membrane ruffles by RacL61, and the FH1 and FH2 domains were both dispensable for the recruitment of FHOD1. In contrast, deletion of the FH2 domain abrogated the association of wt and ΔC FHOD1 with actin filaments in the presence of RacL61 (*panels 3* and *6*). Of note, the recruitment of FHOD1ΔC into membrane ruffles by RacL61 did not affect stress fiber formation but markedly reduced effects of active FHOD1 on cell morphology. Lamellipodia formation was markedly reduced in cells expressing activated FHOD1ΔC. Whereas over 90% of all cells expressing RacL61 displayed prominent lamellipodia, this percentage was reduced to about 35% by the co-expression of FHOD1ΔC, and deletion of either the FH1 or FH2 domain abrogated this effect (Fig. 7*B*). The responsiveness of FHOD1ΔC to active Rac did not correlate with the ability of FHOD1 to associate with the GTPase (Fig. 7*C*). As before, the Rac-FHOD1 interaction was readily detected for FHOD1wt and was not affected by the deletion of the FH2 domain. In contrast, the FH1 deletion significantly reduced the affinity of FHOD1 for Rac. This finding is consistent with the mapping by Westendorf that suggested that the Rac-binding site in FHOD1 partially overlaps with the FH1 domain (31). As for FHOD1ΔC, all other mutants lacking the C-terminal Dia-autoregulatory domain failed to bind to Rac, confirming that FHOD1 no longer associates with the GTPase in its active conformation. Thus, expression of active FHOD1 interfered with Rac-induced lamellipodia formation.

FHOD1 Induces Stress Fibers via the Rho-ROCK Cascade—The formation of thick actin stress fibers by active FHOD1 suggested that activation of the DRF induces the Rho cascade. In fact, co-expression of the Rho inhibitor C3 transferase completely prevented stress fiber formation by FHOD1ΔC (Fig. 8*A*, *panels 3* and *7*). Similarly, inhibition of the ROCK kinase, the major protein involved in actin bundling in the Rho pathway, with the specific inhibitor Y-27632 (*panels 4* and *8*) completely abrogated stress fiber formation by FHOD1ΔC. Of note, formation of membrane ruffles and lamellipodia caused by the activation of Rac upon inhibition of ROCK (11) was delayed in FHODΔC-expressing cells. In contrast, FHOD1ΔC-induced stress fiber formation was independent of the activity of Rac, because this effect was not perturbed by expression of the dominant negative RacN17 (*panels 2* and *6*). These results demonstrate that FHOD1ΔC-mediated stress fiber formation depends on the activity of the Rho-ROCK cascade, suggesting that FHOD1ΔC acts via the activation of this pathway. However, FHOD1 was not recruited to Rho-induced stress fibers and did not interact with Rho (Fig. 6). To test whether FHOD1ΔC would cause activation of Rho resulting in the observed phenotypes, the levels of active Rho were assayed in transiently FHOD1-expressing HeLa cells using a Rho activity pull-down assay with the GTPase-binding domain of Rhotekin as a bait for GTP-loaded Rho (Fig. 8*B*). As positive and negative controls, HeLa cell lysates were incubated with either GTP to ensure GTP loading of Rho or GDP to completely deplete the lysate of Rho-GTP, respectively. As expected, GTP loading resulted in robust amounts of Rho-GTP in the pull-down reaction (*lane 2*), whereas no Rho protein could be detected after pull-down from GDP-treated or untreated cell lysates (*lane 1*). Importantly, expression of FHOD1ΔC did not result in any detectable increase in Rho activity (*lane 4*), despite the presence of pronounced FHOD-actin fibers in these cells (data not shown). Similarly, expression of FHOD1wt did not affect the activity of

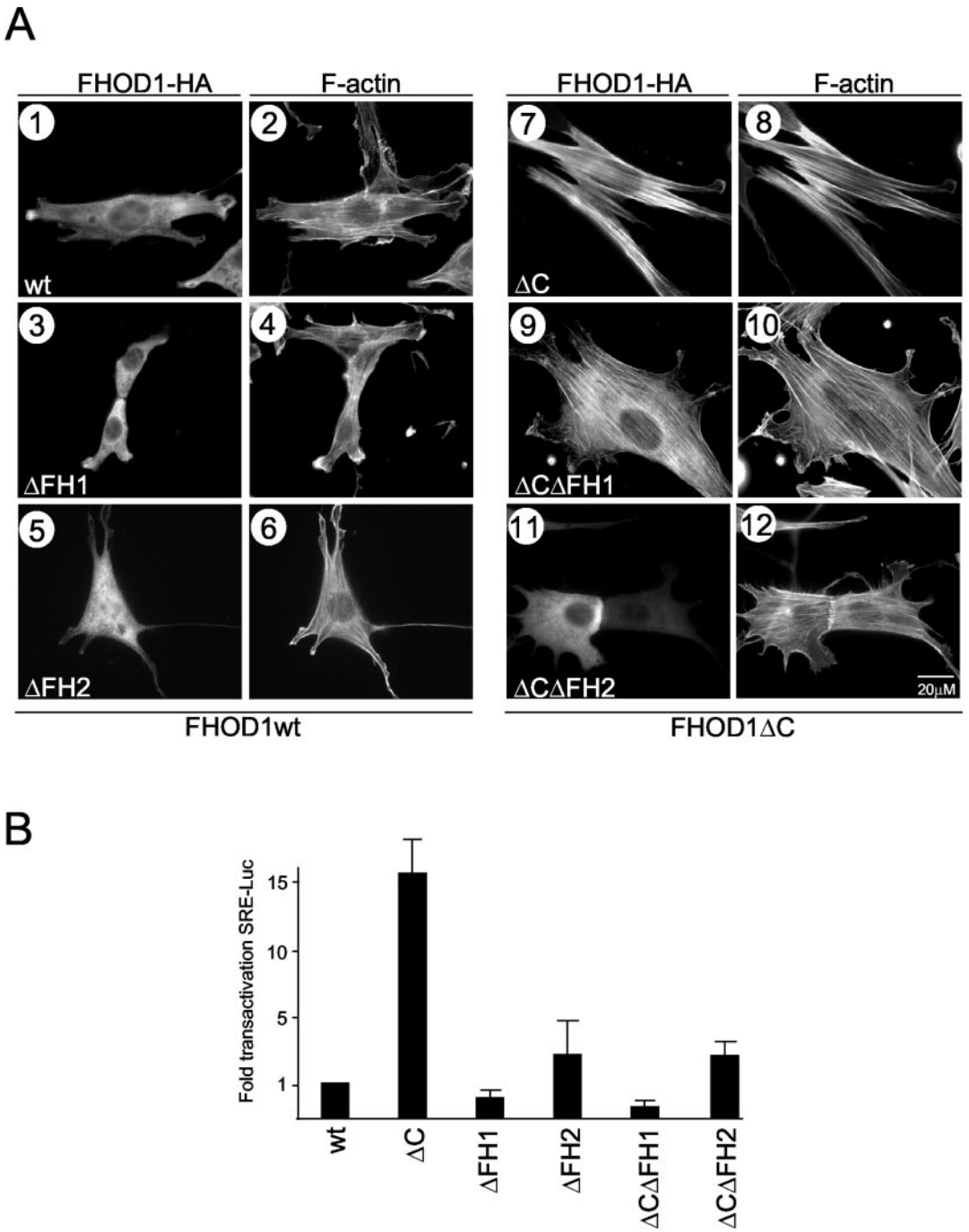

FIG. 5. **The FH1 and FH2 domains of FHOD1 are required for formation of FHOD1-actin fibers and SRE activation.** *A*, subcellular localization and effects on the actin cytoskeleton of the various FHOD1 mutants. NIH3T3 cells were transfected with the indicated FHOD1 expression plasmids. Following fixation, the cells were stained for HA-FHOD1 and F-actin and analyzed by immunofluorescence microscopy. *B*, SRE luciferase reporter assay. Shown are fold transactivation of the SRE luciferase reporter in NIH3T3 cells expressing the indicated FHOD1 variants. Luciferase activity for FHOD1wt-expressing cells was arbitrarily set to 1. Presented are average values from at least three independent experiments with the indicated standard error of the mean.

Rho in these experiments (*lane 3*). Comparable results were obtained by pull-down assays for the activity of the Rac1 GTPase that was also unaffected by the expression of the various FHOD1 constructs (data not shown). We conclude that FHOD1 does not activate Rho but rather acts downstream of the GTPase to induce actin stress fibers.

Given that the formation of FHOD1-actin fibers was inhibited by Y-27632, we reasoned that FHOD1 might act at the level of the Rho effector ROCK. In fact, when the constitutive active form of ROCK (ROCKΔ3) was co-expressed with FHOD1, some of the DRF was recruited to the characteristic star-like actin fibers (Fig. 8*C*, *panels 1* and *3*). Like the recruitment by RacL61, the FH2 domain was essential for the tethering of FHOD1 to actin by ROCK (data not shown). Therefore, ROCK was identified as a second molecule besides Rac that triggers the association of FHOD1 with F-actin. Interestingly, when FHOD1wt was co-expressed with ROCKΔ3 and RacL61, the DRF was recruited to both star-like actin bundles and membrane ruffles (*panels 2* and *4*). Similarly, co-expression of RacL61 and FHOD1wt together with C3 or Y-27632 treatment resulted in recruitment of the DRF into membrane ruffles but not to F-actin in the cytoplasm (data not shown). This indicates

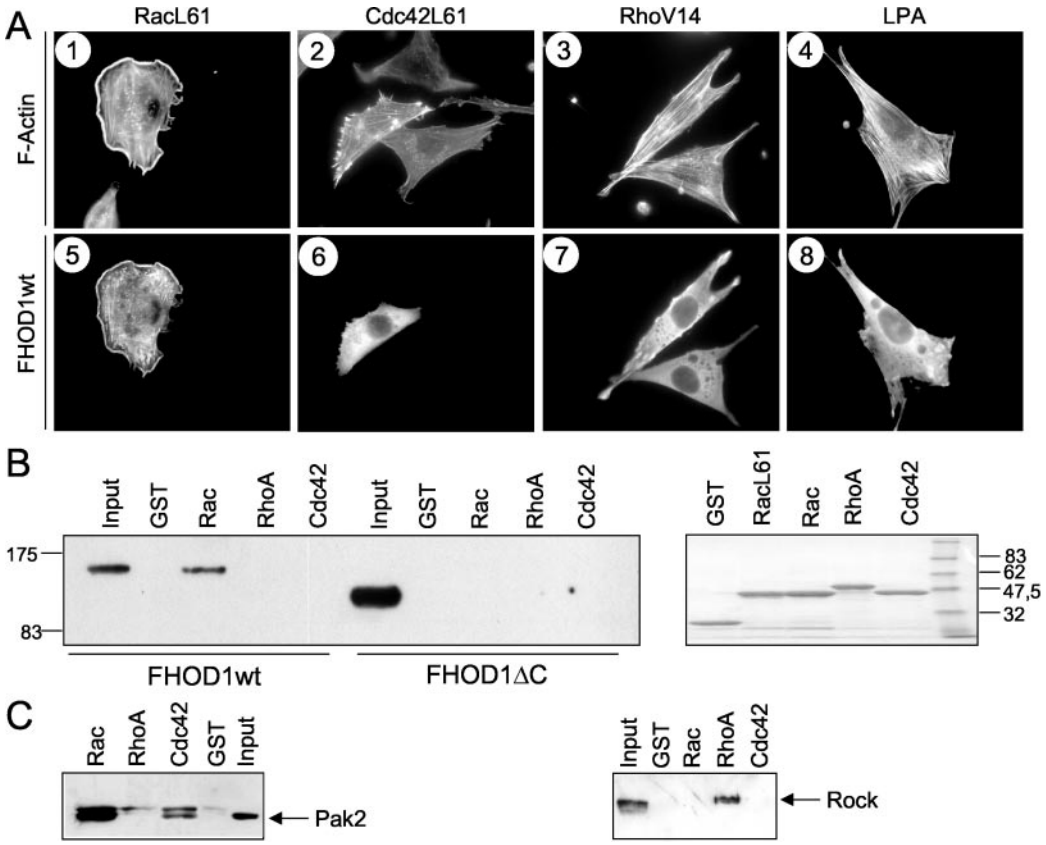

FIG. 6. **FHOD1 is specifically recruited by and binds to Rac in cells.** *A*, NIH3T3 cells kept in 0.1% FCS were microinjected with the expression plasmids for FHOD1wt together with expression plasmids for active Rac (*panels 1* and *5*), Cdc42 (*panels 2* and *6*) and Rho (*panels 3* and *7*), or an empty vector control (*panels 4* and *8*). Expression of the GTPases was verified by GFP positivity of microinjected cells (not shown). For *panels 4* and *8*, the cells were treated with lysophosphatidic acid to induce the Rho cascade. The subcellular distribution of F-actin (*panels 1–4*) was visualized with Alexa660-conjugated phalloidin and FHOD1wt with Alexa568-conjugated anti-HA antibodies. *B*, FHOD1 interacts with Rac in cells. Cytoplasmic lysates from HeLa cells expressing HA-FHOD1wt were incubated with equal amounts of purified GST or the indicated GMP-PNP loaded GST-GTPase fusion proteins immobilized on GST-agarose beads. Bound proteins were resolved by SDS-PAGE, and the association of FHOD1wt was revealed by Western blotting with anti-HA. 10% of the unfractionated cell lysates were run as input controls for FHOD1. The *right panel* shows a Coomassie-stained gel as input control for the GST fusion proteins. *C*, anti-Pak2 (*left panel*) and anti-ROCK (*right panel*) Western blots from control pull-down experiments from HeLa cell lysates with the indicated GST-GTPase proteins showing the specificity of the GTPases for distinct effectors.

that distinct cellular pools of FHOD1 are responsive to the recruitment by Rac and ROCK. We conclude that ROCK activity is likely a target of FHOD1ΔC in the Rho cascade for the induction of FHOD1-actin stress fibers.

DISCUSSION

This study describes that activation of the DRF FHOD1 by removal of the autoinhibitory C terminus induces drastic rearrangements of actin filaments, resulting in the formation of thick actin fibers. In contrast to other cytoskeleton-regulating formins, active FHOD1 decorates these actin fibers, and the kinetic analysis suggests that this association is instrumental for fiber formation. FHOD1-induced changes in cytoskeletal architecture are dependent on the signature FH1 and FH2 domains and correlate with the induction of SRE transcription. Notably, the formation of FHOD1-actin fibers likely occurs independently of *de novo* nucleation by Arp2/3 complex but is paralleled by an increase in total cellular F-actin levels. Thus, the FHOD1 phenotype possibly results from intrinsic nucleation and subsequent bundling of actin filaments into thick fibers. These effects of FHOD1 on actin organization are a classical Rho phenotype and also require the activity of the Rho-ROCK cascade. While this manuscript was under review, similar findings on ROCK-dependent formation of actin stress fibers by deregulated FHOD1 were reported by Koka *et al.* (38). Our analyses indicate that this activation occurs downstream of Rho, possibly at the level of ROCK. However, several lines of evidence identify FHOD1 as a specific effector of Rac. First, FHOD1 associates with Rac but not Rho or Cdc42 in cells, confirming previous *in vitro* data (31). Second, active Rac recruits FHOD1 to F-actin and into membrane ruffles/lamellipodia, whereas the DRF does not associate with actin stress fibers or microspikes induced by Rho or Cdc42, respectively. Third, activation of FHOD1 specifically interferes with the formation of lamellipodia. Together, these results suggest that FHOD1 regulates actin dynamics in a pathway that induces ROCK as a downstream effector of Rac.

In the constitutive active form, FHOD1 triggers the formation of actin fibers via the Rho-ROCK cascade. Importantly, Rho activity was not affected by active FHOD1, but the formation of FHOD1-actin fibers required the filament bundling activity of ROCK for its effects. By these criteria, FHOD1 joins a growing number of Rac effectors involved in the induction of stress fibers. Although activation of the Rho pathway as a late response of Rac activation is a long standing observation in the field (10), only two Rac effectors with potential roles in the cross-talk from Rac to Rho were recently identified. Like FHOD1, the semaphorin Plexin B (39) and the *Caenorhabditis elegans* homologue of ELMO1, CED12 (40), specifically interact with Rac but induce the formation of stress fibers in a Rho-dependent manner. Similar to activated FHOD1, the interaction with Rac is not required for cytoskeletal rearrangements by Plexin B (39). Because activation of Rho by either Plexin B or

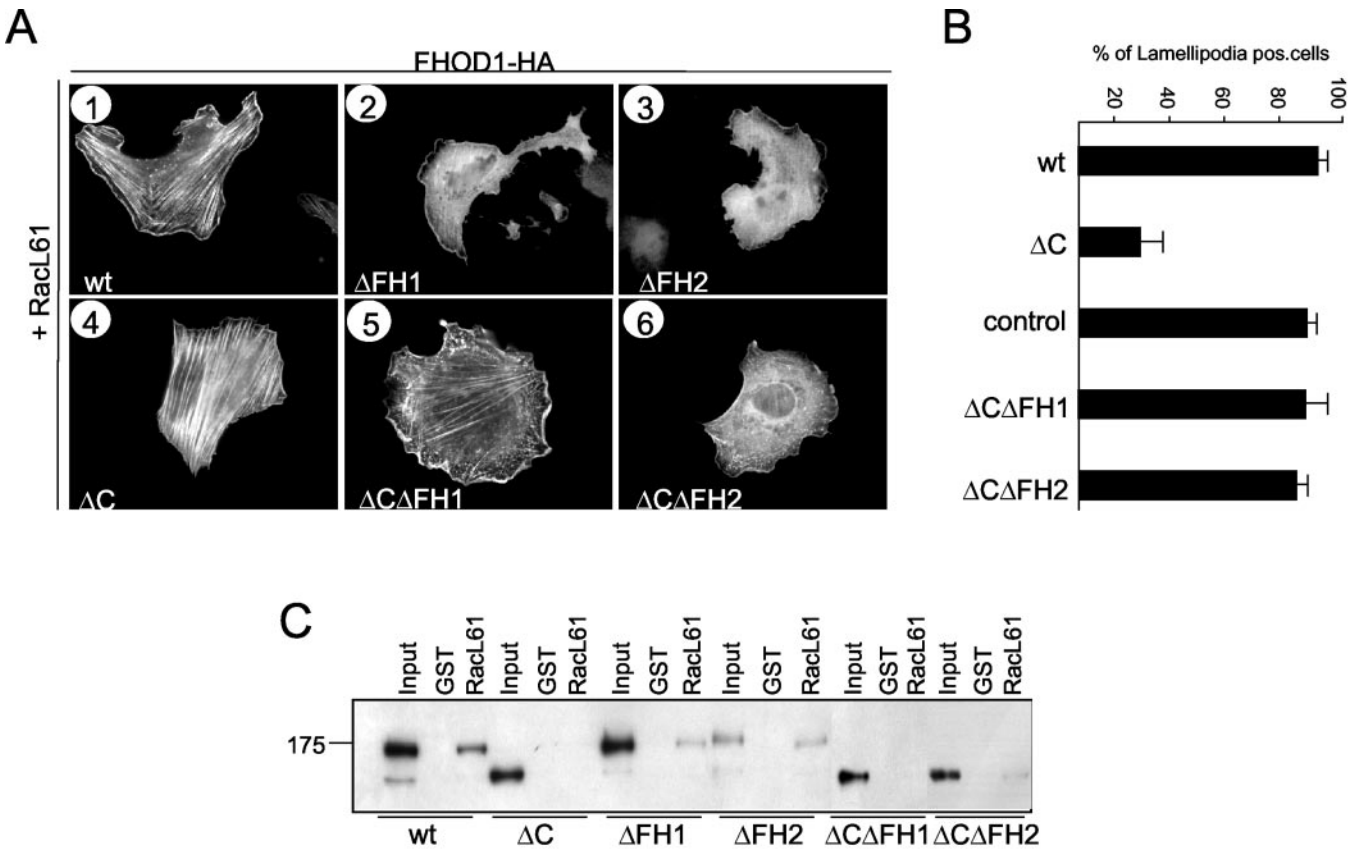

FIG. 7. **FHOD1 participates in Rac-mediated lamellipodia formation.** *A*, recruitment of FHOD1 to actin filaments and the plasma membrane by activated Rac are separable activities. NIH3T3 cells were co-transfected with the indicated FHOD1 expression plasmids and a construct driving the expression of a RacL61·GFP fusion protein. Following fixation, the cells were stained for FHOD1 with anti-HA and analyzed by immunofluorescence microscopy. All depicted FHOD1-positive cells also expressed activated Rac (not shown). *B*, quantification of the inhibitory effect of FHOD1ΔC on the formation of lamellipodia provoked by RacL61. Given is the percentage of cells with prominent lamellipodia upon co-expression of RacL61·GFP and the indicated FHOD1 proteins 24 h post-transfection (such as in *panels 1–3*, *5*, and *6*). The values are the averages of three independent experiments with at least 100 cells each with the indicated standard error of the mean. *C*, GST pull-down from cells expressing wt or mutant FHOD1 proteins performed as in Fig. 5*B* but with GST-RacL61. The *Input lanes* represent 10% of the cell lysates used.

CED12 has not been demonstrated directly, these molecules may act downstream of Rho in a manner similar to FHOD1. Thus, CED12, Plexin B and FHOD1 might be involved in the same pathway of Rac-dependent activation of the Rho cascade. Interestingly, DRFs also regulate Rac activation by Rho. As recently reported (11), Rac is activated indirectly by the Rho effector mDia. This effect of mDia is counteracted by ROCK, suggesting that Rac activity is regulated by a balance between mDia and ROCK. If ROCK is the primary target of FHOD1-mediated activation of the Rho cascade, FHOD1 activation may represent a counterbalance to the Rho-dependent Rac activation. In support of this model, FHOD1ΔC interfered with lamellipodia formation by active Rac. Thus, DRFs emerge as key players for the cross-regulation of individual GTPase pathways.

Our results indicate that in contrast to the deregulated ΔC, FHOD1wt might act downstream of Rac as mediator for the immediate effects of the GTPase such as formation of membrane ruffles and lamellipodia. As a later consequence of Rac activation, the Rho pathway is induced, leading to the formation of stress fibers. Thus, FHOD1 may serve as a general effector for actin rearrangements by Rac. Recruitment of FHOD1 into subcellular microenvironments with high actin remodeling activity should be critical for its function and might result in pleiotropic phenotypes depending on subcellular localization and nature of the recruitment trigger (*i.e.* Rac *versus* ROCK). The recruitment by Rac is indirect because the direct association with Rac is dispensable. Because deletions of the FH1 and FH2 domains, respectively, had no effect, we propose that the relocalization of the DRF is mediated by the interaction with multiple components of the actin remodeling machinery. How specificity for the recruitment of FHOD1 to sites of actin remodelling induced by Rac but not Rho is achieved remains to be determined. Whereas the FH2 domain of FHOD1 mediated its association with polymerized actin, the FH1 domain was required to induce rearrangements of the actin cytoskeleton. These results suggest that stepwise conformational changes of FHOD1 direct interactions with cellular ligands that in turn govern activation and effector functions of FHOD1. In the inactive conformation, the N and C termini form an intramolecular bridge that prevents cytoskeletal as well as transcriptional effects of FHOD1 (Ref. 31; data not shown). In this conformation, Rac binds to FHOD1 independently of its GTP/GDP status (Ref. 31 and Figs. 5, 6, and 7*C*). FHOD1ΔC in turn is fully active and no longer associates with the activating GTPase. Which factors mediate the respective activities of the FH1 and FH2 domains remains unclear. In preliminary experiments we failed to detect the physical and functional interaction of FHOD1 with known FH1 ligands such as Src and profilin (data not shown). Further mapping of the domains of FHOD1 responsible for its translocation and the identification of its respective cellular interaction partners will be the primary goals of future studies.

One important remaining question concerns the mechanism of FHOD1-induced cytoskeletal rearrangements. The temporal coordination of association of FHOD1 with actin filaments and

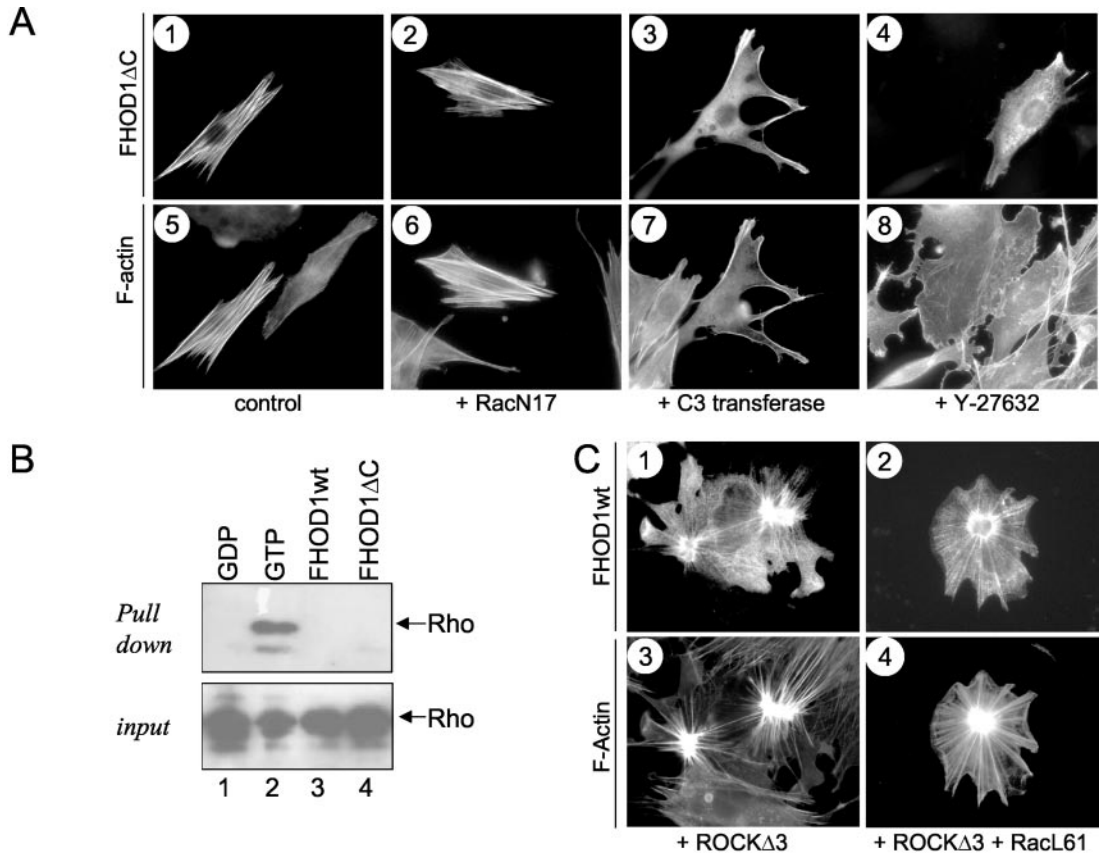

FIG. 8. **The Rho-ROCK activity is required for the formation of FHOD1-actin fibers.** *A*, NIH3T3 cells kept in 0.1% FCS were microinjected with the indicated expression plasmids and then analyzed for the subcellular distribution of FHOD1ΔC and F-actin by immunofluorescence. To assay for the involvement of ROCK in the formation of FHOD1-actin fibers, FHOD1ΔC-expressing cells were treated for 30 min with 30 μM of the ROCK inhibitor Y-27632 (*right panels*). *B*, Rho-GTPase activity pull-down experiment. The *lower panel* (*input*) presents an anti-Rho Western blot from lysates of HeLa cells expressing the indicated FHOD1 derivatives (*lanes 3* and *4*) or an empty control plasmid (*lanes 1* and *2*). GDP and GTP indicates the respective treatment of the lysate to deplete or enrich Rho-GTP as control. The *upper panel* shows an anti-Rho Western blot of the precipitates of GST-Rhotekin pull-down reactions from the same lysates to visualize the amounts of Rho-GTP present. *C*, recruitment of FHOD1wt to stellate actin fibers induced by activated ROCK. NIH3T3 cells were co-transfected with FHOD1wt- and ROCKΔ3-expressing plasmids with or without the RacL61 expression plasmid (*right* and *left panels*, respectively).

the appearance of thick FHOD1-actin fibers indicates that the association of FHOD1 with actin drives fiber formation. Also, preliminary results indicate that FHOD1-actin fibers are more stable than regular actin filaments, possibly because of decoration by the DRF (data not shown). The stabilization of actin filaments by a FHOD1 coat would be in agreement with data reported for the induction of actin cables by Bni1p in yeast (22, 23). Two recent studies, however, raise the intriguing possibility that the FH1-FH2 domains of DRFs possess intrinsic actin nucleation activity for unbranched filaments, leading to the pronounced formation of actin cables (41–43). The net increase in F-actin levels in cells expressing FHOD1ΔC suggests that FHOD1 also induces actin polymerization to cause the formation of actin filaments. ROCK activity might then be needed to bundle these filaments into FHOD1-actin fibers. Additionally, FHOD1 might stabilize these structures, which would be consistent with its tight association with actin fibers. Like the yeast DRFs, FHOD1 exerts its function independently of Arp2/3 complex. Thus, FHOD1 likely possesses *de novo* actin nucleation activity. This activity, however, as for any other mammalian DRF, remains to be demonstrated by *in vitro* nucleation studies. Such an activity would be in line with the requirement for the FH1 and FH2 domains in FHOD1 for the induction of actin rearrangements. However, FH1-FH2 fragments of FHOD1 analogous to those recently reported for mDia (44) failed to recapitulate the FHOD1ΔC phenotype (data not shown). Unraveling the detailed role of these domains will therefore require further experimentation.

In summary, our results demonstrate that FHOD1 is involved in the remodeling of cytoskeletal structures downstream of Rac and that activation of FHOD1 activates the Rho cascade downstream of the GTPase. The ubiquitous expression profile of FHOD1 (30) opens avenues for an involvement in multiple processes triggered by such actin rearrangements. These effects are expected to vary between cell types and stimuli and likely include known effects of Rac such as the regulation of phagocytosis, pinocytosis, intracellular trafficking, and cell cycle progression (45, 46). This study suggests that like other DRFs such as mDia (16, 44, 47), actin remodeling by FHOD1 is directly connected to transcription from the SRE element. Similar to mDia for Rho (16), FHOD1 might therefore provide modular regulation for Rac and integrate cytoskeletal and transcriptional effector functions. In conclusion, our findings emphasize the key regulatory role of DRFs in controlling cell morphology and suggest that the use of individual DRFs by multiple signal transduction pathways is instrumental for the cellular response to a distinct exogenous stimulus.

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