Oligomerization of the diaphanous-related formin FHOD1 requires a coiled-coil motif critical for its cytoskeletal and transcriptional activities

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Abstract The diaphanous-related formin homology 2 domain containing protein 1 (FHOD1) interacts with the Rac GTPase and activates the Rho-ROCK cascade leading to the formation of actin stress fibers. Here, we report the detection of homotypic interactions of FHOD1 in the yeast two-hybrid system, by co-immunoprecipitation and co-localization in mammalian cells. A predicted coiled-coil motif C-terminal to the core FH2 domain, but not the core FH2 domain itself, was critical for self-association of FHOD1. Deletion of both the coiled-coil motif and the core FH2 domain abrogated formation of actin stress fibers and activation of transcription of the serum response element by FHOD1. In contrast, these motifs were dispensable for the physical and functional interaction of FHOD1 with Rac1. Together, these results indicate that oligomerization of FHOD1 via the coiled-coil motif is a critical parameter for its biological activities.

Keywords: Diaphanous-related formin; Formin homology 2 domain containing protein 1; Coiled-coil motif; Oligomerization; Cytoskeleton

1. Introduction

Formins are defined as proteins that contain the formin homology (FH) domains FH1 and FH2. They are evolutionary conserved in eukaryotes and regulate fundamental processes, such as cytokinesis, maintenance of cell polarity and cell migration [1,2]. These activities reflect the ability of formins to modulate cytoskeletal dynamics. While formins also affect microtubules [3,4], their effects on the polymerization state of actin are better understood. Several formins are known to possess actin nucleation activity and can promote de novo formation of actin filaments [5–9]. The nucleation activity of formins is mediated by determinants contained within both, the FH1 and FH2 domains, even if the FH2 domain alone is sufficient to trigger actin nucleation in vitro [6,7,10].

FH1 domains are highly enriched in proline residues that mediate interactions with profilins and SH3 domains of Src tyrosine kinases [11,12]. The FH2 domain is less-well defined and spans up to 500 residues. This large stretch likely encompasses several independent domains that might exert individual activities and interact with select cellular partners. Within the FH2 domain, a highly conserved stretch of around 100 amino acids, including the GNXMN signature motif, comprises the originally defined FH2 domain and is now designated the core FH2 domain [1,13].

Among the formin proteins, the diaphanous-related formins (DRFs) represent a subgroup that physically and functionally interacts with small Rho GTPases. Besides the conserved FH1 and FH2 domains, DRFs contain a specific GTPase-binding domain (GBD) in the N-terminal half of the protein, a diaphanous autoregulatory domain (DAD) at the C-terminal end, and a predicted coiled-coil motif adjacent to the C-terminus of the core FH2 domain [1]. In the inactive state, DRFs are autoinhibited due to the interaction of the GBD with the DAD. This autoinhibition is released upon binding of the GTPase to the GBD, resulting in conformational changes and subsequent activation of the DRF [14]. The role of the coiled-coil motif has not yet been addressed but it could also mediate protein interactions of DRFs with cellular partners or serve to maintain the overall structure of these proteins.

DRFs couple GTPase signaling to cytoskeletal organization to control various cellular processes, including the control of transcription from specific promotors, such as the serum response element (SRE) [12,15,16]. The specificity for the activities of distinct DRFs arises, at least in part, from the selective interaction with a specific GTPase. The DRF FHOD1 (formin homology 2 domain containing protein 1) interacts with the Rac1 GTPase [17,18]. Interestingly, activation of FHOD1 leads to the formation of actin stress fibers and induces SRE transcription [17–20], thus inducing a phenotype rather reminiscent of Rho activation. However, activated FHOD1 acts on Rho signaling downstream of the GTPase at the level of the Rho effector kinase ROCK [17], indicating that FHOD1 might serve as a switch between the Rho and Rac cascades.

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In contrast to other DRFs, activated FHOD1 decorates actin fibers and FHOD1-actin filaments appear thicker than stress fibers induced upon activation of Rho [17]. These effects require both the FH1 and FH2 domains and include the overall elevation of cellular F-actin levels, which is consistent with events driven by FHOD1-mediated actin nucleation [17]. On the other hand, activation of Rac alone is apparently not sufficient to fully induce FHOD1 activity [17,20].

Here, we present evidence for homo-oligomerization as a prerequisite for the activities of the DRF FHOD1. Self-association of FHOD1 was detected in yeast and in mammalian cells and was dependent on the presence of a predicted coiled-coil motif adjacent to the core FH2 domain. Deletion of the coiled-coil motif prevented the formation of actin stress fibers and the induction of SRE transcription by FHOD1. Together, these data indicate that multimerization of FHOD1 is required to mediate its biological activity, suggesting that this property may represent a general feature of DRF proteins.

2. Materials and methods

2.1. Cells and reagents

NIH3T3 and HeLa cells were maintained in standard low and high glucose DMEM, respectively, complemented with 10% (v/v) fetal calf serum, glutamine, penicillin, and streptomycin at standard conditions. Mouse (F-7) or rabbit (Y-11) anti-HA antibodies were obtained from Santa Cruz Biotechnology. Rabbit anti-HA (3F10) and mouse anti-myc (9E10 epitope) antibodies were obtained from Roche. TRITC-labeled phalloidin and fluorescent secondary antibodies (Alexa488 or Alexa568) and Alexa660-conjugated phalloidin were purchased from Sigma and Molecular Probes, respectively. Expression plasmids for GTPases fused to GFP or HA-tagged FHOD1 were described previously [17]. The GFP-tagged version of FHOD1ΔC was generated by PCR and subsequent subcloning into the *Eco*RI site of the pEGFPC2 vector (Clontech). The expression constructs for HA-tagged FHOD1ΔCC and FHOD1ΔCΔCC were generated by PCR using the internal primers described for the yeast two hybrid expression constructs in combination with external primers that allowed subcloning of the PCR fragments via *Afl*III/*Eco*RI into the *Nco*I/*Eco*RI sites of pLinkEF-HA [17].

2.2. Yeast two-hybrid assay

The plasmids for the expression of the full-length FHOD1 and the deletion mutants were generated by cloning the respective PCR amplification products in frame with the LexA operator and the Gal4 activation domain into the pLex12 and pGADGE yeast expression vectors, respectively [21]. For two-hybrid analysis, the L40 yeast reporter strain, containing the two LexA-inducible genes, *His3* and *LacZ*, was co-transformed with vectors for expression of the indicated LexA and Gal4AD hybrids, and plated on selective medium [21]. Double transformants were then assayed for qualitative β-galactosidase (β-gal) activity and histidine auxotrophy.

2.3. Functional assays

Immunofluorescence microscopy, SRE transcription assay and Rac pull-down assay were carried out as described previously [17]. To analyze self-association of FHOD1 by co-immunoprecipitation, HeLa cells (8×10^6 cells) were cotransfected with 12 μg of the indicated plasmid DNA using the electroporation method as previously described [22]. 48 h after transfection, the cells were washed once with PBS and serum starved for 2 h at 37 °C in DMEM supplemented with 0.1% BSA and 10 mM HEPES, pH 7.5. Cells were lysed in assay buffer (50 mM Tris–HCl (pH 7.6), 5 mM EDTA, and 150 mM NaCl) containing 1% Triton for 30 min at 4 °C. The cleared lysate was precipitated with an anti-HA antibody in the presence of protein G–Sepharose and incubated overnight at 4 °C. The immunoprecipitates were then analyzed by Western blotting.

3. Results

3.1. FHOD1 supports both intra- and intermolecular interactions

While activation of FHOD1 is known to cause formation of actin stress fibers, the mechanisms for the regulation of FHOD1 activity are not fully understood. One mechanism consists in the autoinhibition of the DRF via the interaction of its N-terminal GBD with the C-terminal DAD that can be released by GTPase binding (see Fig. 1A) for domain organisation. The ability of FHOD1 to support such an intramolecular interaction was first addressed in the yeast two hybrid

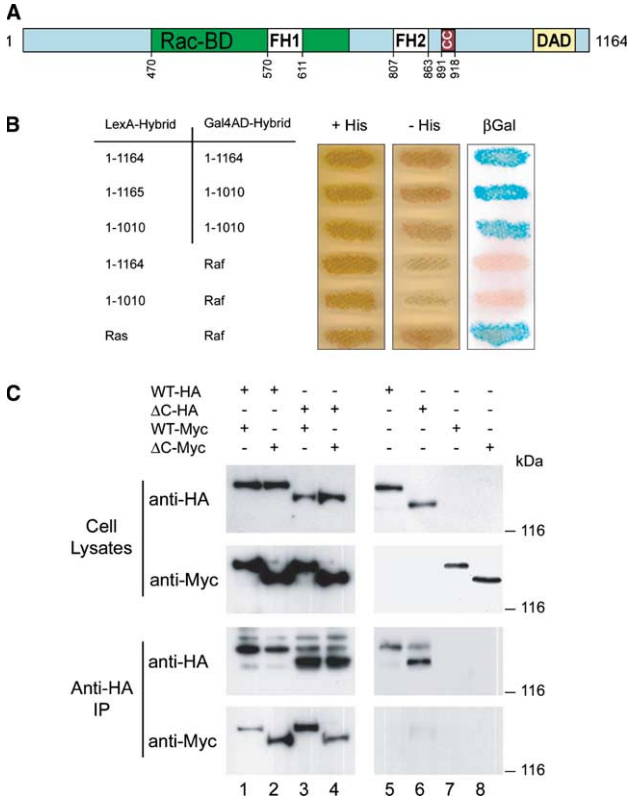

Fig. 1. FHOD1 supports both intra- and intermolecular interactions. (A) Schematic representation of FHOD1 showing the Rac1-binding domain (Rac-BD), the core domains of FH1 and FH2 domains, the DAD and the putative coiled-coil motif (in red). Numbering refers to [18]. (B) Intra- and intermolecular interactions of FHOD1 detected in the yeast two-hybrid system. The L40 yeast strain expressing the indicated pairs of full length FHOD1wt (1–1164) and the FHOD1ΔC mutant (1–1010) fused to LexA (left column) or Gal4AD (right column) was analyzed for histidine auxotrophy and β-gal activity. Double transformants were patched on selective medium with histidine (+His) and were replica plated on medium without histidine (−His) and on Whatman filters for subsequent β-gal assays. Growth in the absence of histidine and expression of β-gal activity indicate interaction between hybrid proteins. The specificity of the binding was verified by the absence of activation of the reporter genes in cells expressing the LexA-FHOD1 or LexA-FHOD1ΔC in combination with the Gal4AD-Raf hybrid. Interaction between LexA-Ras and Gal4AD-Raf hybrids was used as a positive control. (C) Self-association of FHOD1 in HeLa cells analyzed by co-immunoprecipitation. Lysates (upper panels, cell lysates) from HeLa cells expressing the indicated HA- and Myc-tagged FHOD1(WT) or FHOD1ΔC (ΔC) proteins alone (lanes 5–8) or in combination (lanes 1–4) were subjected to immunoprecipitation with the anti-HA antibody (lower panels, anti-HA IP). Cell lysates and immunoprecipitates were separated by SDS–PAGE and analyzed by Western blotting with either anti-HA or anti-Myc antibodies.

system with FHOD1 proteins fused to the LexA operator and the Gal4 activation domain (Gal4AD) (Fig. 1B). As expected, full-length FHOD1 (1–1164, wt) readily reacted with itself as evidenced by growth of the reporter strain on medium without histidine and the expression of β-gal activity. Similarly, FHOD1wt efficiently bound to the activated form of FHOD1 in which the C-terminus was removed (1–1010, FHOD1ΔC). Surprisingly, FHOD1ΔC also self-associated, although these molecules should not be able to form the autoinhibitory N- to C-terminal interaction. Indeed, FHOD1ΔC did not interact with the N-terminal fragment of FHOD1 encompassing the GBD (Fig. 2A, FHOD1/1-863 mutant), whereas the full-length FHOD1 still did (data not shown). In contrast, efficient binding was observed between FHOD1ΔC and C-terminal fragments of the protein (i.e., FHOD1/570-1164 and FHOD1/570-1010), as indicated in Fig. 2. Since FHOD1/570-863 failed to bind FHOD1ΔC in this two-hybrid assay, the minimal region of FHOD1 that is able to support homomeric interaction was delineated between residues 570 and 1010 of the protein (Fig. 2B). Thus, additionally to autoinhibitory intramolecular interactions within individual FHOD1 molecules, the C-termini of at least two FHOD1 molecules can physically interact via a mechanism that does not require the DAD.

We next sought to confirm that FHOD1 was able to mediate homomeric interactions in human cells and attempted to co-immunoprecipitate FHOD1 molecules carrying a Myc or HA epitope tag, respectively. HeLa cells expressing comparable levels of both HA- and Myc-tagged forms of FHOD1wt or FHOD1ΔC (Fig. 1C, cell lysates) were lysed, and subjected to immunoprecipitation with the anti-HA monoclonal antibody. The immunoprecipitates were then separated by SDS–PAGE and analyzed by immunoblotting with an anti-Myc antibody (Fig. 1C, anti-HA IP). Significant amounts of Myc-tagged FHOD1wt or FHOD1ΔC were detected only from cells co-expressing HA-tagged FHOD1wt or FHOD1ΔC molecules (lanes 1–4), but not from control cells expressing either the HA- or Myc-tagged forms of FHOD1 alone (lanes 5–8). This result provides evidence that full-length FHOD1 can form oligomeric complexes in a cellular environment. Again, the C-terminal DAD region of FHOD1 was not involved in the formation of this complex, because the Myc- and HA-tagged forms of the FHOD1ΔC mutant that lack the DAD associated with each other upon co-expression (lane 4). While self-

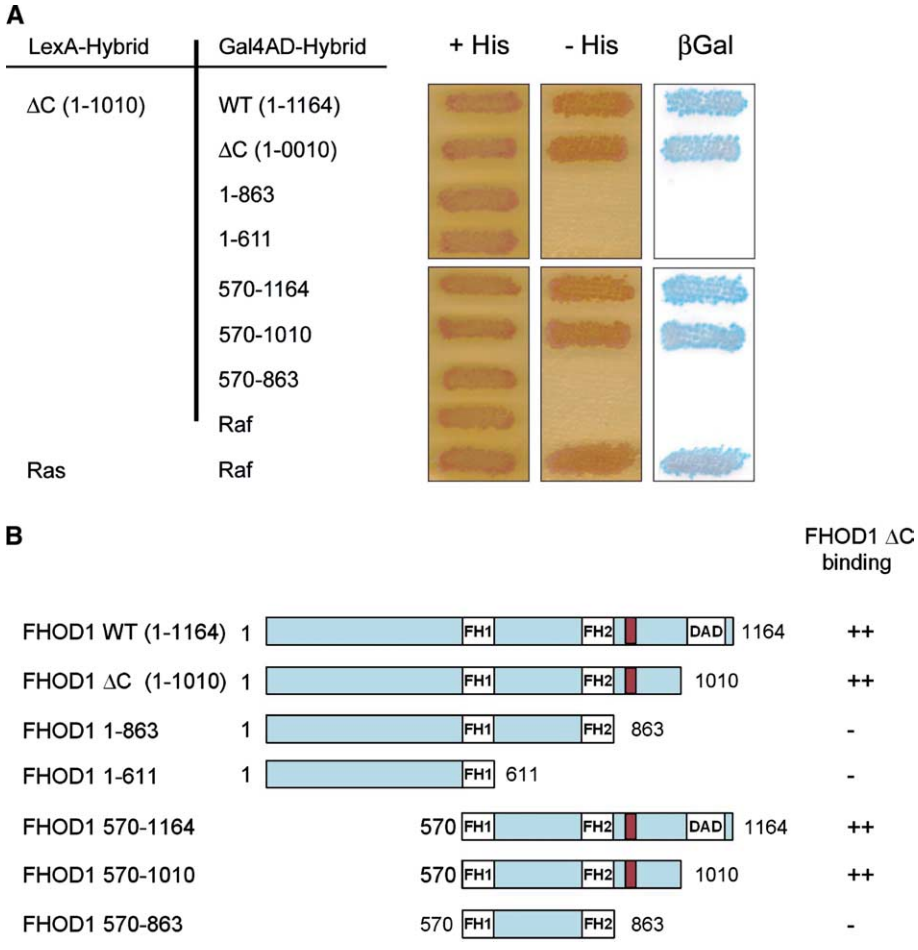

Fig. 2. Characterization of the FHOD1 region required for intermolecular interaction. (A) Intermolecular interactions of FHOD1 assayed in the yeast two-hybrid system. L40 strains expressing FHOD1 ΔC fused to LexA and each of the indicated FHOD1 deletion mutants fused to Gal4AD were analyzed for histidine auxotrophy and β-gal activity as described in Fig. 1B. (B) Schematic representation of FHOD1 deletion mutants assayed in panel (A). A summary of the results is indicated on the right. Interactions are scored as: (++), growth on medium without histidine and development of the β-gal assay within 2 h; (−), no growth on medium without histidine and no β-gal activity.

association of FHOD1ΔC was somewhat more efficient than that of FHOD1wt in this particular experiment, this difference was not consistently observed in all co-immunoprecipitation experiments (see, e.g., Fig. 3C) or by yeast two-hybrid analysis (Fig. 1B). We therefore conclude that deletion of the DAD does not significantly increase FHOD1 homomerization under the experimental conditions used.

3.2. A putative coiled-coil motif adjacent to the core FH2 domain is critical for homo-oligomerization of FHOD1

To further map the determinants for the intermolecular interaction, we focused on the conserved FH1 and FH2 domains found in the C-terminal half of FHOD1. We also investigated the role of a putative coiled-coil motif adjacent to the core FH2 domain (see Fig. 1A), because the initial mapping

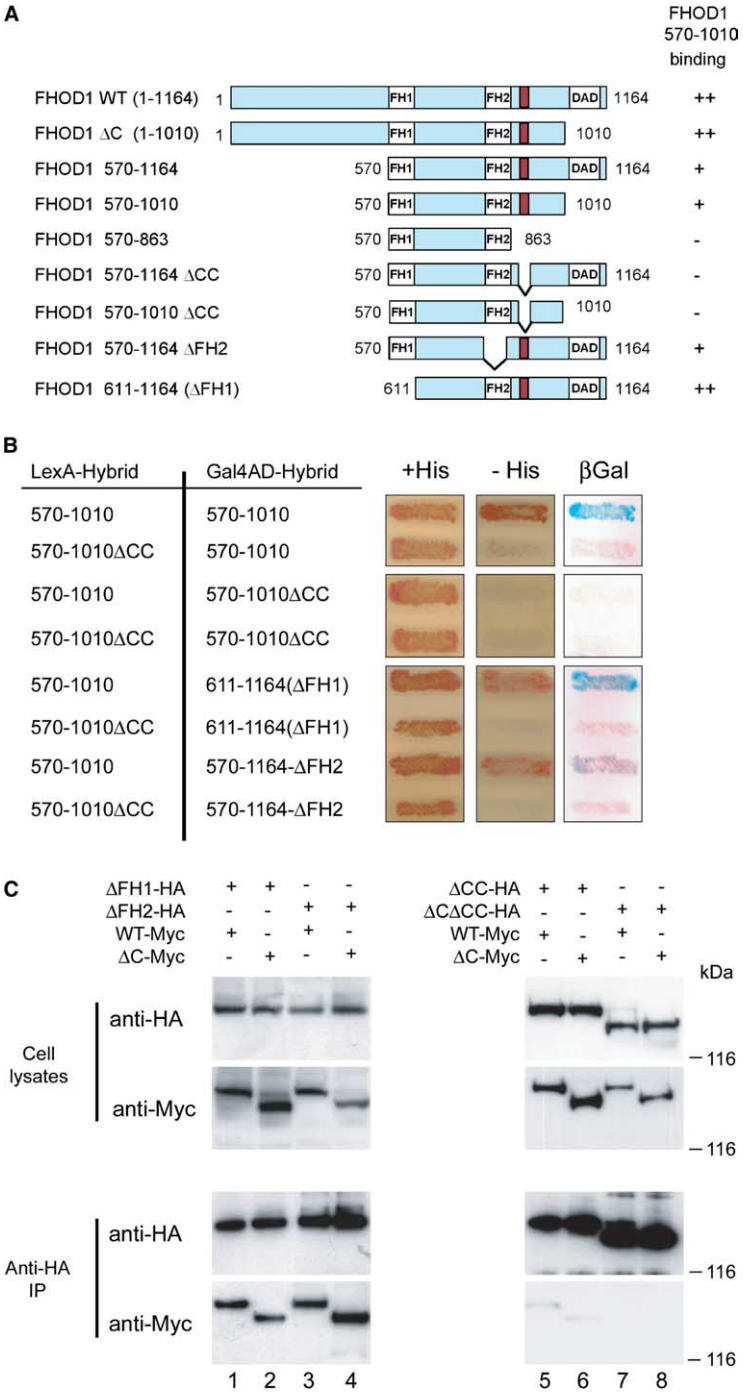

Fig. 3. Role of the FH1/FH2 domains and the coiled-coil motif for FHOD1 homo-oligomerization. (A) Schematic representation of FHOD1 deletion mutants used for two-hybrid analysis in panel (B). Interactions of the respective FHOD1 fragments with FHOD1 570–1010 are indicated on the right and are scored as: (++), growth on medium without histidine and development of the β-gal assay within 2 h; (+), growth on medium without histidine and development of the β-gal assay within 4 h; (−), no growth on medium without histidine and no β-gal activity. (B) Representative data of the interactions are summarized in panel (A). (C) Co-immunoprecipitation analysis from transfected HeLa cells expressing either Myc-tagged FHOD1wt or FHOD1ΔC in combination with the indicated HA-tagged FHOD1 variants (see legend to Fig. 1C for details).

showed that an FHOD1 fragment encompassing aa 570-863 (FHOD1/570–863) failed to bind FHOD1ΔC, whereas FHOD1/570–1010 containing this putative motif still bound (Fig. 2). The FHOD1/570–1010 mutant was the shortest FHOD1 fragment able to support the homomeric interaction in the two-hybrid assay (Figs. 3A and B). Deletion of either the FH1 or the core FH2 domain only caused a slight reduction of the interaction (Fig. 3B). In contrast, removal of the coiled-coil motif completely abolished the homomeric interaction between FHOD1/570–1010 fragments. These results suggested that FHOD1 is able to form homomeric complexes via its C-terminus and that the coiled-coil motif is a critical determinant for these interactions.

Co-immunoprecipitation experiments with mutated full length FHOD1 proteins lacking either the FH1 or the core FH2 domain confirmed that both domains were dispensable for the self-association of FHOD1 in cells (Fig. 3C, lanes 1–4). In contrast, the precipitation of FHOD1 with itself or the activated FHOD1ΔC was significantly reduced upon deletion of the coiled-coil motif (lanes 5 and 6). This effect was even more pronounced when the coiled-coil deletion was introduced in the context of the activated FHOD1ΔC, leading to a complete disruption of the formation of homomeric FHOD1 complexes (lanes 7 and 8). Therefore, we conclude that both wild type and activated FHOD1 are able to form homomeric complexes in cells and that the integrity of the coiled-coil motif adjacent to the core of the FH2 domain is required for FHOD1 oligomerization.

3.3. Activation of FHOD1 leads to the formation of homomeric complexes associated with actin stress fibers

We next addressed whether self-association of FHOD1 can be detected in intact cells, using the property of the FHOD1ΔC activated form to induce formation of and to associate with thick actin stress fibers [17]. FHOD1ΔC fused to GFP was expressed in NIH3T3 cells together with various HA-tagged FHOD1 proteins and their subcellular localization was determined by immunofluorescence. Consistent with the binding studies, FHOD1wt was partially recruited to actin stress fibers coated with GFP.FHOD1ΔC (Fig. 4, panels 1 and 2), presumably as a consequence of the formation of a homomeric complex between both proteins. This recruitment was completely abrogated when the coiled-coil motif was deleted from FHOD1 (panels 3 and 4). In contrast, deletion of the core FH2 domain had no significant effect on the recruitment of FHOD1 to actin stress fibers induced by FHOD1ΔC (panels 5 and 6). Similarly, the self-association of HA-tagged FHOD1ΔC molecules on GFP.FHOD1ΔC-induced stress fibers was dependent on the coiled-coil motif but not on the core FH2 domain (panels 7–12). Thus, FHOD1 molecules oligomerize on FHOD1 induced actin stress fibers in intact cells and the coiled-coil motif is required for this self-assembly.

3.4. Interaction of FHOD1 with Rac1 is independent of the coiled-coil motif

Next, we addressed whether coiled-coil mediated formation of homomeric FHOD1 complexes is required for the functional interaction with the Rac1 GTPase. This was first analyzed in intact NIH3T3 cells expressing wt or mutated FHOD1 proteins in combination with the activated RacL61 mutant fused to GFP (Fig. 5A). As reported previously [17],

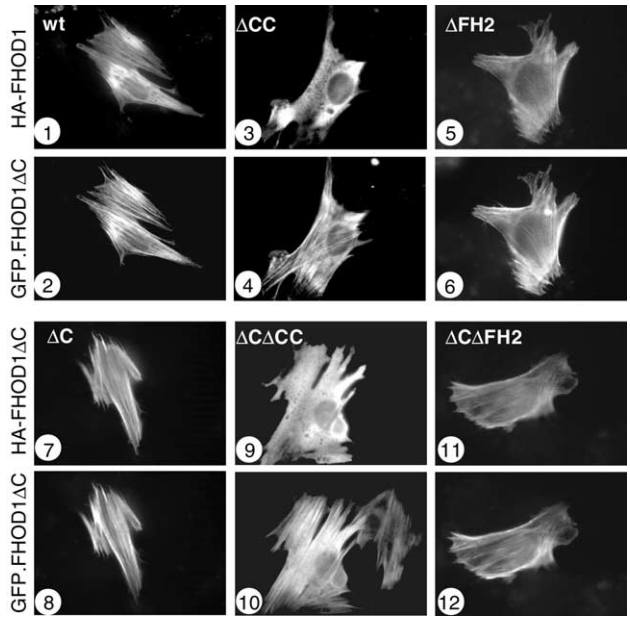

Fig. 4. Formation of homomeric FHOD1 complexes on actin stress fibers in intact cells. NIH3T3 cells were transfected with an expression plasmid for GFP.FHOD1ΔC together with the indicated expression plasmids for various HA-tagged FHOD1 proteins. Following fixation, the cells were stained for HA-FHOD1 with anti-HA and analyzed by immunofluorescence microscopy.

FHOD1wt was targeted to the plasma membrane and to actin stress fibers in the presence of active RacL61 and the cells displayed membrane ruffles and lamellipodia (panel 1) typical for activation of Rac. Activated FHOD1ΔC was also targeted to the plasma membrane in RacL61.GFP expressing cells, but the formation of stress fibers by FHOD1ΔC prevented extensive ruffling and lamellipodia formation (panel 2 and data not shown). Plasma membrane targeting of FHOD1 by active RacL61 was preserved for FHOD1 or FHOD1ΔC mutated proteins lacking the coiled-coil motif (panels 3 and 4) or the core FH2 domain (panels 5 and 6), indicating that they were capable of a functional interaction with the GTPase in cells. However, no recruitment of these mutated proteins to actin filaments was observed upon co-expression of RacL61.GFP. Since plasma membrane recruitment of FHOD1wt by RacL61 does not require the physical interaction with the GTPase [17], we also tested the impact of the coiled-coil motif deletion on binding to the GTPase using GST-RacL61 as a bait for pull down assays from cells expressing FHOD1wt (Fig. 5B). These experiments revealed that deleting the coiled-coil motif in FHOD1 reduced but not abrogated the physical interaction with RacL61, whereas deletion of the FH1 domain completely abolished binding as previously reported [17].

3.5. Coiled-coil mediated oligomerization of FHOD1 correlates with its activity

We next investigated the functional consequences of the disruption of FHOD1 multimerization upon deletion of the coiled-coil motif. First, the ability of FHOD1 to rearrange the actin cytoskeleton into thick stress fibers was analyzed in NIH3T3 cells. Due to the autoinhibition related to the intramolecular interaction, expression of FHOD1wt did not induce actin rearrangements [17], and deletion of the coiled-coil motif or the FH2 core domain had no effect on actin organization

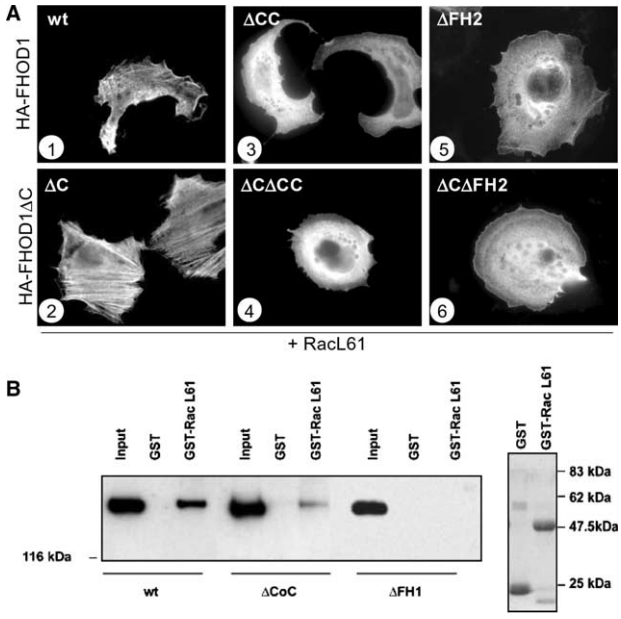

Fig. 5. Role of the coiled-coil motif in the functional and physical interaction of FHOD1 with Rac1. (A) NIH3T3 cells were co-transfected with the indicated HA-tagged FHOD1 expression plasmids and a construct driving the expression of a RacL61.GFP fusion protein. Following fixation, the cells were stained for FHOD1 with anti-HA antibodies and analyzed by immunofluorescence microscopy. All depicted FHOD1-positive cells also expressed RacL61.GFP (not shown). (B) FHOD1 coiled-coil motif is not required for RacL61 binding. Cytoplasmic lysates from HeLa cells expressing HA-tagged FHOD1wt, FHOD1ΔCC or FHOD1ΔFH1 were incubated with equal amounts of purified GST or GST-RacL61 immobilized on GSH–agarose beads. Bound proteins were resolved by SDS–PAGE and revealed by Western blotting with anti-HA antibody. The input lanes represent 10% of the cell lysates used. The right panel shows a Coomassie-stained gel as input control for the GST and GST-RacL61 proteins used in the pull-down reaction.

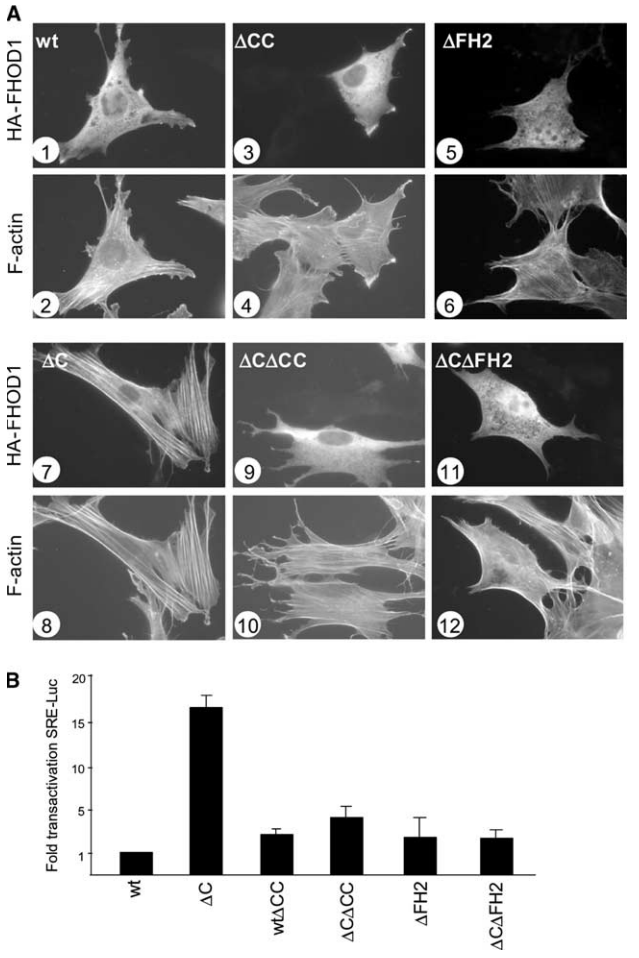

Fig. 6. Correlation between FHOD1 multimerization and biological activity. (A) Subcellular localization and effects on the actin cytoskeleton of the various FHOD1 mutants. NIH3T3 cells were transfected with the indicated FHOD1 expression plasmids. Following fixation, the cells were stained for HA-FHOD1 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.

and on the diffuse cytoplasmic distribution of FHOD1wt (Fig. 6A, panels 1–6). In contrast, the activated FHOD1ΔC induced the formation of thick actin fibers and the DRF was associated with these structures (panels 7 and 8). This phenotype required the presence of both, the coiled-coil motif (panels 9 and 10) and the core FH2 domain (panels 11 and 12). Deletion of either region resulted in the loss of stress fiber induction and prevented the association of FHOD1 with F-actin in cells.

Next, we explored the effect of the coiled-coil motif deletion on the FHOD1-induced activation of SRE transcription. As observed before [17], the induction of stress fibers by FHOD1ΔC coincided with a marked induction of the SRE luciferase reporter in NIH3T3 cells, whereas FHOD1wt had no effect (Fig. 6B). Importantly, the ability of FHOD1ΔC to induce SRE transcription was almost completely abrogated upon deletion of either the coiled-coil motif or the core FH2 domain. Thus, FHOD1ΔC mutants that failed to induce actin stress fibers did not activate the SRE. Together, these results indicate that the coiled-coil motif in FHOD1 represents one critical determinant for FHOD1 activity in cytoskeletal remodeling and transcriptional regulation.

4. Discussion

In this study, we present evidence for the formation of two distinct types of homomeric interactions by the DRF FHOD1. Additionally to the known autoinhibitory interaction of the N- and C-termini within one FHOD1 molecule, we find that FHOD1 molecules self-associate in eukaryotic cells. Formation of intermolecular homomeric complexes by FHOD1 was detected by yeast two-hybrid analysis, co-immunoprecipitation in mammalian cells and by recruitment of FHOD1 to actin stress fibers induced by activated FHOD1 molecules. In all three experimental systems, multimerization of the DRF was strictly dependent on a predicted coiled-coil motif adjacent to the core FH2 domain that is highly conserved among DRFs [1], but not the core FH2 domain itself. Importantly, deletion of the coiled-coil motif in FHOD1 not only prevented its multimerization but also interfered with FHOD1-mediated induction of actin stress fibers and activation of SRE transcription. In contrast, the coiled-coil motif was not required for the physical and functional interaction of FHOD1 with the Rac1 GTPase. Together, these data suggest that coiled-coil-mediated multimerization of FHOD1 molecules is critical for the biological activity of this DRF.

Notably, self-association of FHOD1 molecules was observed by co-immunoprecipitation upon co-expression of two FHOD1 proteins with different epitope tags (see Fig. 1C), but not when two distinct cell lysates expressing these proteins individually were mixed (data not shown). Thus, FHOD1 at least partially resides within tight homomeric complexes in living cells. These results confirm a recent report showing that FHOD1 was able to form a homotypic complex via a fragment encompassing more than the full FH2 domain [20]. The data reported herein extend this observation and identify the coiled-coil motif adjacent to the core FH2 domain as critical determinant for FHOD1 multimerization. Since coiled-coil motifs represent important three dimensional protein structure elements, it has to be considered that the results obtained with the FHOD1 mutant deleted of the coiled-coil motif could arise from the disruption of the overall structure of the protein. While this suspicion cannot be fully excluded, we found that FHOD1 proteins lacking the coiled-coil motif readily formed the autoinhibitory N-to-C terminal interaction (data not shown). Furthermore, deletion of the coiled-coil motif did not impact on FHOD's ability to interact directly with the Rac1 GTPase and to be efficiently targeted to the plasma membrane upon co-expression with activated Rac1.

During preparation of the manuscript, the crystal structures of the FH2 domains of mDia1 and Bni1 were solved, showing an elongated, crescent-shaped molecule consisting of four helical subdomains [23,24]. Interestingly, three different forms of multimer formation were found in the crystal lattice, depending on different domain boundaries or a shortened linker segment. Most informative is the so-called "tethered dimer" formation of Bni1, in which two FH2 domains are tied together at either end by an unusual lasso-linker structure corresponding to the N-terminal 90 residues of each domain. This architecture is proposed to reflect the active conformation of the FH2 domain by allowing a stair-step mechanism on the elongating barbed end of actin filaments [24]. Truncation of the linker segment in Bni1 by only four residues resulted in a markedly different orientation of the two molecules that each turned around from a "face-to-face" assembly to a "back-to-back" structure [24].

Based on multiple sequence alignments between FHOD1, mDia1, and Bni1, we modeled the FH2 domain of FHOD1 to the tethered dimer structure of Bni1 with an automated protein homology modeling approach [25] using a subdomain oriented stepwise strategy (Fig. 7). The FH2 structure is subdivided into the N-terminal lasso-linker segment that exhibits high conformational flexibility and tethers two FH2 molecules together. The succeeding knob subdomain forms a globular section encompassing approximately 100 residues and is tightly integrated into the central three-helix-bundle structure. This segment acts as a scaffold for the entire subdomain assembly. It is interweaving with the C-terminal post domain, which contains the core FH2 motif and a highly conserved lysine residue (K851 in FHOD1) required for actin nucleating activity of the FH2 domain. The deletion mutant of the core FH2 domain (ΔFH2) lacks the first three helices of the post subdomain (hatched green), while the deletion of the coiled-coil region (ΔCC) encompasses the second helix of the three-helix-bundle structure (indicated yellow). Based on this model, it is conceivable that the deletion of the core FH2 domain used in this study is unable to support the intermolecular interactions between the post and linker-lasso

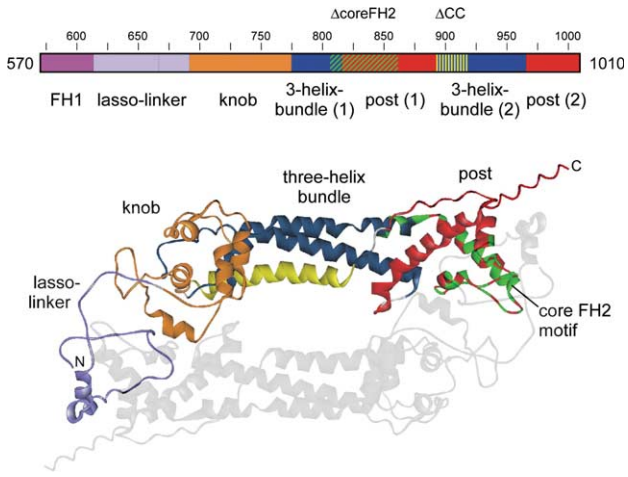

Fig. 7. Model for one possible dimer conformation of the FH2 domain from FHOD1, based on the crystal structure of the resting "back-to-back" dimer of Bni1p [24]. The four subdomains are indicated in different colors: linker-lasso (light-blue), knob (orange), three-helix-bundle (blue) and post (red). The deletion sites of the core FH2 region and the coiled-coil motif in FHOD1ΔFH2 and FHOD1 ΔCC, respectively, are hatched in green and yellow.

domains and the presentation of the catalytic active residues. Structural consequences of the deletion of the coiled-coil motif, however, are less certain and its structural impact on the formation of the post subdomain cannot be predicted.

The functional analysis of multimerization deficient FHOD1 mutants revealed a strong correlation between the ability of FHOD1 to multimerize and its ability to induce the formation of actin stress fibers and activate transcription from the SRE. This correlation is in line with the proposed direct coupling of actin reorganization and SRE transcription induced by the DRF mDia [15,16,26], that also requires multimerization of the DRF [27]. Of note, coiled-coil motif deleted FHOD1 molecules also failed to associate with actin fibers induced by FHOD1ΔC or upon co-expression of active Rac. This suggests that multimerization might be a prerequisite for the efficient association of FHOD1 with polymerized actin in cells. Furthermore, these results indicate that the binding between the N-terminal half of FHOD1 and F-actin recently described in vitro [20] is not the only determinant for the association of the DRF with actin filaments in vivo. However, the loss of FHOD1 function provoked by the deletion of the coiled-coil motif is comparable to that observed by the deletion of the core FH2 domain. Given that our results indicate a clear segregation between the roles of the core FH2 domain and the coiled-coil motif for multimerization, we speculate that multimerization via the coiled-coil motif may facilitate the activity of the adjacent core FH2 domain. In this model, coiled-coil-mediated homomerization is a prerequisite for molecular interactions mediated by the core FH2 domain. This might explain why an intact coiled-coil motif and the core FH2 domain are both necessary but not sufficient for full FHOD1 activity.

Coiled-coil motifs succeeding the core FH2 domain are well conserved among DRFs and the inactivation of mDia in one study might be attributable to experimental disruption of this motif [28]. However, unlike other DRFs, FHOD1 is tightly associated with the actin stress fibers generated upon removal of its DAD. Since multimerization emerges as prerequisite for DRF induced actin polymerization but the relative contribution

of individual multimerization determinants may vary between DRFs, coiled-coil mediated homomers might be particularly well suited for F-actin coating. Such differences between DRF multimerization are also suggested by experiments indicating that the presence of Rac facilitates and/or stabilizes FHOD1 homomerization independently of its GTP status (data not shown), while multimerization of mDia is unaffected by overexpression or inhibition of Rho [27]. Future analyses are warranted to reveal the regulation of DRF multimerization and to reconcile stress fiber coating by FHOD1 with its suggested role as actin nucleator.

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