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The Rho Family GTPase Rif Induces Filopodia through mDia2

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Summary

Eukaryotic cells produce a variety of specialized actin-rich surface protrusions. These include filopodia—thin, highly dynamic projections that help cells to sense their external environment [1]. Filopodia consist of parallel filaments of actin, bundled by actin cross-linking proteins. The filaments are oriented with their rapidly growing "barbed" ends at the protruding tip and their slowly growing "pointed" ends at the base [2]. Extension occurs by polymerization at the tip [3] and is controlled by regulation of filament capping [4]. The Rho GTPase Cdc42 is a key mediator of filopodia formation, which it regulates through binding CRIB domain-containing effectors [2]. Cdc42 binds and activates the WASP proteins, which in turn activate the actin-nucleating complex Arp2/3 [2]. It also binds and activates IRSp53, which recruits the Ena/WASP family protein Mena [5] to the filopodial tip and protects elongating actin filaments from capping [4]. Previously, we identified another Rho family GTPase, Rif, as a potent stimulator of filopodial protrusion through a mechanism that does not require Cdc42 [6]. Here we characterize the differences between filopodia induced by these two small GTPases and show that the Rif effector in this pathway is the Diaphanous-related formin mDia2. Thus, Rif and Cdc42 represent two distinct routes to the induction of filopodia—producing structures with both shared and unique properties.

Results and Discussion

Both Rif and Cdc42 have been shown to induce filopodia; however, when they are compared in the same system, clear differences emerge between the types of filopodia produced.

The filopodial projections induced by Cdc42 are generally short and often arise from the cell periphery (Figure 1 and [7, 8]). Rif-induced filopodia are generally much longer and also project from the apical surface of the cell—the cells present a "hairy" phenotype (Figure 1 and [6]). Rif-induced filopodia are not blocked by expression of a dominant-negative mutant of Cdc42 [6]. We extended these experiments to examine the effects of expression of the WASP CRIB domain, a construct that sequesters active Cdc42 [9]. Expression of the WASP CRIB (Cdc42/Rac interactive binding) domain completely blocked the induction of peripheral filopodia by Cdc42 (Figure 1E) but not by Rif (Figures 1C and 1E). Interestingly, expression of the WASP-CRIB domain alone itself caused an induction of apical filopodia (Figure 1E). Rif-induced filopodia were also not affected by expression of the SCAR-WA domain (Figure 1D), which binds to and sequesters the Arp2/3 complex (see Figure S1 in the Supplemental Data available with this article online; [10]). These data together lead us to conclude that Rif does not access the Cdc42/WASP/Arp2/3 pathway to stimulate filopodial protrusion.

In light of these results, we were interested to determine if Rif utilizes the Diaphanous-related formin mDia2 to induce filopodia. Like the Arp2/3 complex, the Diaphanous-related formins are capable of stimulating actin polymerization; however, unlike Arp2/3, they do so from the "barbed" end of actin filaments and produce unbranched, linear actin filaments in an Arp2/3-independent fashion [11–14]. It is therefore possible to imagine a situation in which an appropriately targeted mDia2 activity could produce the parallel linear actin filaments that form the body of filopodial projections. Indeed, recent work by Alberts and colleagues has shown that mDia2 binds to Cdc42 and that loss of mDia2 activity significantly reduces the ability of Cdc42 to induce filopodia [15].

We used the yeast two-hybrid system to test whether Rif is able to interact with mDia2. Wild-type Rif, as well as inactive Rif-TN and activated Rif-QL mutants, were coexpressed with an mDia2 construct known to interact with GTP bound RhoA [16]. This construct comprises codons 47–800, encompassing the GTPase binding domain (GBD) as well as the formin homology (FH) domains FH3 and FH1, part of the FH2 domain, but not the C-terminal Dia-autoregulatory domain (DAD) (Figure 2B; [17, 18]). Wild-type RhoA, RhoA-TN, and RhoA-QL all bound mDia2 (Figure 2A). Surprisingly, despite the weak similarity between Rif and RhoA [6], wild-type Rif, Rif-TN, and Rif-QL also all interacted strongly with mDia2 in this assay (Figure 2A).

The interaction between the GDP bound RhoA-TN mutant and mDia2 was unexpected but does not contradict published work. The GTP-dependent interaction between RhoA and mDia2 was originally demonstrated with the isolated GBD domain (amino acids 47–257 [16]), and the interaction between RhoA-TN and longer mDia2 constructs was apparently never tested. Moreover, evidence for interaction between GDP bound RhoA and the Diaphanous-related formin mDia1 has been reported [11, 19]. Thus, in a yeast two-hybrid assay, Rif can interact with mDia2 in a similar fashion to RhoA.

The interaction between Rif and mDia2 was further characterized in a series of mDia2 mutants. In the yeast two-hybrid assay, wild-type Rif and Rif-QL, but not Rif-TN, interact with the mDia2 GBD (amino acids 1–297), suggesting that, similar to RhoA [16], Rif interacts with the mDia2 GBD in a nucleotide-dependent fashion (Figure 2B). Cdc42 interacts with mDia2 through a CRIB-like motif within the GBD, and mutation of a critical

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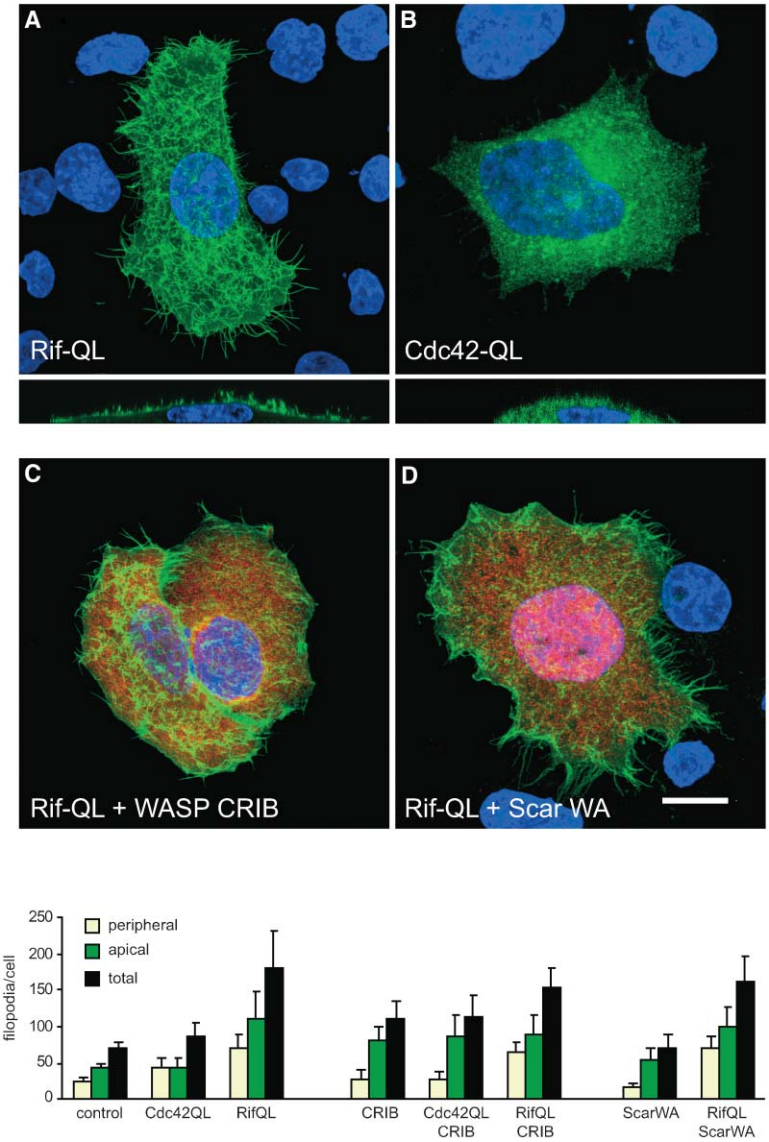

Figure 1. Rif Induces Filopodia Independently from the Cdc42/WASP/Arp2/3 Pathway

HeLa cells were transfected with (A) active Rif-QL (green) or (B) active Cdc42-QL (green) and costained with DAPI to show the nuclei (blue). The panels show projections of confocal xy sections, and a single xz slice through the broadest part of each cell. Both small GTPases induced numerous long filopodia; however, these project more prominently from the apical surface with Rif than with Cdc42. (C) Cells cotransfected with Rif-QL (green) and the WASP CRIB domain (red). (D) Cells cotransfected with Rif-QL (green) and the SCAR-WA domain (red). Scale bars represent 10 μm.

(E) Quantification of filopodial induction and distribution. Rif induced both peripheral and apical filopodia, whereas Cdc42 only significantly induced peripheral filopodia. Expression of the WASP-CRIB domain on its own induced apical filopodia. This construct inhibited the induction of peripheral filopodia by Cdc42 but not by Rif. Expression of the SCAR-WA domain had no effect on Rif-induced filopodia, although it blocked Rac induction of lamellipodia as expected (Figure S1). Data are the mean ± SD of n = 20 cells in each case.

residue in this motif (H160) prevents this interaction [15]. This mutation did not affect the interaction between Rif and the mDia2 GBD (Figure 2B), in agreement with previous evidence that Rif is not capable of binding CRIB motifs [6]. Finally, Rif does not interact with an mDia2 mutant lacking the GBD (amino acids 297–1171; Figure 2B). Taken together, these data suggest that Rif makes a GTP-dependent interaction with the isolated mDia2 GBD. The inactive Rif-TN construct does not interact with the mDia2 GBD domain (amino acids 1–297), nor with the mDia2 ΔGBD construct (amino acids 297–1171), but it does interact with mDia2 (amino acids 47–800). The reasons for this are unclear; one possibility is that a second nucleotide-independent binding site is present in mDia2 and that this binding site is disrupted when the GBD domain is isolated from the rest of the mDia2 protein.

In order to confirm the interaction between Rif and mDia2, we used an immunoprecipitation-based assay. Cos-7 cells were electroporated to introduce expression vectors encoding Myc-tagged mDia2 together with HA-tagged wild-type Rif, HA-Rif-TN, or HA-Rif-QL. Full-length mDia2 failed to coprecipitate with immunoprecipitated Rif (Figure 2C), and we also failed to see an interaction between mDia2 and active RhoA by this method (data not shown). mDia2 makes a strong autoinhibitory interaction between its GBD and its C-terminal DAD domain [16], and disruption of this interaction in vitro requires the addition of a 20-fold molar excess of active RhoA [17]. We therefore generated an mDia2 deletion mutant lacking the C-terminal DAD (mDia2 ΔDAD; amino acids 1–1130) and tested the ability of Rif to interact with this truncated protein. As shown in Figure 2D, Rif coimmunoprecipitates with mDia2 ΔDAD. This interaction is independent of the nucleotide status of Rif, in agreement with the data from the yeast two-hybrid interaction assays. We conclude that the interaction between Rif and mDia2 is strongly competed by the auto-inhibitory DAD domain to the extent that this interaction in not stable under conditions of extraction and immunoprecipitation.

To test the potential involvement of mDia2 in Rif-

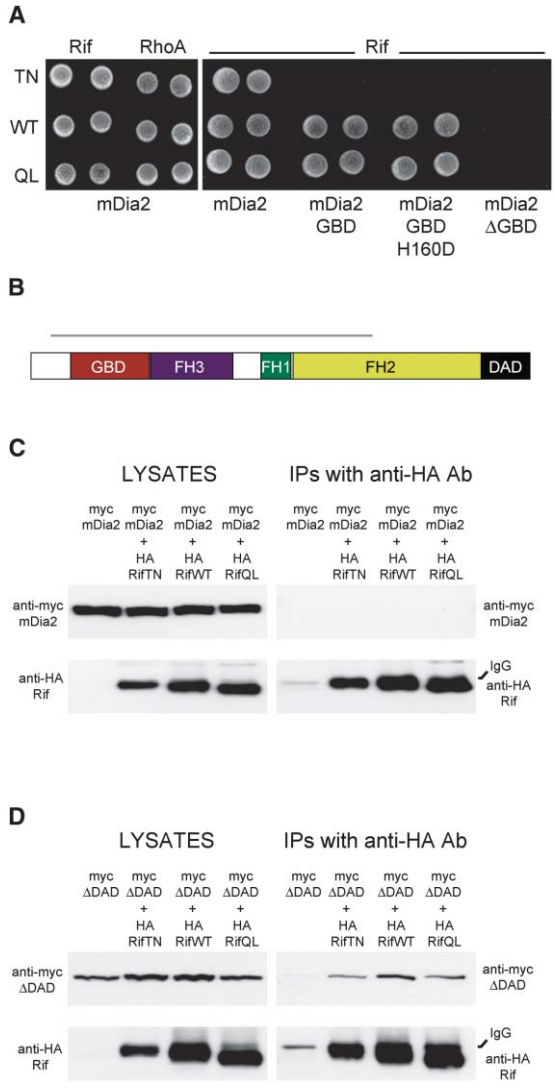

Figure 2. Rif Interacts with mDia2

(A) Interaction of RhoA and Rif with mDia2 was measured with a yeast two-hybrid assay by growth on Trp/Leu/His/Ade-deficient medium. Plasmids encoding constitutively GDP bound (TN), wild-type (WT), or constitutively GTP bound (QL) Rif or RhoA fused to the Gal4-DNA binding domain (BD) were transfected into AH109 yeast cells together with mDia2 fused to the Gal4 activation domain (AD). The mDia2 construct comprises codons 47–800, as shown by the bars drawn above the diagrammatic representations of mDia2 domains (B). Duplicate colonies are shown for each combination. The interaction between Rif-TN, Rif-WT and Rif-QL and various mDia2 mutations and truncations was also determined. The mDia2 constructs are mDia2 (amino acids 47–800), mDia2 GBD (amino acids 1–297), mDia2 GBD H160D, whose CRIB motif has been disrupted by the H160D substitution, and mDia2 ΔGBD (amino acids 297–1171). Duplicate colonies are shown for each combination.

(B) mDia2 domains include the N-terminal GTPase binding domain (GBD), the formin homology (FH) domains FH3, FH1, and FH2, and the C-terminal Dia-autoregulatory domain (DAD).

(C) Myc-tagged full-length mDia2 was expressed in Cos-7 cells together either with empty pcDNA3 vector or with HA-tagged Rif-TN, Rif-WT, or Rif-QL. Samples of cell lysates were taken to confirm expression of the various proteins (left panels). Rif was immunoprecipitated with immobilized anti-HA antibody, and coprecipitating mDia2 was detected by Western blotting (right panels). No interaction could be detected between Rif and full-length mDia2.

(D) Myc-tagged mDia2 truncated to remove its DAD domain was expressed in Cos7 cells together with Rif mutants as above. Samples of cell lysates were taken to confirm expression of the various proteins (left panels). Rif was immunoprecipitated with immobilized anti-HA antibody, and coprecipitating mDia2 was detected by Western blotting (right panels). The truncated mDia2 ΔDAD coprecipitated with all three Rif constructs, including the GDP bound Rif-TN.

induced filopodia, we carried out cotransfection studies in NIH 3T3 cells, which do not express detectable levels of endogenous mDia2 but which do express mDia1 [20]. Expression of active Rif in these cells did not lead to significant filopodia formation; instead, Rif was concentrated to points of focused staining at the apical surface (Figure 3B). When expressed on its own, full-length mDia2 was cytoplasmic and had no significant effect on actin morphology (Figure 3A); however, when active Rif and mDia2 were coexpressed, mDia2 was translocated to the plasma membrane, and the full Rif filopodial phenotype was produced (Figure 3C). In HeLa cells, expression of a dominant-negative mDia2 mutant [15] blocked induction of filopodia by active Rif (Figure S2). We conclude that mDia2 is required for Rif induction of filopodia.

When Rif-QL and full-length mDia2 were coexpressed in NIH 3T3 cells, an enrichment of mDia2 at the tip of filopodia was clearly visible in some cells (Figures 3C and 3D). In other cells, both proteins were evenly distributed along the length of the protrusions (Figure 3E). Localization correlated broadly with expression level; mDia2 was concentrated at the filopodia tip in low-expressing cells and delocalized along the filopodia in high expressers (data not shown). Localization of mDia2 at filopodia tips has been observed previously [15] and correlates well with a model in which mDia2 binds to the "barbed" ends of actin filaments and functions as a high-affinity but partial, or "leaky," cap [21]. Although the inactive Rif-TN mutant bound to mDia2 equally well as the activated Rif-QL (Figure 2), it did not stimulate filopodial protrusion (Figure 3F). We conclude that Rif exhibits nucleotide-independent binding to mDia2 but that full activation of mDia2 requires GTP-Rif.

Finally, we compared the effects of Rif/mDia2 with those of Cdc42/mDia2. In agreement with previous studies [15], mDia2 was seen at the tips of Cdc42-induced filopodia (Figures 3G and S3), but coexpression of an activated Cdc42 mutant with mDia2 did not potentiate filopodia formation (Figure 3G). Furthermore, mutation of the Cdc42 binding site in mDia2 did not affect Rif/mDia2-induced filopodia (Figure 3H). The filopodia observed with activated Cdc42 were markedly different to those induced by Rif—they were short and confined to the periphery of the cell (compare Figures 3G and 3E). Hence, under equivalent cellular conditions, and in the presence of mDia2, Rif and Cdc42 produce morphologically distinct filopodial projections.

We have identified mDia2 as the first downstream effector of the Rif GTPase and shown that it is required for Rif induction of filopodia. We cannot preclude the possibility that other cellular activities also contribute to this process—by analogy with the multiple actions of Cdc42, this would seem highly likely. The mechanisms by which filopodia form are still only partially resolved. Svitkina and colleagues have proposed a model for the

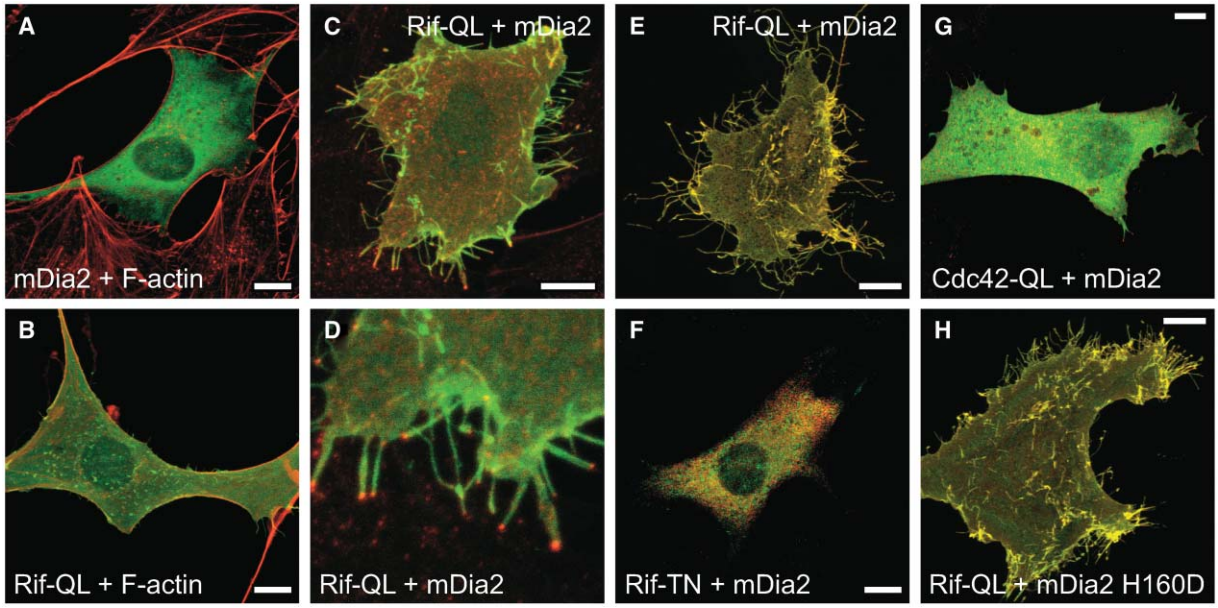

Figure 3. mDia2 Is Required for the Formation Rif-Induced Filopodia

The Figure shows NIH 3T3 cells transfected with the following constructs and then immunostained with the appropriate antibodies:
(A) Full-length mDia2 (green), costained for F-actin (red).
(B) Rif-QL (green), costained for F-actin (red).
(C, D, and E) Rif-QL (green) and full-length mDia2 (red). (D) is a 2.5× magnified section of (C) showing the concentration of mDia2 to filopodia tips seen in cells expressing a low concentration of mDia2. In cells with higher levels of mDia2 (E), Rif and mDia2 were evenly dispersed along the filopodia. Coexpression of active Rif-QL with mDia2 lead to induction of numerous long filopodia in 82% of cells; compared to 32% with Rif-QL alone and 0% with mDia2 alone ($n \geq 30$).
(F) Rif-TN (green) and full-length mDia2 (red).
(G) The constitutively active Cdc42-QL mutant (green) and full-length mDia2 (red).
(H) Rif-QL (green) and mDia2 H160D (red).
The scale bars represent 10 μm.

initiation of the short filopodia, or "microspikes," that arise from lamellipodia at the periphery of the cell [22]. In this model, filaments within the dendritic array of lamellipodial actin coalesce to form a cone-shaped initiation structure. This is followed by binding of proteins such as VASP to the barbed ends of these filaments to protect them from capping and allow elongation. Filament bundling would then occur through recruitment of the actin crosslinking protein Fascin [22]. In this model, the Arp2/3 complex would play an initiating role in filopodia formation because it would be required for production of the dendritic actin array from which these structures arise.

These experimental observations on microspike formation seem to relate better to filopodia induced by Cdc42 than to those induced by Rif. Rif-induced filopodia do not preferentially arise from lamellipodia and are generally associated with the apical cell surface. This would explain their independence from Arp2/3 and suggests that an alternative initiation event is required. The ability of membrane-associated formins such as mDia2 to elongate parallel actin filaments from the barbed end [11–14] suggests one way in which this could happen. In this respect, it is particularly intriguing to see the concentration of mDia2 to the tips of Rif-induced filopodia. One would imagine that conversion of mDia2-induced actin filaments into filopodia (as opposed to, for example, actin stress fibers) would require additional activities—the activities of actin crosslinking proteins, as well as some mechanism to anchor the nascent filaments into the subplasmalemmal actin cytoskeleton.

The key point is that pathways controlled by Rif and Cdc42 produce different kinds of filopodial protrusions. Cells produce a wide range of specialized filopodia and filopodia-like protrusions, from the apical microvilli of absorptive epithelial cells to the highly ordered stereocilia of cochlear hair cells. With each of these structures, the modification of a basic form adapts them to a specific biological function. The differences between Rif and Cdc42 show how such specializations might be controlled. By relating these signaling pathways to biological functions, we can begin to categorize and dissect this family of protrusive structures.

Experimental Procedures

Constructs and Antibodies

The polyclonal anti-Rif antibody has been described [6]. Mouse monoclonal antibodies to the Myc epitope (9E10), HA epitope (HA11), and goat polyclonal anti-Cdc42 antibodies were from Santa Cruz, BAbCO, and Santa Cruz, respectively. Cy2-, Cy3-, and Cy5-conjugated secondary antibodies were from Jackson Laboratories. Mammalian expression vectors encoding the SCAR-WA domain, the WASP CRIB domain, and mDia2 were from Laura Machesky, Kate Nobes, and Art Alberts, respectively. Yeast two-hybrid vectors encoding mDia2 were from Erik Sahai; RhoA vectors were from Pontus Aspenstrom. All other constructs were generated by stan-

dard methods. A full list of constructs is included in the Supplemental Data.

Cell Culture and Immunofluorescence Microscopy

HeLa and NIH 3T3 cells were cultured onto acid-washed glass coverslips in DMEM supplemented with 10% FBS and allowed to adhere overnight. Cells were transfected using Lipofectamine 2000 (HeLa, Invitrogen) or Transfast lipids (NIH 3T3, Promega) according to the manufacturer's protocols. Cells were incubated overnight prior to preparation for confocal epifluorescence microscopy (Supplementary Methods). Where applicable, F-actin was stained using phalloidin labeled with Alexa 488 or 594 (Molecular Probes). All Figures show projected confocal stacks, unless otherwise stated.

Yeast two-hybrid assays

AH109 yeast cells were transfected with RhoA and Rif pYTH6 plasmids as described previously [23]. The pYTH6 plasmids were integrated into the yeast genome and transformants were subsequently transfected with pGAD constructs encoding potential interacting proteins [23]. Double transformants were selected on Leu/Trp deficient agar plates. After 4 days, 2 independent cultures were grown overnight in Leu/Trp deficient medium and plated onto Leu/Trp deficient plates to check for viability and onto Leu/Trp/Ade/His deficient plates to test for protein-protein interaction. Growth on Leu/Trp/Ade/His deficient plates was assessed after 3-5 days.

Immunoprecipitations

Cos-7 cells were transfected by electroporation. After growth overnight, cells (approximately 10^7 cells/condition) were broken on ice with 1ml lysis buffer (20 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 2 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Lysates were clarified by centrifugation at 15,000 $\times$ g for 10 min. Proteins were immuno-precipitated at 4°C by incubation with 1μg anti HA-antibody for 1 hr, followed by incubation with 30 μl Protein G Sepharose beads (packed bead volume) for 1 hr. The beads were washed 3 times in ice-cold lysis buffer and the bound protein eluted by heating at 95°C with SDS-PAGE sample buffer. Immunoprecipitated proteins were analyzed by western blotting.

Supplemental Data

Supplemental Experimental Procedures, three figures, and a list of constructs are available with this article online at http://www.current-biology.com/cgi/content/full/15/2/129/DC1/.

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References

1. Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., and Horwitz, A.R. (2003). Cell migration: Integrating signals from front to back. Science *302*, 1704–1709.
2. Welch, M.D., and Mullins, R.D. (2002). Cellular control of actin nucleation. Annu. Rev. Cell Dev. Biol. *18*, 247–288.
3. Mallavarapu, A., and Mitchison, T. (1999). Regulated actin cytoskeleton assembly at filopodium tips controls their extension and retraction. J. Cell Biol. *146*, 1097–1106.
4. Bear, J.E., Svitkina, T.M., Krause, M., Schafer, D.A., Loureiro, J.J., Strasser, G.A., Maly, I.V., Chaga, O.Y., Cooper, J.A., Borisy, G.G., et al. (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. Cell *109*, 509–521.
5. Krugmann, S., Jordens, I., Gevaert, K., Driessens, M., Vandekerckhove, J., and Hall, A. (2001). Cdc42 induces filopodia by promoting the formation of an IRSp53:Mena complex. Curr. Biol. *11*, 1645–1655.
6. Ellis, S., and Mellor, H. (2000). The novel rho-family GTPase Rif regulates coordinated actin-based membrane rearrangements. Curr. Biol. *10*, 1387–1390.
7. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol. Cell. Biol. *15*, 1942–1952.
8. Nobes, C.D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell *81*, 53–62.
9. Nobes, C.D., and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. *144*, 1235–1244.
10. Machesky, L.M., and Insall, R.H. (1998). Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. Curr. Biol. *8*, 1347–1356.
11. Li, F., and Higgs, H.N. (2003). The mouse formin mDia1 is a potent actin nucleation factor regulated by autoinhibition. Curr. Biol. *13*, 1335–1340.
12. Pruyne, D., Evangelista, M., Yang, C., Bi, E., Zigmond, S., Bretscher, A., and Boone, C. (2002). Role of formins in actin assembly: nucleation and barbed-end association. Science *297*, 612–615.
13. Evangelista, M., Pruyne, D., Amberg, D.C., Boone, C., and Bretscher, A. (2002). Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. Nat. Cell Biol. *4*, 260–269.
14. Zigmond, S.H. (2004). Formin-induced nucleation of actin filaments. Curr. Opin. Cell Biol. *16*, 99–105.
15. Peng, J., Wallar, B.J., Flanders, A., Swiatek, P.J., and Alberts, A.S. (2003). Disruption of the Diaphanous-related formin Drf1 gene encoding mDia1 reveals a role for Drf3 as an effector for Cdc42. Curr. Biol. *13*, 534–545.
16. Alberts, A.S., Bouquin, N., Johnston, L.H., and Treisman, R. (1998). Analysis of RhoA-binding proteins reveals an interaction domain conserved in heterotrimeric G protein beta subunits and the yeast response regulator protein Skn7. J. Biol. Chem. *273*, 8616–8622.
17. Alberts, A.S. (2001). Identification of a carboxyl-terminal diaphanous-related formin homology protein autoregulatory domain. J. Biol. Chem. *276*, 2824–2830.
18. Wallar, B.J., and Alberts, A.S. (2003). The formins: Active scaffolds that remodel the cytoskeleton. Trends Cell Biol. *13*, 435–446.
19. Watanabe, N., Kato, T., Fujita, A., Ishizaki, T., and Narumiya, S. (1999). Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat. Cell Biol. *1*, 136–143.
20. Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S.A., and Alberts, A.S. (2000). Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. Mol. Cell *5*, 13–25.
21. Zigmond, S.H., Evangelista, M., Boone, C., Yang, C., Dar, A.C., Sicheri, F., Forkey, J., and Pring, M. (2003). Formin leaky cap allows elongation in the presence of tight capping proteins. Curr. Biol. *13*, 1820–1823.
22. Svitkina, T.M., Bulanova, E.A., Chaga, O.Y., Vignjevic, D.M., Kojima, S., Vasiliev, J.M., and Borisy, G.G. (2003). Mechanism of filopodia initiation by reorganization of a dendritic network. J. Cell Biol. *160*, 409–421.
23. Aspenstrom, P., and Olson, M.F. (1995). Yeast two-hybrid system to detect protein-protein interactions with Rho GTPases. Methods Enzymol. *256*, 228–241.