The small GTPase Rif is an alternative trigger for the formation of actin stress fibers in epithelial cells

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Summary
Actin stress fibers are fundamental components of the actin cytoskeleton that produce contractile force in non-muscle cells. The formation of stress fibers is controlled by the small GTPase RhoA and two highly related proteins, RhoB and RhoC. Together, this subgroup of actin-regulatory proteins represents the canonical pathway of stress-fiber formation. Here, we show that the Rif GTPase is an alternative trigger of stress-fiber formation in epithelial cells. Rif is distantly related to RhoA; however, we show that the two proteins share a common downstream partner in stress-fiber formation – the Diaphanous-related formin mDia1. Rif-induced stress fibers also depend on the activity of the ROCK protein kinase. Unlike RhoA, Rif does not raise ROCK activity in cells, instead Rif appears to regulate the localization of myosin light chain phosphorylation. This study establishes Rif as a general regulator of Diaphanous-related formins and shows how non-classical Rho family members can access classical Rho pathways to create new signaling interfaces in cytoskeletal regulation.

Key words: Actin stress fibers, Stress fibres, Formin, Rho GTPase, Contractile

Introduction
Actin stress fibers are long contractile bundles of actin filaments and myosin II that span the body of non-muscle cells. Their organization is broadly similar to that of the sarcomeric arrays of muscle cells, but generally loosen and less uniform. Actin stress fibers are linked mechanistically to the extracellular matrix through integrin-rich contacts at the plasma membrane, allowing the cell to apply contractile force to the substratum. This contractile force is used to power retraction of the cell body during migration; however, it also allows cells to remodel the extracellular matrix – a function that is important during development and in wound healing (Pellegrin and Mellor, 2007).

Unlike the sarcomeric actomyosin arrays of muscle cells, actin stress fibers are dynamic and their formation is controlled by a wide range of cellular stimuli, most notably the rigidity of the substratum (Couchman and Rees, 1979; Mohitrate et al., 1991). Early work identified RhoA as a crucial regulator of actin-stress-fiber formation. RhoA is a member of the Rho family of small GTPases, which are signaling proteins with a central role in regulation of the actin cytoskeleton. Expression of active RhoA in cells provokes strong stress-fiber formation (Paterson et al., 1990) and RhoA controls the formation of stress fibers in response to a wide-range of extracellular stimuli (Pellegrin and Mellor, 2007; Ridley and Hall, 1992). In vertebrates, the gene encoding RhoA has undergone duplication to give two additional isoforms: RhoB and RhoC. These two proteins are highly related to RhoA (approximately 85% identity) and also trigger stress-fiber formation (Giry et al., 1995). For simplicity, we will refer to this group as the RhoA-like proteins.

RhoA-like proteins control two activities required for the formation of actin stress fibers – nucleation of actin filaments and phosphorylation of myosin light chain 2 (MLC2). Activated RhoA binds to the Diaphanous-related formin mDia1 (DIAPH1) through its G-protein binding domain (GBD) (Watanabe et al., 1997). Binding to the GBD releases an autoinhibitory interaction in mDia1 and allows nucleation of new F-actin filaments (Alberts, 2001; Watanabe et al., 1999). Activated RhoA also binds to the ROCK/ROK protein kinase (Ishizaki et al., 1996; Leung et al., 1995). ROCK can directly phosphorylate MLC2 at Ser19 (Amano et al., 1996). It also phosphorylates MYPT/MBS, the regulatory subunit of myosin phosphatase. Phosphorylation of MYPT inhibits phosphatase activity, leading to an increase in phosphorylated MLC (Kawano et al., 1999; Kimura et al., 1996). Phosphorylation of MLC increases stress-fiber contractility by increasing myosin ATPase activity (Katoh et al., 2001) and leads to increased bundling of actin filaments in the stress fiber. Activation of ROCK produces thick stress fibers, but these are poorly organized (Leung et al., 1996). Activation of mDia1 produces parallel actin filaments, which are weakly bundled (Watanabe et al., 1997). A combination of both signals is needed for proper stress-fiber formation (Watanabe et al., 1999).

The Rif GTPase is a relatively recent addition to the Rho family; it is found only in chordates and displays a relatively low homology to other family members (Ellis and Mellor, 2000). Previously, we showed that Rif controls the formation of filopodia through the Diaphanous-related formin mDia2 (DIAPH3) (Pellegrin and Mellor, 2005). Filopodia are thin, actin-rich projections that are used by cells to sense their external environment (Mellor, 2009). The canonical pathway for their formation is through the Rho family member Cdc42 (Kozma et al., 1995; Nobes and Hall, 1995). Rif is an alternative trigger for filopodia formation, producing filopodia with different characteristics to those triggered by Cdc42 (Pellegrin and Mellor, 2005). Intriguingly, recent work has shown that Cdc42 and Rif work together in the formation of dendritic spines, which develop from dendritic filopodia (Hotulainen et al., 2009).

Here, we show that Rif also controls the formation of epithelial stress fibers. Rif-induced stress fiber formation depends directly on mDia1 and indirectly on the activity of ROCK. This dual role for Rif demonstrates how non-classical Rho GTPases can interface with
the ancient pathways of cytoskeletal regulation to create new combinations and new morphological outcomes.

**Results**

**Rif stimulates formation of actin stress fibers**

Activated Rif triggers the formation of numerous long filopodia that project from the apical surface of the cell (Ellis and Mellor, 2000). These structures collapse upon fixation, making it difficult to see underlying F-actin structures in the cell body. Confocal sectioning of cells expressing an activated Rif mutant (Rif-QL) revealed strong stimulation of actin stress fiber formation at the base of the cell (Fig. 1A). These stress fibers were indistinguishable from those induced by activated RhoA in these cells (supplementary material Fig. S1); however, they were not dependent on RhoA for formation, because dominant-negative RhoA had no effect on the ability of Rif to induce stress fibers (Fig. 1B). The defining feature of contractile actin stress fibers is the presence of non-muscle myosin II in a loose sarcomeric array (Pellegrin and Mellor, 2007). Rif-induced stress fibers showed clear incorporation of myosin in a characteristic striated pattern (Fig. 1C). At present, there are no known activators of Rif, meaning that we were not able to drive activation of endogenous Rif protein and measure the effect on the actin cytoskeleton; however, silencing of the expression of endogenous Rif using RNA interference led to a significant decrease in actin stress fibers under basal conditions (supplementary material Fig. S2), suggesting that Rif contributes to the formation of actin stress fibers, even in resting epithelial cells.

Rif-induced stress fibers differed from RhoA-induced stress fibers in two ways. Unlike RhoA, the effect of Rif on stress-fiber formation was restricted to cell lines of an epithelial origin, and Rif did not induce stress fibers in fibroblasts (data not shown). This is similar to Rif-induced filopodia, which also are not observed in fibroblasts (Ellis and Mellor, 2000). RhoA stimulates stress fiber formation in parallel with an increase the size and maturity of focal adhesions (Ridley and Hall, 1992). Rif activation had a variable effect on focal adhesion formation, dependent on epithelial cell type, with an increase in the number of focal adhesions in MDCK cells, but no obvious effect in HeLa cells (Fig. 1A).

**Rif-induced stress fibers are dependent on ROCK activity**

ROCK is an important regulator of RhoA-induced stress fibers (Leung et al., 1996). We examined the effect of inhibiting ROCK activity on Rif-induced stress fibers by silencing endogenous ROCK1 and ROCK2 using RNA interference. Reduction of either isoform led to a significant decrease in Rif-induced stress fibers (supplementary material Fig. S2). As we were unable to achieve >50% silencing of the ROCK isoforms, we examined the effects of using the specific ROCK inhibitor Y-27632 (Uehata et al., 1997) to achieve a complete block of ROCK activity. Inhibition of ROCK returned stress-fiber levels to control values in cells expressing either wild-type Rif or the activated Rif-QL mutant (Fig. 2A,B). RhoA activates ROCK by direct interaction with a binding site in the C-terminal region of the protein (Dvorsky et al., 2004; Fujisawa et al., 1996). We therefore examined whether ROCK was also a binding partner for Rif. ROCK1 showed a clear interaction with both wild-type Rif and the activated Rif-QL mutant (Fig. 2C); however, despite many attempts, we were unable to demonstrate an interaction between Rif and ROCK1 by immunoprecipitation (Fig. 2D), or with a truncated mutant of ROCK that lacks the C-terminal autoinhibitory motif (data not shown).

Rif-induced stress fibers showed no obvious ROCK activity under conditions where formation of stress fibers is provoked.

**Fig. 1. Rif triggers formation of stress fibers.** (A) HeLa and MDCK cells were transfected with the activated Rif-QL mutant and stained for Rif (top panels), F-actin (green) and vinculin (red). The top panels show full projections through the cells, the bottom panels show projected images of 4×0.5 μm sections at the base of the cells. Activated Rif stimulated the formation of basal stress fibers in both cell lines. An increase in focal adhesion size was seen in MDCK cells, but not in HeLa cells. (B) Cells were transfected with the activated Rif-QL mutant (green) and dominant-negative RhoA (RhoA-T19N; red). Cells were stained for F-actin using Alexa Fluor 660 Phalloidin (bottom panel). Inhibition of RhoA had no effect on the induction of stress fibers by Rif. (C) Cells were transfected with the activated Rif-QL mutant (blue) and GFP-myosin II (green) and stained for F-actin (red). Myosin II shows a periodic staining pattern along Rif-induced stress fibers. Scale bars: 10 μm.
Rif triggers stress-fiber formation through mDia1

We used RNA interference to test the role of mDia1 in Rif-induced stress fiber formation. We used two independent siRNA oligonucleotides against DIAPH1, the gene encoding mDia1, each of which gave significant downregulation of mDia1 (Fig. 5C). Silencing of DIAPH1 had a drastic effect on the ability of Rif to induce stress fiber formation (Fig. 4A), reducing stress fibers to almost control levels (Fig. 4B). mDia1 has been reported to be a component of focal adhesions, which are the sites of stress fiber initiation and attachment (Hotulainen and Lappalainen, 2006). This has led to the proposal that mDia1 seeds actin stress fibers through nucleation of actin filaments at focal adhesion sites. Activated Rif triggers the translocation of mDia2 to the tips of Rif-induced filopodia (Pellegrin and Mellor, 2005), which is consistent with a model for filopodial extension where mDia2 seeds actin filaments formation from the filopodial tip. We were interested to determine whether activated Rif altered the cellular localization of mDia1, and whether we could see any evidence of co-localization of Rif, mDia1 and stress-fiber formation.

Fig. 3. mDia1 is a Rif binding partner. (A) Interaction of Rif with the GBDs of the three Diaphanous-related formins was measured in a yeast two-hybrid assay by growth on Trp/Leu/His/Ade-deficient medium in the presence 2 mM 3-amino-1,2,4-triazole. Plasmids encoding inactive (TN), wild-type (WT) or activated (QL) Rif or RhoA fused to the Gal4-DNA binding domain were transfected into AH109 yeast cells together with the respective GBD domains fused to the Gal4 activation domain. The interaction between RhoA and the GBD of rhophilin1 was used as a control. Duplicate colonies are shown for each combination. Rif interacted with all three Diaphanous-related formins in this assay, but not with rhophilin1. (B) Cells were transfected with the activated FLAG-epitope tagged mDia1ΔADAD mutant and either empty vector, wild-type Rif, the inactive Rif-TN mutant or the activated Rif-QL mutant. mDia1 was isolated from cell lysates by immunoprecipitation and these samples were probed for co-precipitation of Rif by western blotting. Samples of cell lysates were taken to confirm expression of the various proteins. mDia1 was isolated from cell lysates by immunoprecipitation and these samples were probed for co-precipitation of Rif by western blotting. Samples of cell lysates were taken to confirm expression of the various proteins. mDia1 was isolated from cell lysates by immunoprecipitation and these samples were probed for co-precipitation of Rif by western blotting. Samples of cell lysates were taken to confirm expression of the various proteins.
attachment sites. Co-transfection of mDia1 with activated Rif led to a redistribution of mDia1 (Fig. 4C). We did not observe any concentration of Rif or mDia1 at the stress-fiber attachment site, either when transfected together (Fig. 4D), or separately (data not shown). Instead, we were surprised to see re-localization of mDia1 to Rif-induced filopodia. As with mDia2, we saw localization of mDia1 to filopodia tips. We also occasionally saw punctuate staining of mDia1 along the filopodial shaft (Fig. 4C,D).

**Rif-induced blebbing in the absence of mDia1**

We observed that silencing of DIAPH1 led not only to a loss of Rif-induced stress fibers, but also to profound changes in Rif-induced filopodia. Filopodia were still present; however, they were distended to form bleb-like structures at the apical surface (Fig. 5A). Membrane blebbing is driven by actomyosin contractility in the cell cortex (Charras, 2008). ROCK triggers bleb formation by increasing MLC phosphorylation at the bleb site; it is also a physiological regulator of membrane blebbing during apoptosis (Coleman et al., 2001; Sebbagh et al., 2001) and is involved in cell migration through 3D matrices (Sahai and Marshall, 2003). Interestingly, the Rif-induced blebs showed focussed staining of phosphorylated MLC at their bases (Fig. 5A). Previous studies have shown that there is an antagonistic relationship between mDia1 and ROCK (Sahai and Marshall, 2002). We wondered whether loss of mDia1 was freeing Rif for interaction with ROCK and hence driving bleb formation. In apparent agreement with this, bleb formation by Rif in the absence of mDia1 was dependent on ROCK activity (Fig. 5B). We examined the total cellular activity of ROCK by measuring the phosphorylation of MYPT and MLC2 — the two relevant substrates for ROCK in actomyosin contractility. Rif activation did not increase the total level of phosphorylation of either protein (Fig. 5C), either in the presence of endogenous mDia1 (stress fiber

![Figure 4](image4.png)

**Fig. 4. Rif triggers stress fiber formation through mDia1.** (A) HeLa cells treated with siRNA against DIAPH1 or against a control gene (LMNA, which encodes lamin). The cells were then transfected with the activated Rif-QL mutant. Representative cells are shown stained for F-actin and the positions of the Rif-transfected cells are indicated (arrows). Silencing of DIAPH1 leads to a loss of Rif-induced stress fibers. Scale bar: 10 μm. (B) Cells were treated without siRNA, with control siRNA or with two independent DIAPH1 siRNAs (mDia1A and mDia1B). The cells were then transfected with activated Rif-QL, or with empty vector. Cells were fixed, stained for F-actin and scored (100 cells for each condition) for the presence of actin stress fibers. Data are mean ± s.e.m. (n=3). (C) Localization of mDia1 in cells expressing active Rif. HeLa cells were transfected with mDia1 (green) and the activated Rif-QL mutant (blue) and co-stained for F-actin (red). The panel on the right shows the separate channels at a region of the cell where the stress fibers meet the plasma membrane. There was no concentration of Rif or mDia1 on stress fibers or at their attachment points. Scale bar: 10 μm. (D) Rif causes translocation of mDia1 to filopodia. HeLa cells were transfected with mDia1 (green) either alone (left panel) or together with the activated Rif-QL mutant (red, right panels). The right-hand panels show a magnified region of filopodia showing mDia1 at the tips. In the presence of activated Rif, mDia1 undergoes translocation to the tips of filopodia and to puncta along the filopodial shaft. Scale bar: 10 μm.

![Figure 5](image5.png)

**Fig. 5. Rif-induced blebbing in the absence of mDia1.** (A) HeLa cells were transfected with DIAPH1 siRNA and then with an expression plasmid encoding the Rif-QL mutant. In the absence of mDia1, Rif activation led to the formation of bleb-like projections from the apical surface. These showed concentrated staining for Rif (green) and phosphorylated MLC (red). A-Z projections through the body of the cells show that phosphorylated MLC is concentrated at the base of these apical blebs (top panels). Scale bar: 10 μm. (B) HeLa cells were treated with a control siRNA or with DIAPH1 siRNA. The cells were then transfected with activated Rif-QL, or with empty vector. Cells were then treated with or without 10 μM Y-27632 for 2 hours. Cells were fixed, stained for F-actin and scored (100 cells for each condition) for the presence of apical blebs. Cells were counted as positive for blebs if they contained more than ten blebs and negative if they contained fewer than two blebs. There were no cells that showed intermediate numbers of blebs. Essentially identical results were obtained with a second DIAPH1 siRNA. Data are means ± s.e.m. (n=3). (C) HeLa cells were treated with control siRNA or with two independent DIAPH1 siRNAs. The cells were then transfected with activated Rif-QL, or with empty vector. The phosphorylation of the ROCK1 substrates MYPT and MLC was probed by western blotting of whole-cell extracts. Rif activation did not change the total cellular levels of phosphorylated MYPT or phosphorylated MLC, in the either the presence of absence of mDia1.
RhoA, Rac and Cdc42 are relatively ancient signaling proteins, and all three are present in fungi, worms and flies. One can imagine two ways in which the newer Rho family members might add functionality to cytoskeletal regulation – either through controlling new signaling pathways, or by making novel interactions with the existing pathways. Rif illustrates the second possibility: the pathways that Rif controls are old, but the interface with those pathways is new and different. Many of the complex mechanical movements of cells are created through co-ordinated regulation of actin-regulatory pathways by Rho GTPases. The emergence of the non-classical human Rho GTPases parallels the development of the adaptive immune system, increasing complexity of the nervous system and a host of other processes requiring complex regulation of cell shape and cell migration. It seems highly likely that it is in these processes that we will find non-classical Rho GTPases, such as Rif.

**Materials and Methods**

**Reagents**

A polyclonal antibody against Rif was raised in sheep using the synthetic peptide MDAPALGATAPGPIKGKLNC-NH₂ corresponding to the N-terminus of the human sequence. A polyclonal antibody against the myc epitope tag was raised against the synthetic peptide MEQKLISEEDLGC-NH₂. In each case, the antibodies were affinity purified using the immunising peptide coupled to SulfoLink resin (Pierce). Monoclonal antibodies against vinculin (VIN-11-5), Flag epitope tag (M2) and tubulin (B-5-1-2) were from Sigma. Polyclonal rabbit anti-phospho-MLC (Ser19) was from Cell Signaling Technologies. Polyclonal rabbit anti-phospho-MYPT (Thr696) was from Upstate. Rabbit polyclonal anti-mDia1 was from Abcam. Rabbit polyclonal anti-HA epitope tag and monoclonal anti-myc epitope antibodies (9E10) were from Santa Cruz. Y-27632 was from Calbiochem. siRNA oligonucleotides duplexes were from Eurofins. Fluorescent secondary antibodies were raised in donkey and purchased from Invitrogen (Alexa Fluor conjugated) or Stratech Scientific (Cy conjugated). Alexa Fluor 488-phallolidin was from Invitrogen. A full list of cDNA constructs is included in supplementary material Table S1.

**Cell culture and transfection**

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated bovine fetal calf serum, 100 μg/ml streptomycin sulfate and 100 μM benzylpenicillin. Cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. siRNA oligonucleotides were introduced into HeLa cells using calcium phosphate. Briefly, cells were cultured in six-well dishes until 70% confluent (approximately 10⁶ cells). Transfection solutions were prepared by mixing 4 μl of 20 μM siRNA in 100 μl 0.25 M CaCl₂ solution with 100 μl 50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na₃HPO₄. After a 15 minute incubation, this suspension was added drop-wise to the dish. Samples were incubated at 37°C and 3% CO₂ overnight. The medium was then replaced twice and samples returned to 37°C and 5% CO₂ for a further 48 hours. The sequences of the siRNA oligonucleotides were as follows: Lamin A/C, GGUGGUGACGACUCUGGGCUC; mDia1A, GAAGUUGGUUCGUGUAGAGA; mDia1B, AGAAuccGAGACuAGuGACuA; Rif, GCuGACGAGuGiuGiuCUCuA; ROCK1, GCuAACuGUuACuACAGuGAT; ROCK2, GCAuAAGuUAuAAuA-ACuGTT.

**Immunofluorescence microscopy**

Cells were prepared for confocal immunofluorescence microscopy by fixation with paraformaldehyde and permeabilization with Triton X-100, as described previously (Pellegrin and Mellor, 2005). Confocal microscopy was performed using a Leica AOBSP SB confocal laser-scanning microscope with an attached Leica DMIRE2 inverted epifluorescence microscope under a Plan Apo BL ×63/1.4 NA oil-immersion objective. Fluoresphers were excited using the 405 nm line of a diode laser, the 488 nm line of a Kr/Ar laser and the 543, 594 and 605 nm lines of an HeNe laser, as appropriate. A series of images was taken at 0.5 μm intervals between the Z-plane of the cell and processed to form a projected image. For imaging of actin stress fibers, projections were from 4×0.5 μm sections taken at the base of the cell.

**Immunoprecipitation assays**

Cells were transfected as described. After growth overnight, cells (approximately 10⁶ cells per condition) were broken on ice with 1 ml lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche)). lysates were rotated for 20 minutes at 4°C and then clarified by centrifugation at 15,000 g for 15 minutes. Proteins were immunoprecipitated at 4°C by incubation for 1 hour with 1 μg anti-FLAG antibody or anti-myc antibody, as appropriate, followed by incubation with 30 μl Protein-G-Sepharose beads (packed bead volume) for 1 hour. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated bovine fetal calf serum, 100 μg/ml streptomycin sulfate and 100 μM benzylpenicillin. Cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. siRNA oligonucleotides were introduced into HeLa cells using calcium phosphate. Briefly, cells were cultured in six-well dishes until 70% confluent (approximately 10⁶ cells). Transfection solutions were prepared by mixing 4 μl of 20 μM siRNA in 100 μl 0.25 M CaCl₂ solution with 100 μl 50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na₃HPO₄. After a 15 minute incubation, this suspension was added drop-wise to the dish. Samples were incubated at 37°C and 3% CO₂ overnight. The medium was then replaced twice and samples returned to 37°C and 5% CO₂ for a further 48 hours. The sequences of the siRNA oligonucleotides were as follows: Lamin A/C, GGUGGUGACGACUCUGGGCUC; mDia1A, GAAGUUGGUUCGUGUAGAGA; mDia1B, AGAAuccGAGACuAGuGACuA; Rif, GCuGACGAGuGiuGiuCUCuA; ROCK1, GCuAACuGUuACuACAGuGAT; ROCK2, GCAuAAGuUAuAAuA-ACuGTT. Humans have 21 Rho family members, most of which date back to the division of the chordate phylum (Wherlock and Mellor, 2002).
Yeasts two-hybrid assays
cDNAs against mRNA encoding RhoA and Rif were expressed as fusions with the GAL4 DNA-binding domain in the yeast expression plasmid pGBK7T (Clontech). ROCK1 and the GBD domains of the Diaphanous-related forms were expressed as fusion proteins with the GAL4 activation domain in pGADT7 (Clontech). Potential interactions were tested by co-transformation of plasmids into AH109 yeast cells. Double transformants were selected on Leu/Trp-deficient agar plates. After 4 days, two independent cultures were picked and plated onto 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/Aden/His-deficient plates was assessed after 5-7 days. For high-stringency assays, growth was assessed on Leu/Trp/His-deficient plates in the presence 2 mM 3-amino-1,2,4-triazole.

Statistical analysis
Cells were counted blind. Unless stated otherwise, data are given as means ± s.e.m. of three separate experiments.

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Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/8/1247/DC1

References