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Inhibition of RhoA-mediated SRF activation by p116^{Rip}

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Abstract p116^{Rip}, originally identified as a binding partner of activated RhoA, is an actin-binding protein that interacts with the regulatory myosin-binding subunit (MBS) of myosin-II phosphatase and is essential for Rho-regulated cytoskeletal contractility. Here, we have examined the role of p116^{Rip} in RhoA-mediated activation of the transcription factor SRF. We show that p116^{Rip} oligomerizes via its C-terminal coiled-coil domain and, when overexpressed, inhibits RhoA-induced SRF activation without affecting RhoA-GTP levels. Mutant forms of p116^{Rip} that fail to oligomerize or bind to MBS are still capable of inhibiting SRF activity. Our results suggest that p116^{Rip} interferes with RhoA-mediated transcription through its ability to disassemble the actomyosin cytoskeleton downstream of RhoA.

Keywords: Actin-binding protein; Cytoskeleton; RhoA; SRF; Coiled-coil

1. Introduction

Rho GTPases are involved in a wide range of cellular responses, ranging from regulation of the actin cytoskeleton and cell morphology to transcriptional activation and cell cycle progression [1–3]. Thus, activation of RhoA leads not only to actomyosin-driven contraction [4,5], but also to activation of the transcription factor SRF [6]. Changes in actin dynamics are both necessary and sufficient for serum response factor (SRF) activation [7]. SRF activation is mediated by two distinct downstream effectors of RhoA, Rho-kinase (ROCK) and mDia, which act cooperatively to induce F-actin assembly and stabilization [8,9]; the resulting depletion of G-actin triggers SRF activation [10]. Activated ROCK phosphorylates the regulatory myosin-binding subunit (MBS) of myosin light-chain (MLC) phosphatase, thereby stimulating MLC phosphorylation and actomyosin contractility [11,12].

Using a yeast two-hybrid screen, we have previously identified a ubiquitously expressed protein of 116 kDa, named p116^{Rip}, that interacted with activated V14RhoA [13]. Subsequent studies revealed that p116^{Rip} is an F-actin binding protein with actin-bundling properties in vitro [14]. Overexpression of p116^{Rip} disrupts the actin cytoskeleton and thereby inhibits contractility [13,14], whereas knockdown of p116^{Rip} maintains cells in a rounded state [15]. We and others found that the C-terminal coiled-coil domain of p116^{Rip} interacts directly with the regulatory MBS of myosin II phosphatase [15–17]. The availabe evidence suggests that p116^{Rip} acts as a scaffold that links the MLC phosphatase complex to F-actin [15]. Thus, p116^{Rip} may interfere with RhoA signaling at multiple levels.

In the present study, we set out to examine the effect of p116^{Rip} on RhoA-regulated SRF activity, with emphasis on the role of the coiled-coil domain. We show that: (i) the coiled-coil domain of p116^{Rip} mediates homo-oligomerization; and (ii) overexpression of p116^{Rip} inhibits RhoA-mediated SRF activation without affecting RhoA-GTP levels; (iii) oligomerization and MBS binding are not required for p116^{Rip} to inhibit SRF activation. We conclude that p116^{Rip} acts downstream of ROCK and abrogates RhoA-induced SRF activation through its ability to disassemble the actin cytoskeleton.

2. Materials and methods

2.1. Plasmids

pcDNA3-HA-ΔNp116^{Rip}, pcDNA3-HA-FLp116^{Rip}, pMT2sm-FLp116^{Rip}-GST, pcDNA3-HA-NTp116^{Rip}, pcDNA3-HA-CTp116^{Rip} and pcDNA3-HA-RBDp116^{Rip} have been described elsewhere [13–15]. SRE.L-luciferase plasmids, GST-C21, pcDNA3-HA-p190RhoGEF and pMT2sm-HA-p190RhoGEF-DH/PH are described previously [18]. The dominant-active RhoA pMT2sm-myc construct has been described previously [13]. pcDNA3-HA-PHp116^{Rip} (aa 383–488) was generated by PCR. pMT2sm-ΔNp116^{Rip}-GST was constructed by ligation of a *Bst*XI–*Sal*I, blunt-ended fragment out of FLp116^{Rip}-peGFPN1 into *Sma*I cut pMT2sm-GST. pMT2sm-ΔCp116^{Rip}-GST was generated by digestion of pMT2sm-FLp116^{Rip}-GST with *Sma*I and ligation into *Sma*I–*Not*I, blunt-ended pMT2sm-GST. The p116^{Rip} mutants (L857P/L864P, L905P/I912P, and I919P/L926P) were made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the p116^{Rip} mutants (L857P, L905P, and I919P [15]) as a template.

pXJ40-FLAG-p85 (MBS) and pEFBOS-myc-MBS130 were kindly provided by Dr. T. Leung and Dr. K. Kaibuchi, respectively.

2.2. Western blotting, immunoprecipitation, and pull-down assays

COS-7 and HEK293 cells were grown and transfected as described before [14,15]. COS-7 cells co-expressing various constructs were scraped in lysis buffer A (1% NP-40, 50 mM Tris, pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 10% glycerol, supplemented with protease inhibitors). Immunoprecipitation using hemagglutinin (HA) mAb (12CA5, ATCC) was performed as described [15]. Western blotting was performed using myc (9E10, ATCC), HA, or FLAG mAbs (M2, Sigma).

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Abbreviations: GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; LPA, lysophosphatidic acid; MLC, myosin light chain; MBS, myosin-binding subunit of MLC phosphatase; ROCK, Rho-kinase; SRE, serum response element; SRF, serum response factor

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Glutathione *S*-transferase (GST) pull-downs in COS-7 cells were performed as follows. GST fusion proteins were prepared as described [14]. COS-7 cell lysates were prepared as above, clarified by centrifugation, and incubated for 3 h at 4 °C with GSH-sepharose coupled GST fusion proteins. Beads were washed, eluted in Laemmli sample buffer and separated by SDS–PAGE and analyzed by Western blotting using myc or GST mAbs (2F3, ATCC).

2.3. SRF-mediated luciferase gene transcription

HEK293 cells were transfected with pCMV-β-gal (0.2 μg), pSRE.luc (0.2 μg) and indicated plasmids. The total DNA was 3 μg per well using pcDNA3 and/or pMT2sm empty vectors. 24 h after transfection DMEM containing 0.5% FCS was added to the cells. 48 h after transfection, cells were lyzed in 200 μl Reporter Lysis Buffer (25 mM Tris–HCl, pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, and 1% Triton X-100). Luciferase activity was measured at 495 nm using 20 μl of lysate and 50 μl of Luciferase Substrate (Promega). β-Galactosidase measurements were used to normalise transfection efficiency. Luciferase and β-galactosidase activity were quantified using a Victor luminometer (Wallac, Turku, Finland). Equal amounts of lysate were subjected to SDS–PAGE and analyzed by western blotting. Experiments were repeated at least three times.

2.4. RhoA activation assay

RhoA activity in transfected COS-7 cells was analyzed by pull-down assays using GST-C21 containing the RhoA-binding domain of Rhotekin as described [18]. 1-oleoyl-LPA was purchased from Sigma.

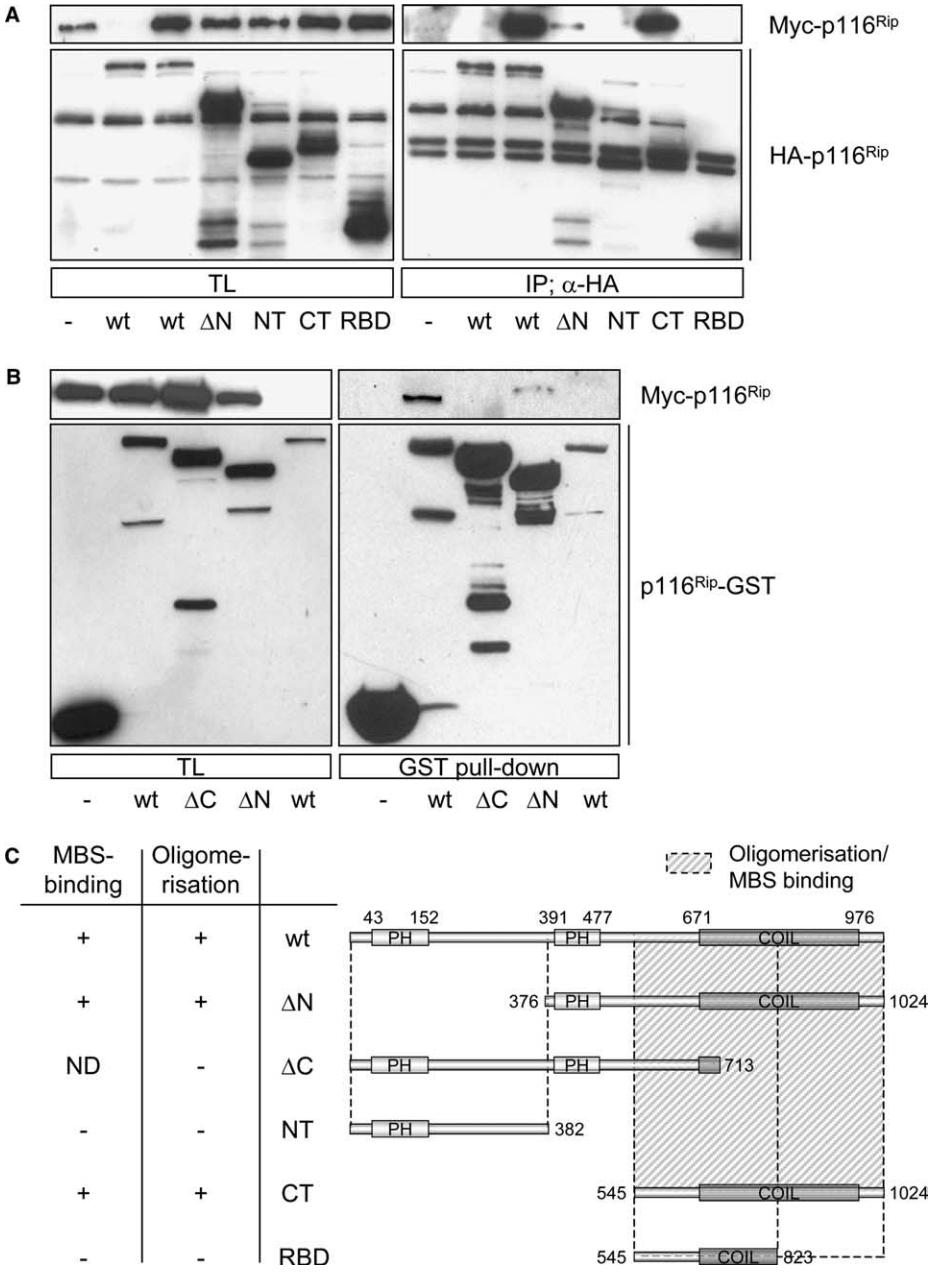

Fig. 1. The coiled-coil domain of p116^{Rip} mediates p116^{Rip} oligomerization. (A) COS-7 cells were transfected with indicated plasmids. Total lysates (TL, left panel) and immunoprecipitates (IP, right panel) were analyzed by immunoblotting (IB) using α-HA and α-Myc (B) COS-7 cells were transfected with indicated plasmids. TL (left panel) and GST precipitates (right panel) were analyzed using α-GST and α-Myc. (C) Schematic representation of the oligomerization via the coiled-coil domain of p116^{Rip}; +denotes p116^{Rip} oligomerization or MBS binding [15]. –, empty vector; wt, wild-type p116^{Rip}; NT, N-terminal actin-binding domain [14]; CT, C-terminal region; RBD, 'Rho-binding' domain [13]; ΔN and and ΔC lacks the N-terminal and C-terminal regions, respectively; ND, not determined.

2.5. Confocal microscopy

Transfected NIH3T3 cells were analyzed for the presence of stress fibres as described [14].

3. Results and discussion

3.1. The coiled-coil of p116^{Rip} mediates homo-oligomerization

The C-terminal domain of p116^{Rip} (aa 600–1024) contains six coiled-coil regions, as predicted by the MULTICOIL program [19] (Fig. S1). This domain was previously found to interact with the leucine-zipper domains of MBS (MBS85 and MBS130) [15,17]. We observed that, in COS-7 cell lysates, Myc-tagged p116^{Rip} co-precipitated with HA-tagged p116^{Rip}, indicative of oligomerization (Fig. 1A). To investigate whether oligmerization is mediated by the coiled-coil domain, we expressed various truncation mutants in COS-7 cells. As shown in Fig. 1A, N-terminally truncated versions of p116^{Rip} (HA-tagged) could precipitate Myc-p116^{Rip}, whereas deletion mutants lacking the coiled-coil region could not. Similarly, Myc-p116^{Rip} was pulled down by a GST fusion protein of p116^{Rip} lacking the N-terminal region (ΔN), but not by a GST-p116^{Rip} fusion protein lacking the coiled-coil (ΔC; Fig. 1B). Thus, the p116^{Rip} coiled-coil mediates both homo-oligomerization and MBS binding (Fig. 1C).

Mutagenesis of (iso)leucine residues (to alanine, valine or proline) affects coiled-coil stability and abolishes protein–protein interaction [20,21]. Using various (iso)leucine mutants (L857P, L905P, I919P, L857P/L864P, L905P/I912P, and I919P/L926P; Fig. 2A), we found that double mutants L905P/I912P and I919P/L926P failed to oligomerize (Fig. 2B), indicating that I(912) and L(926) are critical for oligomerization. All double mutants failed to interact with MBS (Fig. 2C), indicating that, in addition to L(857) and I(919)[15], residue I(912) is critical for interaction with MBS. We conclude that an overlapping region within the p116^{Rip} coiled-coil domain mediates both p116^{Rip}–p116^{Rip} and p116^{Rip}-MBS oligomerization.

3.2. p116^{Rip} inhibits RhoA-mediated activation of SRF

Rho GTPases activate the serum response factor (SRF) [6] through their ability to induce actin polymerization [10]. Since overexpression of p116^{Rip} interferes with RhoA-induced cytoskeletal changes [13,14], we examined how p116^{Rip} may affect RhoA-mediated SRF activation. We used a reporter assay in which the luciferase gene is driven by a c-Fos serum-responsive promoter element (pSRE.L) that specifically reports Rho

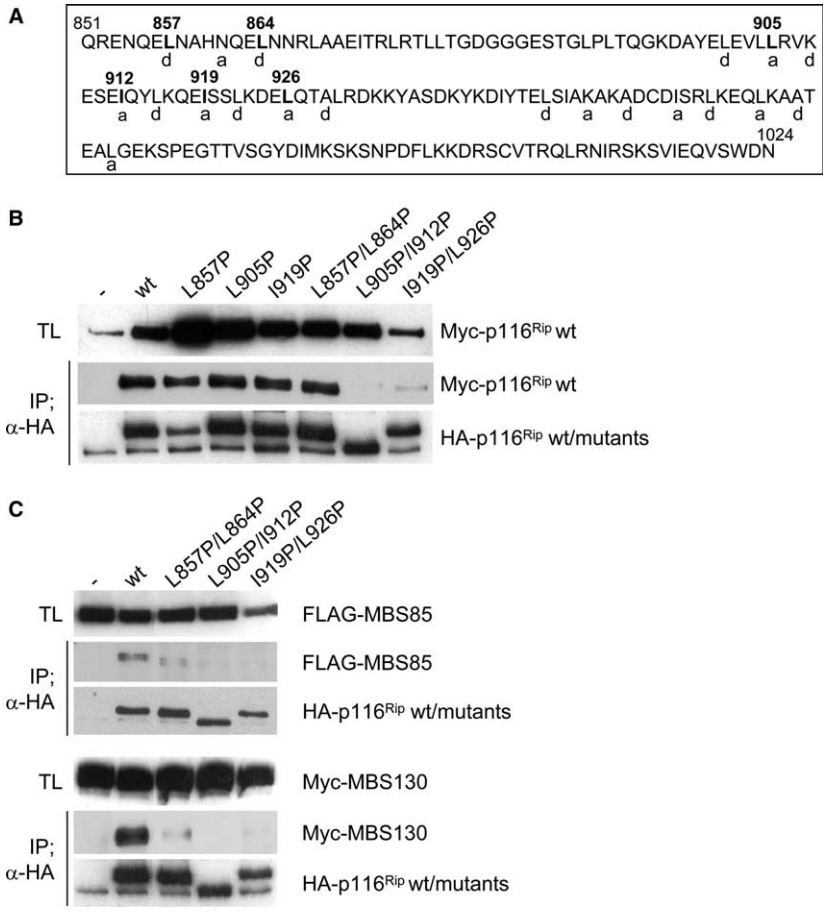

Fig. 2. (Iso)leucine residues in the coiled-coil of p116^{Rip} mediate homo-oligomerization. (A) Representation of residues 851–1024 of the coiled-coil domain of p116^{Rip}. (Iso)leucines in bold were mutated to proline. (B) COS-7 cells were transfected with indicated plasmids. –, empty HA-tagged plasmid; wt, wild-type p116^{Rip}; TL, total lysates (upper panels); IP, immunoprecipitation using α-HA. Note that the double mutant L905P/I912P has a higher mobility, consistent with distortion of the coiled-coil structure. (C) (Iso)leucine residues in the coiled-coil of p116^{Rip} mediate binding to MBS85 and MBS130. COS-7 cells were transfected with plasmids encoding either FLAG-MBS85 (upper) or Myc-MBS130 (lower) and indicated HA-tagged plasmids. Immunoprecipitation was performed as in B.

signaling [22]. HEK293 cells were co-transfected with pSRE.L, RhoV14 or the Rho-specific exchange factor p190RhoGEF [18] together with p116^{Rip}. As shown in Fig. 3A and B, expression of p116^{Rip} markedly inhibits SRF activation induced by either RhoV14 or p190RhoGEF.

3.3. p116^{Rip} does not affect RhoA activation

Given that overexpression of p116^{Rip} inhibits SRF activation and induces a "dominant-negative" RhoA phenotype [13,14], we examined the activation state of RhoA in p116^{Rip}-expressing COS-7 cells using lysophosphatidic acid (LPA) as an agonist and p190RhoGEF (DH/PH) [18] as a positive control. Basal Rho-GTP levels were unaffected by overexpression of p116^{Rip} (Fig. 3C), whilst RhoA was activated by LPA in both control and p116^{Rip}-transfected cells (Fig. 3C). Thus, overexpressed p116^{Rip} does not interfere with LPA-induced RhoA activation.

3.4. Inhibition of SRF activity by p116^{Rip} does not depend on its oligomerization or interaction with MBS

We next examined whether inhibition of SRF activity depends on the ability of p116^{Rip} to homo-oligomerize or/and to associate with MBS. Wild-type p116^{Rip}, (iso)leucine single mutants that no longer bind MBS, or (iso)leucine double mutants that fail to interact with both MBS and p116^{Rip} were co-expressed with activated RhoA in HEK293 cells and SRF activation was determined. Since the ΔN and ΔC truncation mutants of p116^{Rip} yielded inconclusive results in the luciferase read-out (data not shown), a p116^{Rip} deletion mutant containing only the second PH domain was used as a control. As

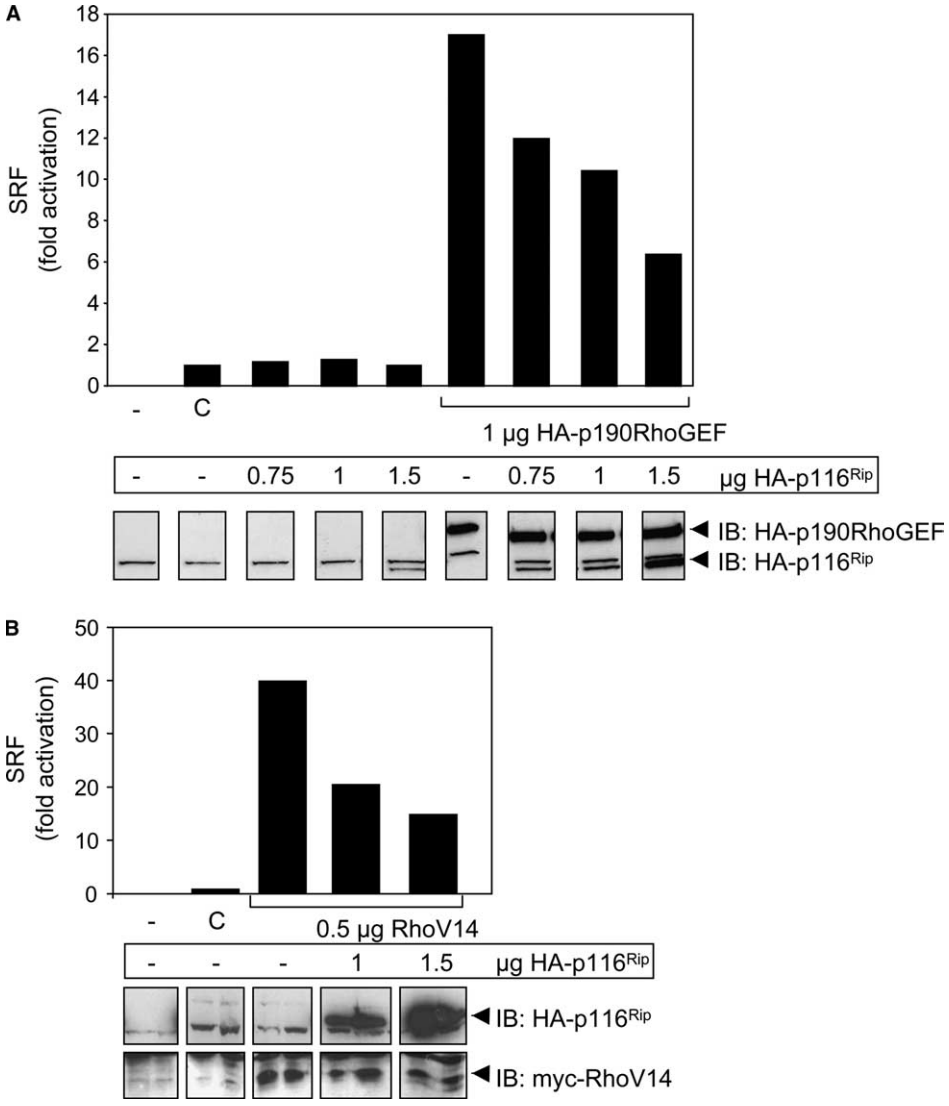

Fig. 3. p116^{Rip} expression interferes with RhoA-mediated SRF activation. (A,B) HEK293 cells were transfected with SRE.L-luciferase reporter plasmid, β-galactosidase control plasmid, HA-p116^{Rip} and either HA-p190RhoGEF (A), or myc-RhoV14 (B). As negative controls, empty vector was transfected into cells (−) or cells were transfected with SRE.L-luciferase reporter-, β-galactosidase plasmids, and empty vector (control, C). Lower panels show the expression of transfected proteins in total cell lysates. (C) p116^{Rip} overexpression does not interfere with RhoA activity. COS-7 cells were transfected with empty vector (−), HA-p190RhoGEF-DH/PH, or p116^{Rip}-myc. Cells were serum-starved overnight and stimulated with 1 μM LPA for 3 min. Samples were analyzed for levels of activated RhoA (GTP-RhoA) by immunoblotting using α-RhoA. TL, total lysates analyzed with α-RhoA, α-myc, and α-HA. Lines indicate duplicate samples. (D) HEK293 cells were transiently transfected as in B, with or without myc-RhoV14 and increasing amounts of HA-p116^{Rip}wt/HA-p116^{Rip} mutants. HA-PHp116^{Rip} was used as a negative control.

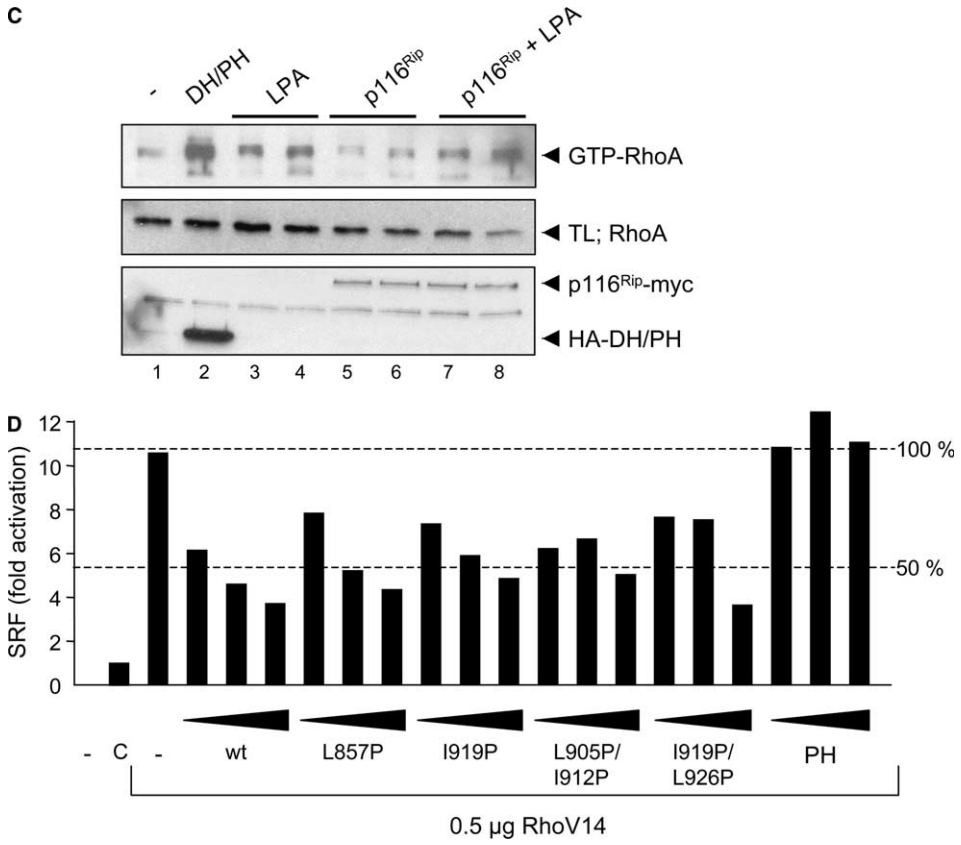

Fig. 3 (*continued*)

Table 1
Summary

	SRF activity (%)[a]	MBS interaction[b]	p116^{Rip} interaction[b]	Loss of stress fibres[c]
RhoV14	100			
RhoV14 + p116^{Rip} wt	35	++	++	Yes
RhoV14 + p116^{Rip} L857P	40	±	++	Yes
RhoV14 + p116^{Rip} I919P	46	±	++	Yes
RhoV14 + p116^{Rip} L905P/I912P	47	–	–	Yes
RhoV14 + p116^{Rip} I919P/L926P	35	–	–	Yes
RhoV14 + PH-p116^{Rip}	105	ND	ND	ND

ND, not determined.
[a]SRF activity induced by RhoA(V14) in HEK293 cells using 1.5 µg of p116^{Rip} plasmid.
[b]Interaction with p116^{Rip} in COS-7 cells: ++, strong interaction; ±, weak interaction; –, no interaction.
[c]Determined in NIH3T3 cells upon transfection of p116^{Rip} wt or mutants (see Fig. 4).

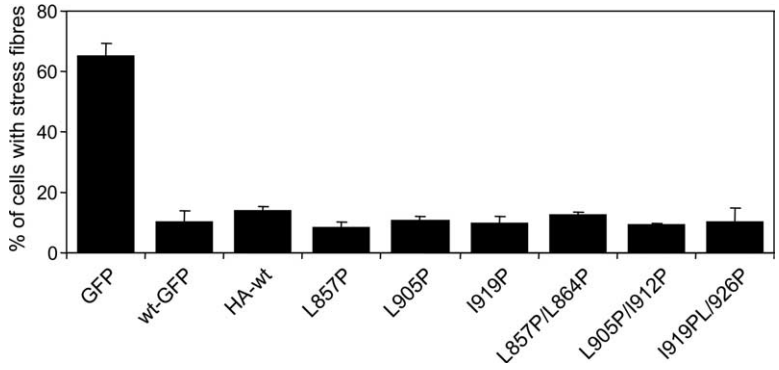

Fig. 4. Expression of p116^{Rip} (iso)leucine mutants induces loss of stress fibres. NIH3T3 cells were transfected with HA-tagged p116^{Rip} wild-type (wt), HA-tagged p116^{Rip} (iso)leucine mutants, GFP or p116^{Rip}-GFP constructs. Stress fibres were analyzed as described [14].

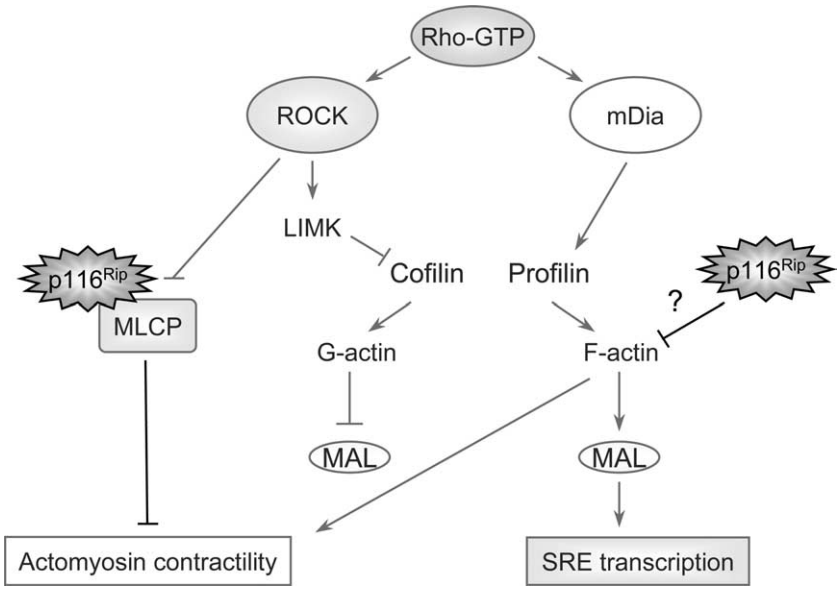

Fig. 5. Model of p116^{Rip} counteracting the RhoA pathway. RhoA activation leads to reorganization of the actin cytoskeleton through the activation of its effectors ROCK and mDia, causing accumulation and stabilization of F-actin and increased actomyosin contractility. Consequently, MAL is no longer sequestered by G-actin and relocates to the nucleus, where it activates SRE-mediated gene expression [10]. p116^{Rip} may interfere with the RhoA pathway at multiple levels: (i) by direct affecting F-actin leading to inhibition of actomyosin contractility and gene transcription; (ii) by acting in parallel with MLC phosphatase resulting in reduced actomyosin contractility [15]. SRF, serum response factor; SRE, serum response element; MAL, megakaryocytic acute leukaemia protein, ROCK, Rho-kinase; LIMK, LIM-kinase; MLC, myosin light chain; MLCP, MLC-phosphatase.

shown in Fig. 3D and summarized in Table 1, wild-type p116^{Rip} and the (iso)leucine mutants were all capable of inhibiting RhoA-induced SRF activation, indicating that the effect of p116^{Rip} on SRF activation does not depend on oligomerization or MBS binding.

3.5. Inhibition of SRF activity by p116^{Rip} correlates with its capacity to disrupt the cytoskeleton

To examine whether p116^{Rip} modulates Rho-induced SRF activation through its effect on the F-actin cytoskeleton [14], we studied the cytoskeletal organization of NIH3T3 cells expressing the (iso)leucine mutants of p116^{Rip}. Expression of all mutants resulted in a dramatic loss of stress fibres, similar to what is observed with wild-type p116^{Rip} (Fig. 4). Less than 15% of the p116^{Rip} wild-type or (iso)leucine mutant-transfected NIH3T3 cells contain stress fibres, compared to >60% of green fluorescent protein (GFP)-expressing control cells. Thus, the effect of p116^{Rip} overexpression on SRF activation closely correlates with its ability to disrupt the actin cytoskeleton (Table 1).

4. Conclusion

We have shown that p116^{Rip}, through its C-terminal coiled-coil domain, homo-oligomerizes with itself and hetero-oligomerizes with MBS. How oligomerization may affect the function of p116^{Rip} remains to be examined. The present data show that p116^{Rip} expression abrogates RhoA signaling to the nucleus, which is consistent with our previous studies showing that p116^{Rip} expression inversely correlates with an activated RhoA phenotype [14,15]. Through mutational analysis we have ruled out the possibility that p116^{Rip} oligomerization or MBS-p116^{Rip} interaction plays a role in the transcriptional inhibition by p116^{Rip}. Of note, p116^{Rip} overexpression does not affect RhoA-GTP levels, which argues against a scenario in which p116^{Rip} might have intrinsic GAP activity [16].

In conclusion, our studies show that p116^{Rip} overexpression antagonizes RhoA signaling, not only at the level of cytoskeletal regulation (as evidenced by loss of stress fibres and induction of neurite outgrowth [13,14]) but also, as reported here, at the level of gene transcription. However, it has been unclear at which step in the RhoA signaling pathway p116^{Rip} acts. Our studies indicate that p116^{Rip} acts downstream of the RhoA-ROCK pathway, because p116^{Rip} overexpression does not affect either basal or LPA-induced levels of RhoA-GTP (Fig. 3C) and, furthermore, knockdown of p116^{Rip} prevents cell spreading and neurite outgrowth in response to the ROCK inhibitor Y-27632 [15]. Recent evidence indicates that activation of SRF is regulated by its ability to sense G-actin levels [7,10]. Since inhibition of SRF activity by p116^{Rip} correlates with the ability of p116^{Rip} to disrupt the actin-based cytoskeleton, our data suggest a model in which p116^{Rip} has the potential to disassemble the F-actin cytoskeleton with a concomitant increase in G-actin levels serving as a trigger for decreased SRF activity. If and how p116^{Rip} may affect the balance between G- and F-actin remains to be examined.

Our previous studies suggested that p116^{Rip} acts as a scaffold to target MBS to the actin cytoskeleton [15]. Whilst the RhoA/ROCK pathway leads to phosphorylation of MLC, the MBS complex dephoshorylates MLC and thereby antagonizes RhoA/ROCK signaling [23–25]. As schematicaly illustrated in Fig. 5, our data support a model in which p116^{Rip} acts downstream of the Rho/ROCK pathway, acting in concert with MLC phosphatase and F-actin, to negatively regulate the RhoA-actin pathway and to attenuate actomyosin contractility and SRF activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.09.083.

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