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Research article

The Rif GTPase regulates cytoskeletal signaling from plexinA4 to promote neurite retraction

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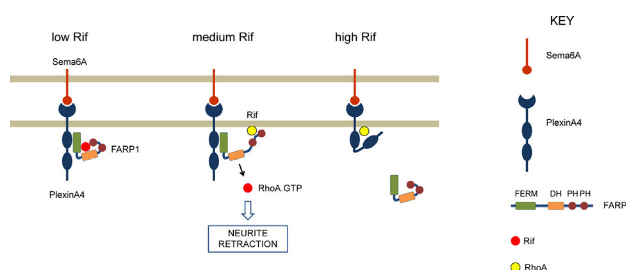
HIGHLIGHTS

- Rif interacts with the Rho GTPases activator FARP1.
- Rif interacts with the semaphorin receptor plexin A4.
- Binding of Rif to plexin A4 controls its association with FARP1.
- Binding of Rif to FARP1 regulates activation of RhoA.
- This regulation of the plexin A4/FARP1 complex by Rif controls neurite retraction in response to Sema6A.

GRAPHICAL ABSTRACT



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ABSTRACT

The small GTPase Rif is required for the early stages of dendritic spine formation in neurons, acting through the formin mDia2 to control actin polymerization. Rif is expressed at high levels in the brain, suggesting broader roles in neuronal function. We screened a yeast two-hybrid cDNA library to identify additional binding partners for Rif of potential relevance to neuronal function. We found that Rif interacts with FARP1, a neuronal activator of the RhoA GTPase. We show that Rif has two separate roles in FARP1 regulation–in controlling its association with plexinA4, and in releasing active RhoA from a plexinA4/FARP1 complex. The regulation of FARP1 by Rif promotes neurite retraction in cells stimulated with the semaphorin Sema6A.

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1. Introduction

The Rho family of small GTPases are critical regulators of neuronal morphology and migration, which they control through dynamic regulation of the actin cytoskeleton. The human Rho GTPase family contains 20 members, of which the best characterized are RhoA, Rac1 and Cdc42 [1]. Previously, we have characterized the Rif GTPase, a Rho family member that is distantly related to RhoA, Rac1 and Cdc42. In cultured cells, Rif controls the formation of filopodia – thin actin-rich protrusions from the cell surface that are involved in sensing the external environment [2,3]. The physiological role of Rif is currently unclear. Rif is highly expressed in brain and neural tissue [2], suggesting functions in cytoskeletal regulation in neurons. In support of this, recent studies have shown that Rif is required for the early stages of dendritic spine formation in neurons, acting in collaboration with Cdc42 [4]. Here we identify the RhoA guanine nucleotide exchange factor (GEF) FARP1 (FERM, RhoGEF, and pleckstrin homology domain protein 1) as a novel downstream signaling partner of Rif. FARP1 is a regulator of semaphorin signaling in neurons [5]. We show that Rif

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controls release of active RhoA from FARP1 in a pathway that links Rif to the regulation of repulsive signaling from the semaphorin Sema6A.

2. Materials and methods

2.1. Materials

Monoclonal anti-HA antibody was from Covance, monoclonal antibodies to myc epitope and to RhoA were from Santa Cruz Biotechnology, Rabbit anti-FARP1 was from Orbigen. The sheep polyclonal anti-Rif antibody has been described previously [3]. Plasmids encoding N-terminally epitope-tagged human Rif in pcDNA3 (Clontech) were as described previously [2,3,6], including the constitutively-active Q77L mutant, and the inactive T33N mutant. A full list of plasmids is given in Supplementary materials.

2.2. Cell culture

HEK293 cells were maintained in DMEM containing 10% FBS. PC12 cells were maintained in RPMI-1640 containing 5% FBS, 5% horse serum.

2.3. Yeast two-hybrid screening

Human Rif-QL (activated Q77L mutant) cDNA was cloned into pGBKT7 (Clontech) and used to screen a human liver cDNA library according to the manufacturer's instructions (Clontech).

2.4. Immunoprecipitation

HEK293 cells were transfected using calcium phosphate [7]. Cells were lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM DTT and protease inhibitor cocktail (Roche)). Lysates were placed on a rotator for 20 min at 4 °C and then clarified by centrifugation at 15,000× *g* for 15 min. Lysates were incubated with the relevant antibody for 1 h. Protein complexes were collected on protein-G Sepharose beads (GE Lifesciences), washed, and eluted in SDS-PAGE sample buffer.

2.5. Preparation of recombinant Rif

Escherichia coli were transformed with GST-tagged Rif. Cells were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Cells were lysed in ice-cold extraction buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM GDP, 1 mM DTT) by nebulization. The lysate was clarified by centrifugation at 17,400× *g* for 30 min and loaded onto gluthathione-coupled Sepharose beads. For nucleotide loading, GST-Rif beads were washed 3 times with exchange buffer (20 mM Hepes pH 7.5, 10 mM EDTA, 100 mM NaCl and 1 mM DTT). The solution was then made to a final concentration of 1 mM GDP or GTP and incubated at 4 °C for 30 min. The exchange process was terminated by adding $MgCl_2$ to 60 mM.

2.6. RhoA activation assays

RhoA activation assays were carried out as described [8].

2.7. Neurite formation assays

PC12 cells were transfected using Lipofectamine 2000 (ThermoFisher). After 24 h, the cells were changed into fresh serum-free media and stimulated as described for another 24 h. Cells were then fixed for 15 min in 4% paraformaldehyde and processed for immunofluorescence. For quantification of neurites, >100 cells

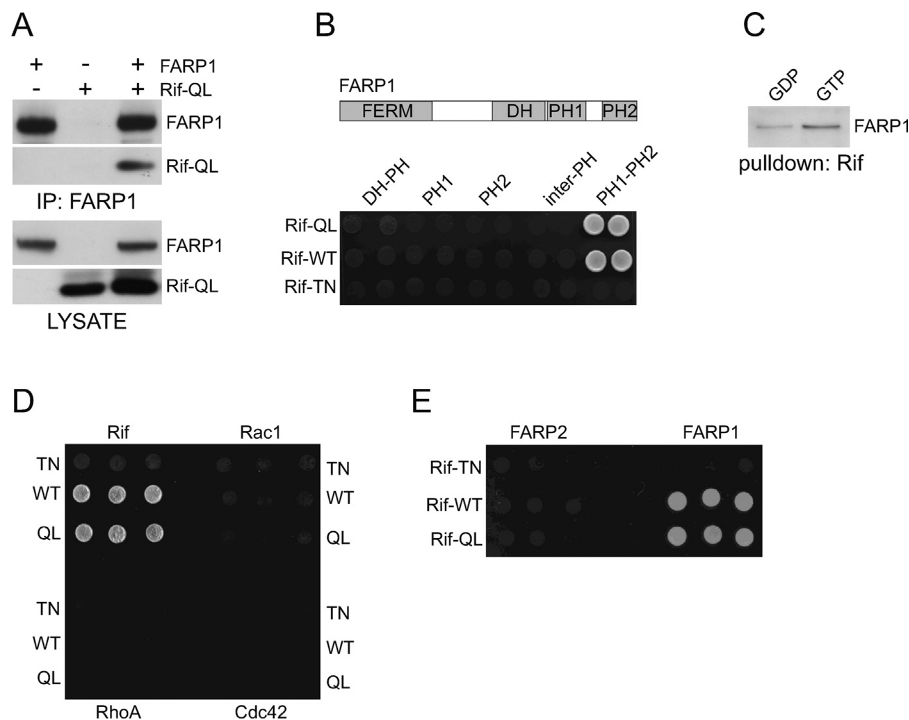

Fig. 1. Rif interacts with FARP1. (A) HEK293 cells were co-transfected with HA-tagged FARP1 and Rif-QL as indicated. FARP1 was isolated from cell lysates by immunoprecipitation and these samples were probed for co-precipitation of Rif by western blotting. (B) Interaction of Rif with different domains of FARP1 was measured in a yeast two-hybrid assay. Plasmids encoding inactive (TN), wild-type (WT) or activated (QL) Rif were transfected into AH109 yeast cells together with domains of FARP1. Rif interacted with the PH1-PH2 region of FARP1. (C) 5 μg of purified recombinant GST-tagged Rif was loaded with either GDP or GTP and then incubated with a Jurkat cell lysate. Complexes were isolated on GSH-Sepharose beads and analyzed by western blotting. (D) The interaction of Rac1, Cdc42 and RhoA with twin PH domains of FARP1 was tested by yeast two-hybrid assay. Only Rif interacted with FARP1. (E) The interaction of Rif with the twin PH domains of FARP2 was tested by yeast two-hybrid assay. Rif only interacted with FARP1.

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