

Characterization of a Novel GTPase-activating Protein Associated with Focal Adhesions and the Actin Cytoskeleton*

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In the present study we characterize a novel RhoGAP protein (RC-GAP72) that interacts with actin stress fibers, focal adhesions, and cell-cell adherens junctions via its 185-amino acid C-terminal region. Overexpression of RC-GAP72 in fibroblasts induces cell rounding with partial or complete disruption of actin stress fibers and formation of membrane ruffles, lamellipodia, and filopodia. RC-GAP72 mutant truncated downstream of the GTPase-activating protein (GAP) domain retains the ability to stimulate membrane protrusions but fails to affect stress fiber integrity or induce cell retraction. A mutant protein consisting of the C terminus of RC-GAP72 and lacking the GAP domain does not exert any visible effect on cellular morphology. Inactivation of the GAP domain by a point mutation does not abolish the effect of RC-GAP72 on actin stress fibers but moderates its capability to induce membrane protrusions. Our data imply that the cytoskeletal localization of RC-GAP72 and its interaction with GTPases are essential for its effect on the integrity of actin stress fibers, whereas the induction of lamellipodia and filopodia depends on the activity of the GAP domain irrespective of binding to the actin cytoskeleton. We propose that RC-GAP72 affects cellular morphology by targeting activated Cdc42 and Rac1 GTPases to specific subcellular sites, triggering local morphological changes. The overall physiological functions of RC-GAP72 are presently unknown, yet our data suggest that RC-GAP72 plays a role in regulating cell morphology and cytoskeletal organization.

Rho-family GTPases are regulators of diverse biological activities including the organization of the actin cytoskeleton. The best known, “classical” members of this family are RhoA, Rac1, and Cdc42. Activation of RhoA in fibroblasts induces the formation of stress fibers and focal adhesion, whereas active Rac1 and Cdc42 induce the extension of lamellipodia and filopodia, respectively (1–4). Each of these proteins exerts its particular cytoskeletal effect via a variety of target proteins that affect actin polymerization or actomyosin contractility. In addition, Rho GTPases affect each other’s activity, and the coor-

ordinated interplay between them is crucial for proper cell adhesion and migration (5).

Rho GTPases cycle between an active, GTP-bound, and inactive GDP-bound states. These transitions are regulated by three types of proteins: GDP/GTP exchange factors, which catalyze the exchange of bound GDP with GTP; GDP dissociation inhibitors, which block the release of GDP from the G-protein; GTPase-activating proteins (GAPs),¹ which stimulate GTPase activity by increasing the hydrolysis of bound GTP (6).

The RhoGAP family consists of multiple members, characterized by the presence of a conserved RhoGAP domain (also known as BH domain) of ~150 amino acids that binds to GTP-bound Rho-family proteins and accelerates their GTPase activity (6, 7). A conserved arginine residue present in most RasGAP and RhoGAP proteins functions to stabilize the conformation needed for GTPase acceleration, and its mutation results in loss of GAP activity (8–12). Outside their GAP domain RhoGAPs exhibit high sequence diversity and have various signaling motifs that can target them to specific subcellular sites, including SH2, SH3, pleckstrin homology or phorbol ester binding domains (13–18).

A recent genome-wide bioinformatics search revealed about 80 human cDNA-encoding potential RhoGAPs that can be divided into subfamilies according to their primary structure (19, 20). Interestingly, various GAPs exhibit overlapping substrate specificities, *i.e.* they often affect more than one GTPase, and a single GTPase may serve as a target for several different GAPs. For example, oligophrenin1, PARG1, and ARHGAP9 have been shown to enhance the GTPase activity of RhoA, Rac1, and Cdc42 (16, 21, 22), GRAF and srGAP1 affect RhoA and Cdc42 (14, 23), N-chimaerin and RICH1 have specific GAP activity toward Rac1 and Cdc42 (24, 25), and p190RhoGAP and ARHGAP6 are highly specific for RhoA (12, 26).

RhoGAP activity can be regulated in a multitude of fashions. GAP activity of p190 RhoGAP and GRAF is modulated by phosphorylation (27–30), GAP activity of chimaerin can be regulated by phospholipids (31, 32), whereas GAP activity of CdGAP has been shown to be regulated by protein-protein interactions (33). Various RhoGAP proteins can also display diverse tissue-specific expression patterns, some ubiquitous and others displaying narrow tissue specificity (22, 25, 34, 35), thus contributing to the differential regulation of Rho GTPase activity in different cell types.

In the present study we characterized a novel member of the RhoGAP family, denoted RC-GAP72, which is associated with the actin cytoskeleton and specifically suppresses Rac1 and Cdc42 activity. This molecule has an N-terminal GAP domain

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¹ The abbreviations used are: GAP, GTPase-activating protein; TRITC, tetramethylrhodamine isothiocyanate; MDCK, Madin-Darby canine kidney cells; PAK, p21-activated protein kinase; GRAF, the GTPase regulator associated with focal adhesion kin; YFP, yellow fluorescent protein.

and binds to focal adhesions and stress fibers via its C-terminal region. Ectopic expression of RC-GAP72 in cultured fibroblasts induces disruption of actin stress fibers and extension of membrane ruffles, lamellipodia, and filopodia. These data as well as the effect of overexpression of different RC-GAP72 mutants indicate that the cytoskeletal localization of this protein and its interaction with Rac1 and/or Cdc-42 are essential for the disruption of stress fibers, whereas the induction of filopodia depends on the GAP activity irrespective of the cytoskeletal localization of the molecule.

MATERIALS AND METHODS

Cells and Transfections—REF52 fibroblasts, HeLa cells, and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. HeLa and 293T cells were transfected using the standard calcium phosphate method, whereas the REF52 cells were transfected using 2 μ g of plasmid DNA complexed with 1 mM polyethyleneimine reagent *M*, 25,000 (Sigma) in culture medium.

Reverse Transcription-PCR Analysis—Total RNA from murine tissues was extracted with TRI reagent (Molecular Research Center, Inc.) according to the company's recommended protocol. First-strand cDNA was synthesized using 1 μ g of total RNA as a template, oligo(dT) as a primer, and Moloney murine leukemia virus reverse transcriptase (Invitrogen). A PCR master mix was added to yield 1 μ M specific primers, 200 μ M dNTPs, 2.5 units of BIO-X-ACT DNA polymerase (Bioline), and BIO-X-ACT buffer containing 1.5 mM MgCl₂. PCR was performed through 23 cycles (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s). The primer sequences used were as follows: RC-GAP72 (based on the mouse cDNA sequence, accession number BC023344) forward 5'-AGAGAAG-CAGCGTGGACAAT-3', reverse 5'-TGGACATTGTCGTAGGTGGA-3'; glyceraldehyde-3-phosphate dehydrogenase, forward 5'-ACCACAGTC-CATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The expected amplification products were 460 bp for the RC-GAP72 and 451 bp for glyceraldehyde-3-phosphate dehydrogenase.

DNA Constructs—A human cDNA clone containing a RhoGAP homology domain in pYFP-gen vector (DKFZp564B1162 cDNA clone, GenBank™ accession number AL136646) was kindly provided by Dr. R. Pepperkok (EMBL, Heidelberg, Germany) following a microscopy-based screening of expression libraries constructed from human brain by the group of S. Wiemann (Molecular Genome Analysis, German Cancer Research Centre, Heidelberg, Germany) (36). The plasmid pEXV.V12rac1, encoding the Myc-tagged constitutively active version of the rac1, was obtained from Dr. A Hall (1). The RC-GAP72 mutants including Δ C RC-GAP72 construct (residues 1–470) bearing the RhoGAP domain and the Δ N RC-GAP72 construct (residues 291–655) (see the schematic representation in Fig. 5, I) were generated by PCR of the coding cDNA and subsequent subcloning of the PCR fragments into the BglIII and SalI sites of pEYFP-C1 vector (Clontech) to be expressed as YFP fusions. R82K mutant, which bears an arginine-to-lysine mutation at the predicted catalytic site of the RhoGAP domain, was generated by introducing a point mutation using PCR-based site-directed mutagenesis by overlap extension (37, 38) and was cloned into pEYFP-C1 vector.

Preparation of Antibodies—Antibodies to human RC-GAP72 were prepared by immunizing rabbits with a synthetic peptide (SWDK-SESPQRSSMNN) corresponding to amino acids 282–296 of human RC-GAP72 conjugated to keyhole limpet hemocyanin (Pierce). Antibodies were evaluated by Western blotting and immunofluorescence labeling.

Biochemical Procedures—For Western blot analysis 293T and HeLa cells were lysed with radioimmune precipitation assay buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0, containing a mixture of protease inhibitors). Protein extracts were subjected to 8% SDS-PAGE, transferred to Hybond-C membrane (Amersham Biosciences), and immunoblotted. For *in vivo* GTPase activity assay, constructs expressing the Rho binding domain of Rhotekin (39) and p21 binding domain of PAK1 (40), both containing glutathione *S*-transferase tags in the pGEX-5 bacterial expression vector, were kindly provided by B. Liu and K. Burrige (University of North Carolina at Chapel Hill). For determination of the intrinsic and GAP-stimulated GTPase activities of Cdc42, Rac1, and RhoA, HeLa cells were transfected with YFP-encoding plasmid as a control and vectors encoding YFP-tagged wild type or mutated RC-GAP72, and the assay was performed as described (39, 40). The amount of RhoA-GTP, Rac1-GTP, or Cdc42-GTP that was pulled down by the bead-associated GTPase binding domain of PAK1 or Rhotekin as well as the total

amount of these G-proteins in the cell lysates was determined by immunoblotting using monoclonal antibodies against RhoA (Santa Cruz Biotechnology), Rac1, or Cdc42 (Transduction Laboratories). The amounts of YFP-RC-GAP72, and its mutant forms were estimated by Western blotting using anti-GFP antibody (Roche Applied Science). To measure the interaction between RC-GAP72 and Rac1/Cdc42, 293T cells were co-transfected with constructs encoding RC-GAP72 and wild type Rac1 or Cdc42. The amount of YFP-fused RC-GAP72 and its mutants co-precipitated with an active form of the GTPases and pulled down by the bead-associated p21 binding domain of PAK1 was estimated using anti-GFP (Roche Applied Science) and anti-RC-GAP72 antibodies.

Immunofluorescence Microscopy—Cells were cultured on glass coverslips, fixed-permeabilized for 2 min in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 3% formaldehyde, and post-fixed with 3% formaldehyde in PBS for 30 min. The cells were then rinsed with phosphate-buffered saline and stained with anti-paxillin (Transduction Laboratories), anti- α -actinin (Sigma), or anti-Myc epitope 9E10 (Santa Cruz Biotechnology) monoclonal antibodies for 1 h, washed, and further incubated with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). For actin staining cells were incubated with TRITC-conjugated phalloidin for 20 min and mounted in Elvanol (Moviol 4–88, Hoechst). Images were acquired using the DeltaVision system (Applied Precision Inc.) on an Axiovert 125 TV inverted microscope (Zeiss). Image acquisition and processing were controlled by a Silicon Graphics work station model O₂ (Mountain View) using the Resolve3D and Priism software packages (Applied Precision Inc.).

RESULTS

Characterization of RC-GAP72—A bioinformatics search using the BLAST and NCBI Conserved Domain Search programs indicated that a new cDNA clone (GenBank™ accession number AL136646, also known as ARHGAP24 according to the new nomenclature classification (HUGO Gene Nomenclature Committee)) encodes a putative protein of 655 amino acids. This protein contains an N-terminal-located RhoGAP domain (residues 60–233) in which the critical residues required for GAP activity, including a highly conserved arginine (Arg-82), are present. This protein functions as a GAP specific for Rac1 and Cdc42 (see next paragraph) and was, therefore, named RC-GAP72 (Rac1/Cdc42-specific GAP with a predicted molecular mass of 72 of kDa). The RhoGAP domain of RC-GAP72 shows 90 and 77% sequence similarity to the corresponding regions of RhoGAP2 (GenBank™ accession number NP_067049) and KIAA0053 (XP_376060), with a lower similarity to other RhoGAPs (e.g. N-chimaerin, oligophrenin1, RICH-1, GRAF, and p190ARhoGAP). Outside the RhoGAP domain the cDNA sequence of RC-GAP72 diverges from other RhoGAPs, except RhoGAP2, to which it shows an overall protein sequence similarity of 53%. Using human cDNA sequences, we identified the mouse ortholog of RC-GAP72 (GenBank™ accession number BC023344). Both the human and the mouse genes are composed of seven exons, and the mouse RC-GAP72 shows 85% sequence identity to the human RC-GAP72 cDNA and 87% identity to the human protein.

To elucidate the function of the N-terminal RhoGAP domain of RC-GAP72, we determined its effect on the activation of Rho-family GTPases in cultured cells. The constructs encoding wild-type RC-GAP72-YFP or YFP alone, used as a control, were expressed in HeLa cells, and the levels of activated RhoA, Rac1, and Cdc42 were determined. As shown in Fig. 1, the levels of active Rac1 and Cdc42 were significantly reduced in the presence of wild-type RC-GAP72 compared with cells expressing YFP alone. The levels of RhoA-GTP, on the other hand, were not affected, suggesting that RC-GAP72 enhances GTPase activity of Rac1 and Cdc42 but not RhoA.

Analysis of RC-GAP72 mRNA and Protein Expression—The expression pattern of RC-GAP72 in different murine tissues was studied using mRNA from adult mouse tissues. RC-GAP72 mRNA was ubiquitously expressed in all tissues

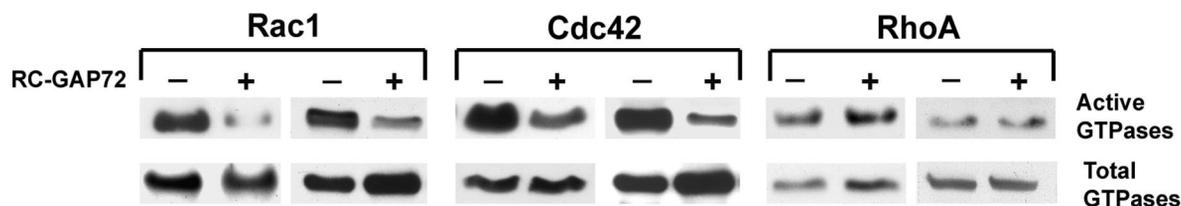
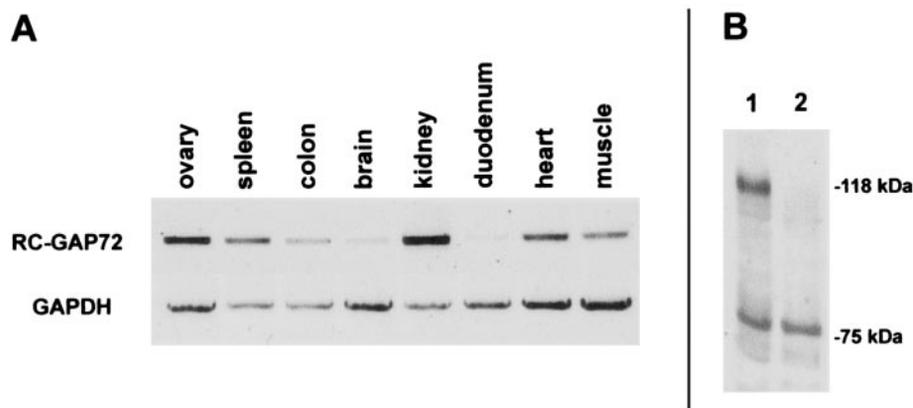


FIG. 1. GAP activity of RC-GAP72 on Rho family GTPases in cultured cells. The constructs encoding wild-type RC-GAP72 fused to YFP as well as YFP alone as a control were expressed in HeLa cells, and the levels of activated RhoA, Rac1, and Cdc42 were determined. The levels of active Rac1 and Cdc42 were significantly reduced in cells expressing wild-type RC-GAP72 compared with cells expressing YFP alone. The level of RhoA-GTP was not affected by RC-GAP72.

FIG. 2. Expression of RC-GAP72 mRNA and protein. *A*, the expression pattern of RC-GAP72 mRNA in adult murine tissues. RC-GAP72 mRNA is ubiquitously expressed in all tissues analyzed, with particularly high expression levels in the kidney. *B*, immunoblotting analysis of RC-GAP72 protein in HeLa cells. Lane 1, HeLa cells transfected with RC-GAP72-YFP cDNA; lane 2, non-transfected cells. The band of ~120 kDa corresponds to the exogenous YFP-RC-GAP; the 80-kDa band corresponds to the endogenous RC-GAP72. GAPDH, glyceraldehyde-3-phosphate dehydrogenase



analyzed; however, the expression was particularly high in the kidney (Fig. 2A). RNA and protein extracts from several human cell lines were also analyzed, including human foreskin fibroblasts, human embryonic kidney 293T cells, prostate cancer cell lines PC-3, LNCaP, and DU-15, colon carcinoma SW480, renal carcinoma KTCTL60, and lung non-small-cell carcinoma H1299 cell lines. Of these only HeLa and H1299 cells expressed detectable levels of RC-GAP72 mRNA and protein (data not shown).

To determine the expression pattern and size of the endogenous RC-GAP72 protein, we generated a polyclonal antibody against a polypeptide corresponding to amino acids 282–296 of human RC-GAP72 and tested its reactivity with RC-GAP72-YFP expressed in HeLa cells (Fig. 2B, lane 1). Immunoblotting analysis revealed two immunoreactive bands of ~120 kDa, corresponding to the exogenous RC-GAP72-YFP, and 80 kDa, consistent with the predicted molecular mass of RC-GAP72. Non-transfected HeLa cells (Fig. 2B, lane 2) showed only the 80-kDa band.

To determine the cellular localization of RC-GAP72, REF52 fibroblasts and MDCK (epithelial) cells were transiently transfected with a plasmid encoding RC-GAP72 fused to YFP. In REF52 cells fluorescent RC-GAP72 was associated with the actin stress fibers (Fig. 3, A–C) with particular enrichment in the associated focal adhesion. In that area RC-GAP72 co-localized with several markers of focal adhesion including paxillin (Fig. 3, D–F) and vinculin (not shown). RC-GAP72 was also associated with the actin-rich lamellipodium (Fig. 3A, arrow), suggesting that this protein plays a role in regulating cell adhesion and motility. In MDCK cells RC-GAP72 co-localized primarily along the α -actinin-containing adherens junctions as well as in the nucleus (Fig. 3, G–I). Interestingly, the relative intensity of RC-GAP72 along stress fibers compared with focal adhesions was affected by the level of protein expression in the cell. Thus, in cells expressing low levels of the protein, fluorescent RC-GAP72 concentrated primarily in focal adhesions (Fig. 4A), whereas at high expression levels the protein was widely distributed along the entire stress fiber system (Fig. 4B).

To investigate the involvement of different structural do-

main of RC-GAP72 in its particular subcellular localization, we transfected cells with different mutant forms of the protein (Fig. 5, 1). In contrast to the full-length RC-GAP72, which concentrates on stress fibers and focal adhesions (Fig. 5, 2A), the N-terminal domain corresponding to amino acids 1–470 (Δ C RC-GAP72), which includes the RhoGAP domain, was diffusely dispersed in the cytoplasm (Fig. 5, 2B), suggesting that the N-terminal domain does not contain the cytoskeleton-binding sites of RC-GAP72. On the other hand the C-terminal 364-amino acid RC-GAP72 mutant (Δ N RC-GAP72) lacking the RhoGAP domain co-localized with actin stress fibers and focal adhesions just like the full-length protein (Fig. 5, 2C). Expression of the central region of RC-GAP72 (amino acids 291–470) resulted in diffuse cytoplasmic fluorescence (not shown), suggesting that the domain that determines cytoskeletal localization of the protein is located within the 185 C-terminal amino acids of RC-GAP72. We further examined a full-length mutant of RC-GAP72 bearing an Arg to Lys mutation at position 82. Arginine in this location is known to be essential for efficient catalytic activity toward Rho family proteins but does not affect binding to the target proteins (41–43). As expected, mutation of the essential arginine resulted in RC-GAP72 inactivation (data not shown), and it had no apparent effect on the subcellular localization of the protein (Fig. 5, 2D).

The Effect of RC-GAP72 Overexpression on the Organization of the Actin Cytoskeleton—The ability of RC-GAP72 to regulate actin organization was tested by overexpressing the full-length protein in REF52 fibroblasts and visualized by staining actin filaments with fluorescent phalloidin. Transfected cells displayed striking differences in cell shape and cytoskeletal architecture, manifested by the partial or complete disruption of actin stress fibers and the formation of membrane ruffles, extended lamellipodia and long filopodia-like protrusions (Fig. 6A). About 60–70% of the transfected cells showed loss of adhesion to the coverslip and rounding of the cell body. Cell rounding and loss of stress fibers were accompanied by a decrease in the number of focal adhesions with no effect on their size.

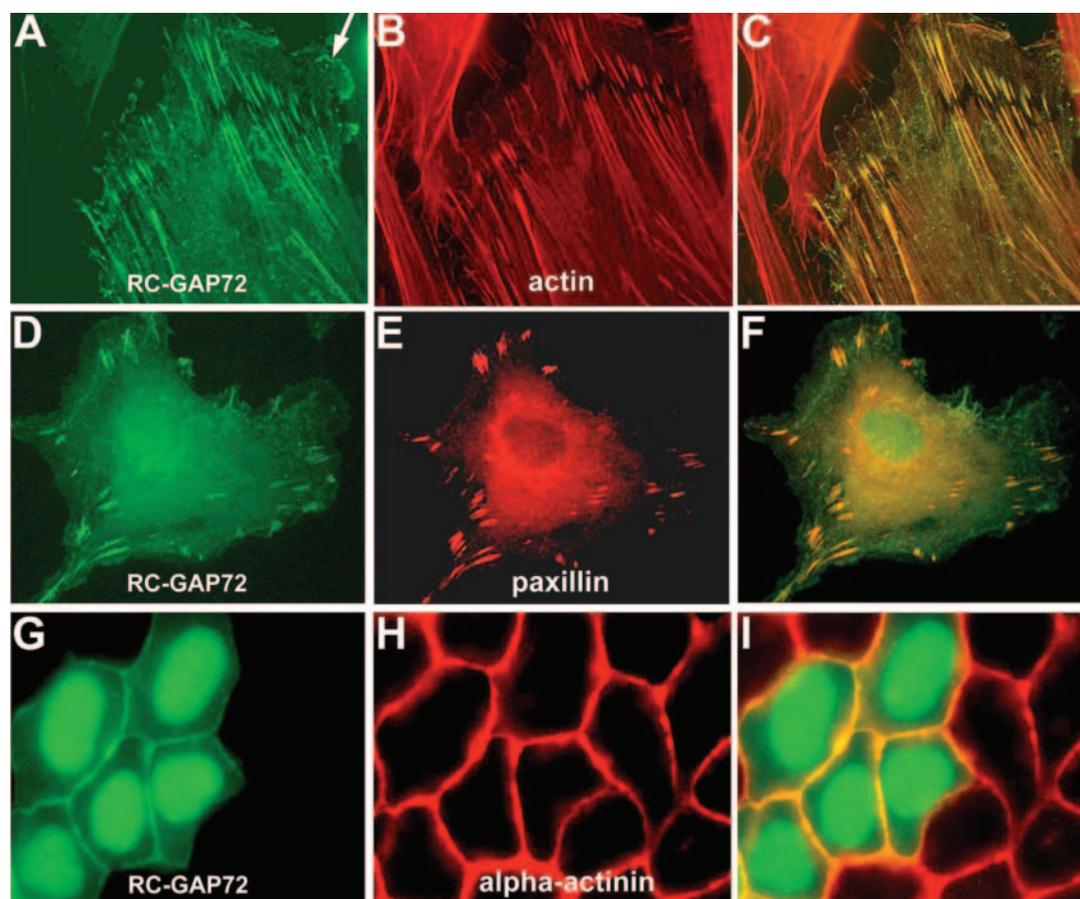


FIG. 3. **Subcellular localization of RC-GAP72 in cultured cells.** REF52 fibroblasts (A–F) and MDCK epithelial cells (G–I) were transiently transfected with a plasmid encoding full-length RC-GAP72-YFP and stained for actin (A–C), paxillin (D–F), or α -actinin (G–I). In REF52 cells fluorescent RC-GAP72 (A and D) co-localized with the actin stress fibers (B) and with paxillin-rich focal adhesions (E). In MDCK cells, RC-GAP72 (G) localized primarily along the α -actinin-containing adherens junctions (H) as well as in the nucleus. Co-localization of the two labels is shown in merge images C, F, and I.

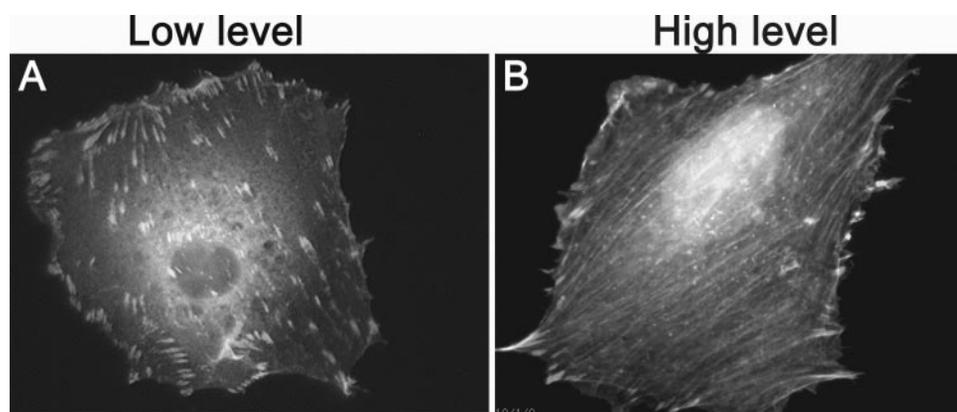


FIG. 4. **Differential association of RC-GAP72 with stress fibers and focal adhesions is affected by the level of protein expression in the cell.** RC-GAP72 is concentrated primarily in focal adhesions in cells expressing low levels of the protein (A), whereas at high expression levels the protein is widely distributed along the entire stress fiber system (B).

To determine the role of the different structural domains of RC-GAP72 in the induction of these morphological effects, different RC-GAP72 mutants were expressed in REF52 cells. Expression of the Δ N RC-GAP72 did not exert any visible effect on cellular morphology or cytoskeletal organization (Fig. 6B), whereas truncated downstream of the RhoGAP domain Δ C RC-GAP72 mutant induced formation of numerous filopodia without affecting stress fiber integrity or cell retraction (Fig. 6C). Surprisingly, overexpression of the R82K RC-GAP72 mutant resulted in the disruption of actin stress fibers and retrac-

tion of the cell body (Fig. 6D). This mutant lacks RhoGAP activity and is localized along stress fibers and focal adhesions.

To explore the mechanism underlying the effect of RC-GAP72 on the actin system we next examined binding of RC-GAP72 and its mutants to the GTPases. Co-transfection of RC-GAP72 and an Myc-tagged constitutively active mutant of Rac1 (V12Rac1) followed by immunoprecipitation with anti-Myc 9E10 antibody (44) indicated that the two molecules are associated with each other (Fig. 7A). Furthermore, the R82K RC-GAP72 as well as the Δ C mutant was pulled down with the

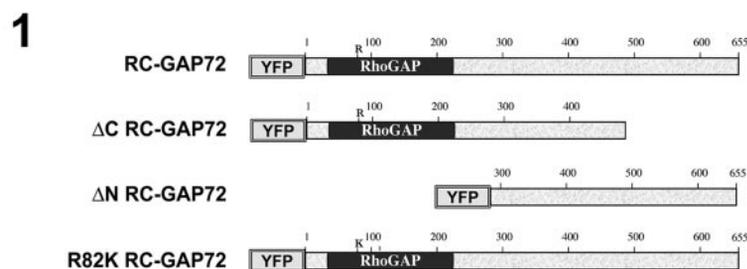


FIG. 5. Involvement of different structural domains of RC-GAP72 in its subcellular localization. 1, a schematic representation of RC-GAP72 mutants. 2, subcellular distribution of RC-GAP72 mutants in REF52 cells. The cells were transfected with different mutant forms of RC-GAP72. The full-length RC-GAP72 concentrates on stress fibers and focal adhesions (A); the ΔC RC-GAP72 mutant is diffusely distributed in the cytoplasm (B); the ΔN RC-GAP72 is co-localized with actin stress fibers and focal adhesions (C); the R82K RC-GAP72 mutant exhibits the same subcellular localization as wild type protein (D). *wt*, wild type.

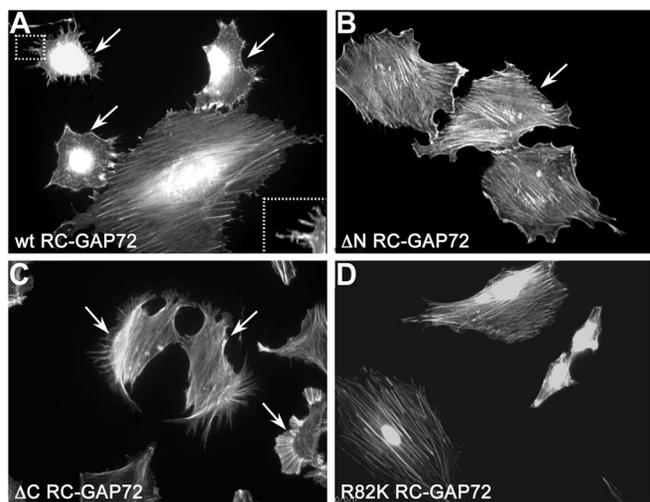
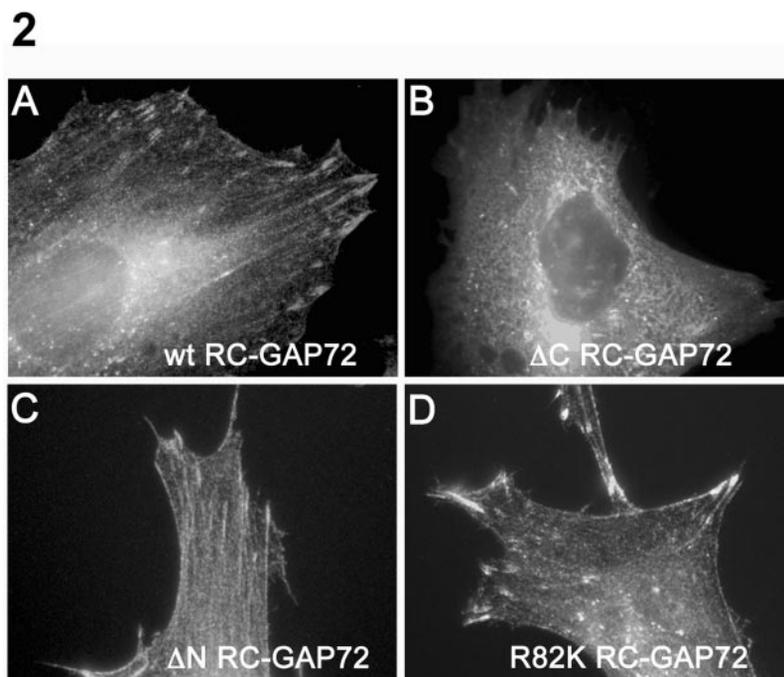


FIG. 6. The effect of RC-GAP72 on the actin cytoskeleton. RC-GAP72 was overexpressed in REF52 fibroblasts, and actin filaments were stained with fluorescent phalloidin. Transfected cells are marked with arrows. A, wild-type (*wt*) RC-GAP72 causes striking differences in cell shape and cytoskeletal architecture. B, ΔN RC-GAP72 does not exert any visible effect on cellular morphology. C, ΔC RC-GAP72 induces the formation of numerous filopodia without affecting stress fiber integrity or cell retraction. D, R82K RC-GAP72 mutant causes disruption of the actin stress fibers and retraction of the cell body.

glutathione *S*-transferase-fused GTPase binding domain of PAK1 associated with activated Rac1 and/or cdc42, similarly to the wild-type form of RC-GAP72. The ΔN mutant, lacking the

RhoGAP domain, failed to bind to either GTPases (Fig. 7B).

To confirm these data in live cells, Myc-tagged V12Rac1 was co-expressed in REF52 fibroblasts with RC-GAP72-YFP (Fig. 8A, B, and F) or YFP as a control (Fig. 8, C, D, and E). Overexpression of V12Rac1 led to a distinct phenotype characterized by the extension of numerous lamellipodia, membrane ruffles, and the formation of large vesicles. In the cells co-expressing V12Rac1 and RC-GAP72 partial colocalization of these proteins was observed on the stress fibers (Fig. 8, A–B) as well as on the surface of the vesicles (not shown), in contrast to the cells expressing V12Rac1 alone (Fig. 8, C–D), where V12Rac1 was localized mainly to the surface of the vesicles and along the plasma membrane as described previously (1). It should be mentioned that some enrichment of Rac1 along the stress fibers was also detected in some of the control cells. On the other hand co-expression of V12Rac1 with RC-GAP72 caused a severe alteration of actin organization, including a dramatic reduction in the number and size of lamellipodia and ruffles (Fig. 8F) relative to the cells expressing V12Rac1 alone (Fig. 8E). This suppression of the Rac1 "membrane" effect cannot be attributed to the GAP activity of RC-GAP72 because the Gly-to-Val mutation at amino acid 12 decreases the intrinsic GTPase activity of Rac1 and makes it resistant to GAP proteins (24). Furthermore, this co-transfection resulted in almost total destruction of the actin stress fibers and partial rounding of the cells, suggesting that RC-GAP72 is capable of recruiting activated Rac1 to stress fibers, inducing stress fiber disassembly and reducing the peripheral effect of Rac1 on the development of membrane protrusions. These results confirm our hypothesis that cytoskeletal localization and binding to the

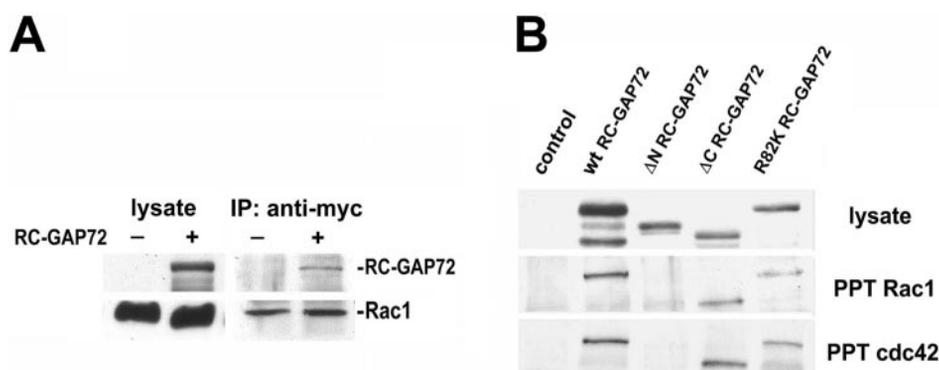
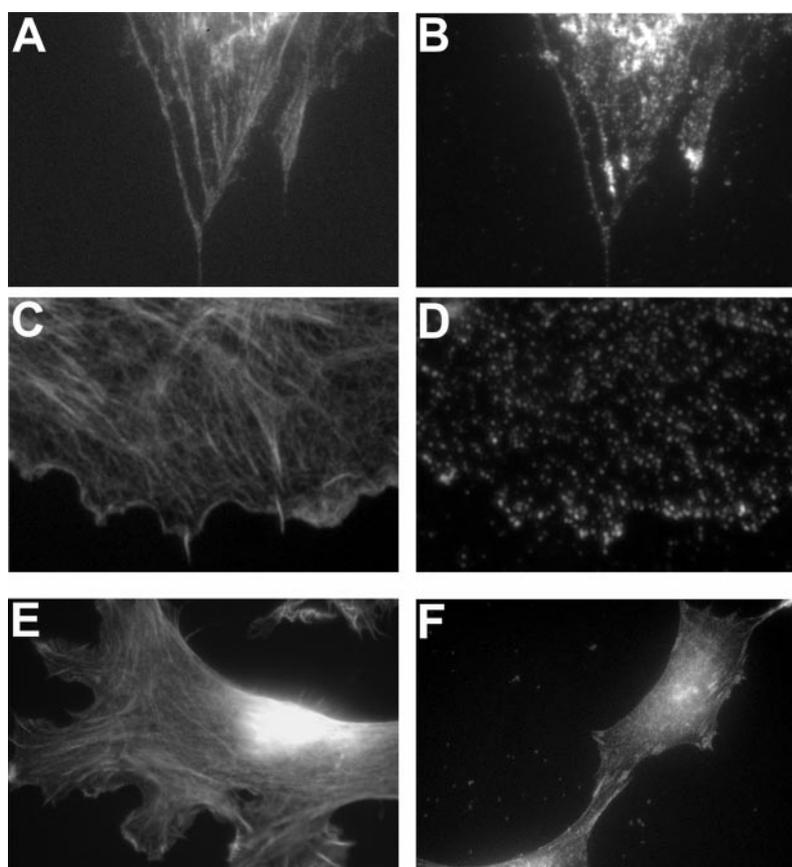


FIG. 7. The R82K mutant of RC-GAP72 retains the ability to interact with the active form of Cdc42 or Rac1. *A*, RC-GAP72 forms a complex with Rac1. 293T cells were co-transfected with RC-GAP72 and the Myc-tagged constitutively active mutant of Rac1. Immunoprecipitation (IP) was performed with anti-Myc antibodies, and a Western blot was carried out using anti-Rac1 and anti-GFP antibodies. *B*, R82K RC-GAP72 mutant associates with activated Rac1 similar to the wild-type (*wt*) protein and Δ C RC-GAP72 containing the RhoGAP domain, whereas the Δ N RC-GAP72, lacking the GAP domain, fails to bind to the two GTPases. 293T cells were co-transfected with RC-GAP72 or its mutants and wild type of either Rac1 or Cdc42. The active forms of the two GTPases were pulled down with glutathione *S*-transferase fusion of the GTPase binding domain of PAK1. RC-GAP72 associated with that complex was detected by Western blotting using anti-GFP antibodies. *PPT*, precipitate.

FIG. 8. RC-GAP72 was co-localized with the active form of Rac1 in living cells and alters its effect on cell morphology.

A–D, constitutively active mutant of Rac1 (V12Rac1) tagged with an Myc epitope was co-expressed in REF52 fibroblasts with RC-GAP72-YFP (*A* and *B*) or with YFP as a control (*C* and *D*). RC-GAP72 (*A*) and V12Rac1 (*B*) are co-localized on the stress fibers. In control cells V12Rac1 (*D*) does not show localization on the stress fibers (*C*, phalloidin staining). *E* and *F*, coexpression of V12Rac1 with RC-GAP72 caused a dramatic reduction in number and size of lamellipodia and cell body retraction (*F*) compared with the cells expressing V12Rac1 alone (*E*).



active form of GTPases but not GTP hydrolysis are essential for the cytoskeletal perturbations caused by RC-GAP72.

DISCUSSION

In this paper we describe a novel, uncharacterized member of the RhoGAP family that was isolated in a microscopy-based screen due to its association with focal contacts and stress fibers. This protein belongs to a new family of RhoGAPs that, based on sequence similarities, includes at least three members; RC-GAP72 and its splice variants AK098193 and AK091196, RhoGAP2, which shows 53% overall protein sequence similarity and 90% similarity to the RhoGAP domain, and KIAA0053, whose RhoGAP domain shows 77% similarity to that of RC-GAP72 but has no homology to other parts of the protein. Like some other RhoGAP proteins (*e.g.* GRAF, olig-

ofrenin, ArhGAP8, ArhGAP9, ArhGAP15, and PSGAP), RhoGAP2, KIAA0053, and RC-GAP72 splice variants AK098193 and AK091196 have a PH domain in the N-terminal region. The PH domains of AK091196 show 75 and 66% similarity to RhoGAP2 and KIAA0053, respectively, and lower similarity to other PH domain-containing proteins.

Biochemical characterization of RC-GAP72 demonstrates that it specifically activates GTP hydrolysis on Rac1 and Cdc42 but not on RhoA and that the conserved catalytic arginine residue in its RhoGAP domain is necessary for GAP activity. In the present study we aimed at establishing whether Rac1 and Cdc42 GAP activity of RC-GAP72 or its unique subcellular localization are responsible for the observed cellular effects.

RhoGAPs are characterized by diverse subcellular localiza-

tion. For example, diffuse cytoplasmic localization has been reported for RICH-1 (25); β -chimaerin, ARHGAP4, and IQGAP are associated with the Golgi apparatus (45–47); IQGAP and nadrins have been observed in the nucleus (47, 48); and ARHGAP4 and PSGAP show perinuclear localization (46, 49). Some RhoGAP proteins co-localize with different structures of the actin cytoskeleton, which is their primary cytoskeletal target. ArhGAP15, GRAF, and Grit/RICS/p200RhoGAP are associated with cortical actin (14, 18, 50), p190RhoGAP, ARHGAP6, CdGAP, and IQGAP colocalize with filopodia and lamellipodia (12, 33, 35, 51), and Grit/RICS/p200RhoGAP, nadrins, and oligophrenin are associated with neurite tips (48, 50, 52). The co-localization of RhoGAP proteins to stress fibers has been reported for *N*-chimaerin, oligophrenin1, and GRAF (14, 52, 53). GRAF and IQGAP have been specifically observed in cell-matrix and cell-cell adhesions, respectively (14, 35, 54). The diversity in the cellular distributions of Rho GAPs may reflect the important role of the location in their function. The targeting of different Rho GAPs to diverse subcellular compartments may also explain the need for more than 80 human RhoGAPs compared with only 20 Rho-family GTPases, enabling site-specific regulation of the same GTPases.

In this study we showed that RC-GAP72 is associated with actin stress fibers and focal contacts in fibroblasts and, in addition, with cell-cell adherens junctions in epithelial (MDCK) cells. This localization suggests that at least one of the functions of RC-GAP72 is regulation of the cytoskeleton. The interaction with actin is mediated via the C-terminal region of the molecule, and characterization of different mutants indicates that the binding site is located within the 185 C-terminal amino acids. Although the C terminus is required for RC-GAP72 localization to the cytoskeleton, it is not yet clear whether it binds directly to actin or to some actin-binding protein.

Functional analysis of RC-GAP72 in cultured cells identified several unique characteristics of this novel RhoGAP. Transfected cells lost actin stress fibers, retracted from the coverslip, and extended long filopodia, ruffles, and lamellipodia. Such a destructive effect on the actin cytoskeleton has been shown for several RhoGAP proteins (11, 12, 48, 53, 55). One of the accepted explanations for this phenomenon is inactivation of Rho family GTPases by GAP proteins, based on the established physiological roles of Rho GTPases in the regulation of the actin cytoskeleton. Consequently, regulators and effectors of Rho GTPases including RhoGAPs are supposed to play key roles in regulating actin architecture. However, the morphological effect of RC-GAP72 is rather surprising since disruption of actin stress fibers and formation of membrane ruffles, extended lamellipodia, and filopodia would be expected for excessive activation of Rac1 and cdc42, not for GAP-mediated inhibition of these GTPases. On the other hand, similar effects have been reported to be induced by other Rac1/cdc42-specific RhoGAPs such as *N*-chimaerin, ArhGAP15, and nadrin (18, 53, 56), indicating that regulation of the actin cytoskeleton by these proteins may be partially or completely independent of their RhoGAP action.

Some RhoGAPs (p190, GRAF) containing a mutation in the GAP domain, which renders the protein enzymatically inactive, have also been reported to lose their effect on cellular morphology (10, 11). The same mutation was reported to disrupt the ARHGAP6 ability to remodel the actin cytoskeleton without any significant effect on the extension of cytoplasmic processes (12). Interestingly, all of these proteins acted as GAP toward RhoA. The GAP-deficient mutant of *N*-chimaerin (brain Rac1/Cdc42-specific RhoGAP) was able to induce the formation of lamellipodia and filopodia similar to the wild-type protein (53).

Inactivation of the RhoGAP domain does not abolish the capacity of RC-GAP72 to disrupt stress fibers; however, this mutation specifically eliminates its ability to induce membrane protrusions. These results suggest that RC-GAP72 has at least two unrelated actin-organizing effects that are mediated via different domains of the RC-GAP72 molecule, and one of them is independent of its RhoGAP activity.

The truncated forms of RC-GAP72, including Δ C-RC-GAP72, containing the GAP domain and lacking the cytoskeleton-binding site and the GAP domain-deficient Δ N-RC-GAP72 with cytoskeletal binding property, were ineffective in inducing stress fiber disruption. The notion that the effect on stress fibers requires proper subcellular targeting is indirectly corroborated by the failure of the Δ C-RC-GAP72 mutant to disrupt stress fibers while exerting a full membrane effect. Thus, RC-GAP72 requires both the GAP domain (active or inactive) with its GTPase binding property and the C-domain, necessary for localization, to induce stress fiber destruction.

The exact mechanism underlying the effect of RC-GAP72 on the actin cytoskeleton is still largely unknown. It is, however, worth noting that the point mutant lacking GAP activity is capable of binding to Rac1 and cdc42 and to target them to stress fibers just like the wild-type molecule. This observation raises the possibility that RC-GAP72 modulates the activity of the respective GTPases not only by promoting their inactivation but also by re-localizing them in their active state to specific subcellular domains (such as stress fibers and focal adhesions) to serve as a cytoskeletal anchor for activated Cdc42 and Rac1 GTPases, whose recruitment triggers morphological changes. Thus, co-expression of RC-GAP72 with a constitutively active mutant of Rac1 shifts the focus of Rac1 effect on actin from the membrane (where it induces the formation of protrusions) to the stress fibers (where it induces disassembly). This intriguing model requires that the rate of GTP hydrolysis induced by the GAP molecule be rather slow and that the GTPase be able to interact with and affect downstream targets while being associated with the GAP domain. The results shown here, namely the capacity of PAK1 to pull down the RC-GAP72-Rac1 and/or RC-GAP72-Cdc42 complex, support the suggested model, although that is in conflict with the notion that PAK and GAPs bind to overlapping sites on Rac1 and Cdc42, and thus, their binding is expected to be mutually exclusive. We can offer a few possible explanations for the present findings, including the formation of a multimolecular complex containing a few molecules of GTPase, where different GTPase molecules bear either PAK or RC-GAP72. Other possibilities are that RC-GAP72 might directly bind PAK or that RC-GAP72 can bind to the respective GTPases without interfering with the binding to their downstream targets. All these possibilities are currently being investigated. Whether the mechanism suggested here whereby the effect of GAP protein on the respective GTPases is regulated by its subcellular localization may be a more general characteristic shared by other GAP molecules remains to be determined.

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