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Expression of α_1-Chimaerin (*rac-1* GAP) Alters the Cytoskeletal and Adhesive Properties of Fibroblasts

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Abstract The small GTP-binding protein *rac-1*, a member of the *ras* gene superfamily of GTPases, is thought to be a key component of a signal transduction pathway that mediates cell membrane ruffling and actin stress fiber formation induced by growth factors. *rac-1* protein is regulated by the interplay of several activities: proteins that enhance GDP dissociation (GDP Dissociation Stimulator, GDS), inhibit nucleotide exchange (GDP Dissociation Inhibitor, GDI), or accelerate GTP hydrolysis (GTPase Activating Protein, GAP). We have assessed the relative contribution of the *rac-1*/GAP interactions to the overall activity of *rac-1* by expressing α_1-chimaerin, a *rac-1*-specific GAP, in fibroblasts. NIH 3T3 cells were transfected with α_1-chimaerin cDNA-containing expression vector and stable clones were established. Extracts prepared from α_1-chimaerin-expressing cells showed *rac-1* GAP activity that was regulated by phosphatidylserine and phorbol ester.

The cells expressing α_1-chimaerin showed a distinct phenotype. They had altered adhesive properties as measured by their ability to bind to a fibronectin-coated glass surface, suggesting that the expression of a *rac-1* GAP alters the assembly of integrin receptors, actin and cytoskeletal proteins such as vinculin and talin. Direct demonstration of this phenomenon was achieved by studying the organization of actin stress fiber and formation of focal adhesions in the α_1-chimaerin expressing cells following stimulation by growth factors. Mock transfected cells, upon serum or lysophosphatidic acid stimulation, organize actin as a dense array of parallel fibers running the length of the cell. This process did not take place in the cells expressing *rac-1* GAP. Similarly, the formation of focal adhesions as measured by the appearance of vinculin clusters was impaired in the α_1-chimaerin expressing cells. These results demonstrate that expression of a GAP for *rac-1* in fibroblasts produces profound changes in the cytoskeletal organization and suggest that GAP activity negatively regulates *rac-1* function.

Key words: GTP-binding proteins, GTPases, *ras*-related proteins, GAPs, actin organization

The *ras* gene family encodes proteins (i.e., the *ras, rho,* and *rab* subfamily) that play crucial roles in cell-cycle progression, cytoskeletal organization, cell differentiation, protein trafficking, and secretion [Bourne et al., 1990; Valencia et al., 1991; Chardin, 1991; Hall, 1990]. These low-molecular weight GTPases share several common features: they exhibit GDP/GTP binding, and their active GTP-bound form is controlled by a switch mechanism involving the activity of nucleotide exchange(s) factors (GDS) and GTPase activating proteins (GAPs). These small GTP-binding proteins often undergo post-translational modifications that are essential for their biological activity [Bourne et al., 1990; Chardin, 1991; Gibbs, 1991; Marshall, 1993].

The regulatory cycle that operates in the control of *rac-1* activity, a member of the *rho* subfamily present in a variety of tissues [Didsbury et al., 1989], has been described. The conversion of the GDP-bound *rac-1* to the GTP-bound form is thought to involve two separate events: (1) dissociation of *rac-1* from GDI, a protein that forms a cytosolic complex with the postranslationally modified *rac-1* [Hiraoka et al., 1992; Abo et al., 1991; Kikuchi et al., 1992; Chuang et al., 1993; Hancock and Hall, 1993]; and (2)

Abbreviations used: DMEM, Dulbecco's minimal essential medium; PBS, phosphate-buffered saline; HEPES, N-2 hydroxyethylpiperazine-N-2 ethanosulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GAP, GTPase activating protein; GDS, GDP dissociation stimulator; GDI, GDP dissociation inhibitor; GST, glutathione S-transferase; DTT, dithiothreitol; BSA, bovine serum albumin; LPA, lysophosphatidic acid; PS, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate.

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interaction with GDS, a protein(s) that facilitate(s) the dissociation of GDP and enhances the binding of GTP to the *rac-1* protein [Hiraoka et al., 1992; Kikuchi et al., 1992]. It is believed that this GTP bound form of the protein would then be capable of interacting with the effector that mediates its biological activity. Like many of the small GTP-binding proteins, the *rac-1* protein exhibits an intrinsic GTPase activity [Ménard et al., 1992]. Stimulation of this activity by GAP proteins is considered the inactivation step.

Several multidomain proteins are known to be GAPs for *rac-1*. These include the BCR gene product, a protein that has both *rac-1* GAP and serine kinase activities [Diekman et al., 1991; Maru and Witte, 1991], the *ras* GAP protein p190 [Settleman et al., 1992], and the α_2-chimaerin protein that contains SH2- and phorbol ester.

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A biological role for *rac-1* has been deduced by Hall and colleagues [Ridley et al., 1992]. They have shown that growth factor induced accumulation of actin filament at the plasma membrane could be blocked by a negative dominant *rac-1* protein. They have also shown that the formation of stress fiber is dependent on the activity of *rac-1*. This latter effect is mediated by a *rac-1*-dependent activation of *rho*, a small GTP-binding protein that plays a key role in the organization of polymerized actin [Rubin et al., 1988; Chardin et al., 1989; Paterson et al., 1990; Ridley and Hall, 1992; Ridley et al., 1992].

In this study, we have determined the relative contribution of one of the components of the cycle that regulates the activity of *rac-1* in NIH 3T3 fibroblasts by expressing α_1-chimaerin, a specific *rac-1* GAP. We show here that *rac-1* GAP expression causes marked changes in the shape and adhesive properties of transfected cells.

MATERIALS AND METHODS

Cloning and Expression of α_1-Chimaerin

The α_1-chimaerin cDNA [Hall et al., 1990] was tagged at the 5′ end with a DNA sequence encoding a myc epitope (MEQKLISEEDL) by PCR cloning from a human brain cDNA library (Clontech Laboratories, Inc., Palo Alto, CA). The PCR reaction was carried out according to the manufacturer's recommended conditions (Perkin Elmer Cetus, Norwalk, CT). The amplified cDNA was sequenced and subcloned into the expression vector pCep4 (Invitrogen, San Diego, CA) to generate the *rac-1* GAP/pCep4 vector. NIH 3T3 cells (American Type Culture Collection, Rockville, MD) were transfected using Transfectam (Promega, Madison, WI) with either pCep4 or *rac-1* GAP/pCep4 and stable clones were obtained by hygromicin selection (100 U/ml Sigma Chemicals, St. Louis, MO). Expression of α_1-chimaerin was detected by immunoprecipitation using the monoclonal anti-myc antibody 9E10 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Expression of GST–*rac-1* Fusion Protein

The murine *rac-1* cDNA [Moll et al., 1991] was obtained by PCR reaction from a mouse brain cDNA library (Clontech Laboratories, Inc.) and cloned into pGEX-3X to generate the glutathione S-transferase fusion protein (GST; Pharmacia, Piscataway, NJ). Protein induction and purification were carried out as previously described [Frangioni and Neel, 1993]. The purified protein had an intrinsic GTPase activity of 0.08 mmol/min/mmol of GTP bound at 15°C.

Immunoprecipitation

Cells were labeled with tran^{35}S-Label (100 μCi/ml, ICN Biomedical, Inc., Irvine, CA) in DMEM and 10% fetal calf serum for 5 h. Cell extracts were prepared by lysing the labeled cells in the following buffer: 1% Triton X-100; 50 mM HEPES, pH 7.5; 0.2% SDS; 0.15 M NaCl; and 2 μg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor. Lysed cell were cleared by centrifugation and immunoprecipitated with antibody 9E10 (2 μg) for 2 h at 4°C. The immune complex was collected by incubation with Protein G-Plus/Protein A Agarose (Oncogene

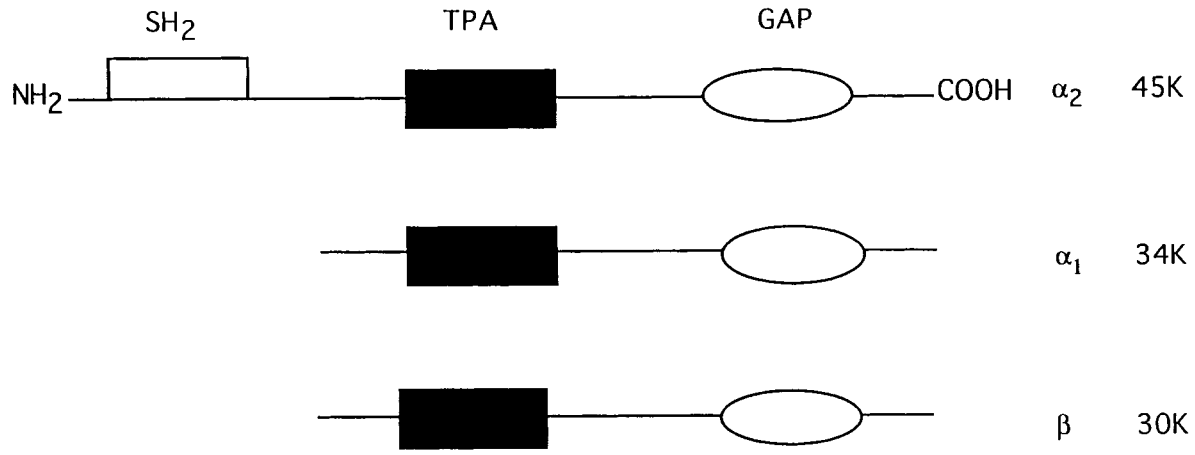

Fig. 1. Schematic representation of the three forms of chimaerin (α_1, α_2, β). The relative location of each of the functional domains is shown. The open rectangle depicts the location of an SH2 binding domain in α_2 chimaerin only. The solid rectangle shows the existence of the phorbol ester binding (TPA) in all three isoforms. The catalytic domain (open circles; GAP) resides on the carboxy terminus of each isoform.

Science, Uniondale, NY), washed with lysis buffer and analyzed by PAGE–SDS.

Immunoprecipitates used for measuring *rac-1* GAP activity were prepared by lysing the cells in a buffer containing 1% Triton X-100; 50 mM HEPES pH 7.5; 5 mM $MgCl_2$ and the protease inhibitors indicated above. Immunocomplexes were collected with Protein G-plus/Protein A Agarose by centrifugation and washed in the same buffer.

rac-1 Gap Activity

α_1-Chimaerin activity was assayed by incubating immunoprecipitated protein prepared from NIH 3T3-C8 cells with a GST*rac-1* fusion protein bound to ^{32}P-GTP. Binding of GTP to GST-*rac-1* was carried out as described in Garrett et al., [1989], using ^{32}P-labeled GTP (10 μM, 60 μCi/nmol). The intrinsic and α_1-chimaerin-stimulated GTPase activity of *rac-1* was measured by incubating the GTP-loaded protein in 100 μl of 50 mM Tris–HCl Buffer, pH 7.5; 5 mM $MgCl_2$; 0.5 mM DTT and BSA (100 μg/ml) at 15°C. The subsequent release of ^{32}P was measured as described in Kikuchi et al. [1988]. All the reactions were carried out for 10 min unless otherwise indicated. Under our conditions, the GTPase assay is linear up to 30 min. The effect of ligands on the activity of α_1-chimaerin was carried out as described [Ahmed et al., 1993] with the modifications indicated above.

Fluorescent Detection of Actin and Vinculin

Cells were grown in a Lab-Tek Chamber Slide (Nunc, Inc., Naperville, IL) coated with a fibronectin solution (500 μg/ml in PBS) and serum starved for 8 hr prior to stimulation, as indicated in figure 1. After stimulation with the indicated ligands, cells were rinsed three times with PBS and fixed in 3% neutral-buffered paraformaldehyde as described in Ridley and Hall [1992a]. For actin localization, the fixed cells were stained with 0.1 μg/ml TRITC-labeled phalloidin (Sigma Chemical Co., St. Louis, MO). For localization of vinculin, the fixed cells were incubated with an antivinculin mouse antibody (1:250; Sigma Chemical Co.) for 2 h, followed by incubation with an antimouse IgG FITC-labeled goat antiserum (1:400; Sigma Chemical Co.) for 40 min at room temperature and rinsed in PBS. Cells were viewed on a Zeiss 135 Axiovert microscope using the appropriate filters and photographed with Ektachrome film (100 ASA, Rochester, NY).

Fibronectin Binding

A serum-free cell suspension (10^5 cells) of either NIH 3T3/pCep4 or NIH 3T3-C8 cells were seeded on a glass surface (Lab-Tech Chamber Slide) previously been coated with different concentrations of fibronectin (Sigma Chemical Co.), as indicated in Figure 4. After 1 h of

incubation in a serum-free medium at 37°C, the cell suspension was removed and the slides were rinsed twice with PBS. The bound cells were removed by incubation with a trypsin-EDTA solution and quantified using a hemocytometer. Each measurement was done in triplicate for a given fibronectin concentration.

RESULTS

Expression of Epitope-Tagged α_1-Chimaerin in NIH 3T3 Cells

There are three known forms of the protein chimaerin (Fig. 1). The α_1 form is expressed in neural tissues. The α_2 form is expressed both in neurons and testis [Hall et al., 1993], while the β form is expressed in late stages of spermatocyte maturation [Leung et al., 1993]. All three forms contain a phorbol ester-binding domain at their N-terminus and a *rac-1* GAP catalytic domain at their COOH-terminus. The α_2 form contains, in addition, a src-homology 2 region (SH2 domain).

We have transfected NIH 3T3 cells with a mammalian expression vector containing α_1-chimaerin cDNA. The protein carries at its NH_2-terminus a short peptide sequence that is recognized by the monoclonal antibody 9E10 (see Materials and Methods). Cell clones resistant to hygromycin were isolated and analyzed for the expression of this tagged protein by immunoprecipitation of ^{35}S-labeled cell extracts. Of the clones obtained, four were characterized. A representative clone (NIH 3T3-C8) is depicted in Figure 2A. An ^{35}S-labeled extract prepared from mock (lane 1) or α_1-chimaerin (lane 2) transfected cells was incubated with the antibody directed to the N-terminus epitope and the immunoprecipitate analyzed by SDS–PAGE. The antibody precipitated a protein from NIH 3T3-C8 cells, but not from mock transfected cells (NIH 3T3/pCep4), that migrates in a gel at the predicted molecular weight of α_1-chimaerin (34 kD) [Hall et al., 1990].

To confirm that this protein was, indeed, α_1-chimaerin, we carried out a GTPase assay using a GST*rac-1* fusion protein as a target. Incubation of an immunoprecipitate prepared from NIH 3T3-C8 cells with GST*rac-1* protein resulted in a stimulation of the intrinsic GTPase activity of GST*rac-1*. This effect was not detected when GST*rac-1* was incubated with an immunoprecipitate prepared from mock transfected cells (Fig 2B). Under our conditions, the

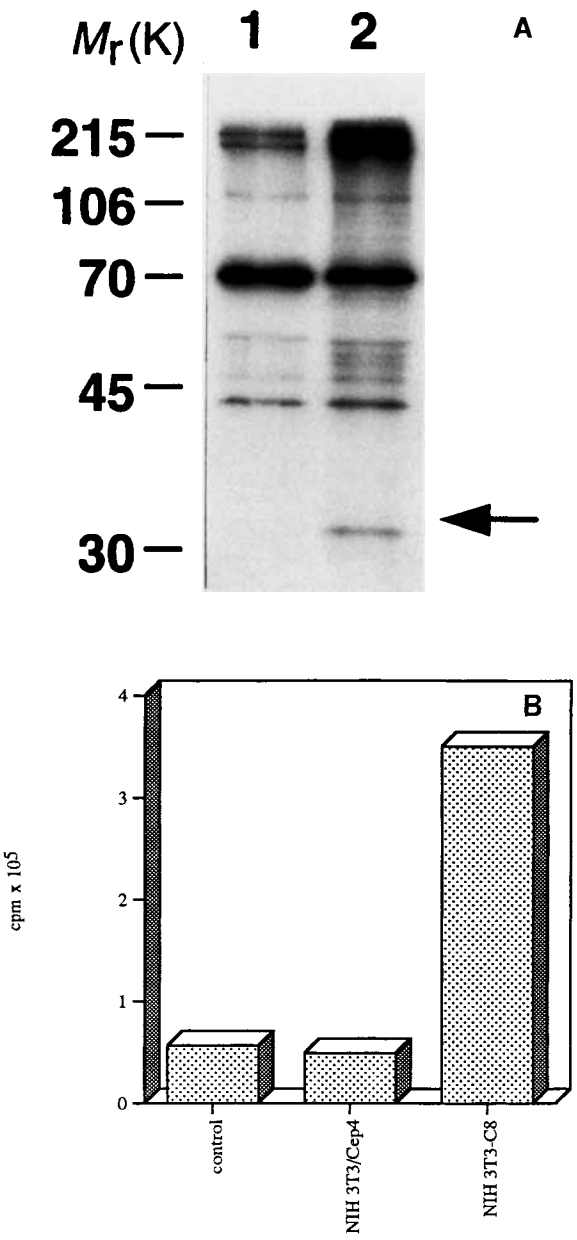

Fig. 2. **A:** Expression of α_1-chimaerin in NIH 3T3 cells. ^{35}S-labeled proteins were immunoprecipitated from either mock-transfected (*lane 1*) or *rac-1* GAP-transfected NIH 3T3 cells (*lane 2*) and analyzed using SDS–PAGE with a 10% gel. An autoradiogram produced from an overnight exposure to film is shown. The arrow indicates a specific protein having a molecular weight of about 34 kD. Cell labeling and immunoprecipitation were carried out as described in Materials and Methods. **B:** In vitro *rac1* GAP activity of α_1-chimaerin expressed in NIH 3T3 cells. Cell extracts prepared from NIH 3T3/Cep4 (mock-transfected) or NIH 3T3-C8 (α_1-chimaerin-transfected) cells were immunoprecipitated with the anti-myc epitope antibody and the immunoprecipitate were analyzed for *rac1* GAP activity using ^{32}P-GTP-loaded GST–rac1 fusion protein (50 nM) as described in Materials and Methods. Control represents basal GTPase activity of the GST-rac1 protein.

activation of *rac-1* GTPase activity by α_1-chimaerin varied from four- to eightfold.

Properties of α_1-Chimaerin Expressed in NIH 3T3 Cells

The catalytic activity of α_1-chimaerin is located at its COOH-terminus [Diekman et al., 1991]. The NH_2-terminus contains a specificbinding site for phorbolesters [Ahmed et al., 1990]. Recently, it has been proposed that the phorbol ester binding site has an inhibitory effect on the intrinsic catalytic activity of the protein [Ahmed et al., 1993]. We have compared the in vitro *rac-1* GAP activity of α_1-chimaerin immunoprecipitated from treated or untreated NIH 3T3-C8 cells with PMA. The α_1-chimaerin prepared from treated or untreated cells showed similar activity toward GST*rac-1* fusion protein (data not shown). However, α_1-chimaerin prepared from untreated cells could be readily activated in vitro by incubation with PS or PMA or a combination of both (Fig. 3). These results suggest that the activity of the α_1-chimaerin protein could be subject to regulation by metabolites generated by activation of specific phospholipases.

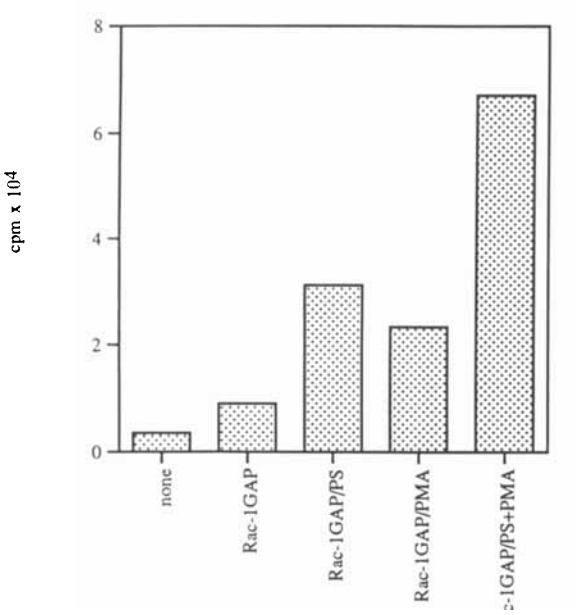

Fig. 3. In vitro activation of α_1-chimaerin. Immunoprecipitates containing α_1-chimaerin activity (*rac-1* GAP) were obtained from cell extracts prepared from NIH 3T3-C8 cells as described in Materials and Methods. The immune complexes were incubated with a ^{32}P-GTP-loaded GST*rac1* protein (5 nM) in the absence or presence of PS (100 μg/ml), PMA (100 nM) or both (15 μg/ml PS and 35 nM PMA). None indicates the basal GTPase activity of the GST*rac1* fusion protein in the presence of both PS and PMA.

NIH 3T3 Cells Expressing α_1-Chimaerin Have Altered Adhesive Properties

During the isolation of the hygromycin-resistant clone (C8), we observed that the transfected cells had the tendency to detach from the dish surface, especially at low serum concentrations. This prompted us to analyze the adhesive properties of the NIH 3T3-C8 clone. Thus, we studied the binding of the transfected cells to a surface that had been coated with different concentrations of fibronectin. As shown in Figure 4, to reach a binding efficiency of 50%, the NIH 3T3-C8 cells required a 20-fold higher concentration of fibronectin-coated surface as compared to the binding of the mock transfected cells. This result suggests that a high expression of α_1-chimaerin alters the cytoskeletal properties of the cell upon which adhesion depends.

NIH3T3 Cells Expressing α_1-Chimaerin Show Altered Cell Shape and Organization of Actin Filaments

The biological role for the chimaerins is unknown. However, its intracellular target, *rac-1*,

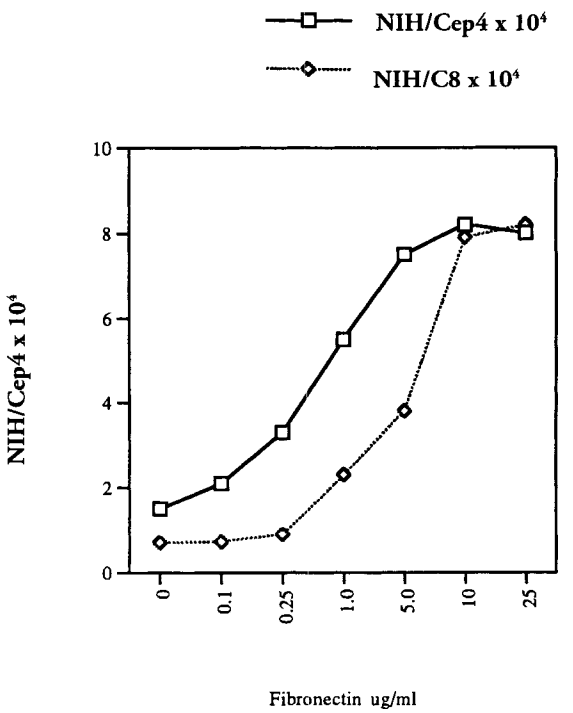

Fig. 4. Fibronectin binding of mock or α_1-chimaerin transfected cells. The binding of the cells to a fibronectin-coated surface was carried out as described in Materials and Methods. The number of cells bound versus the indicated concentration of fibronectin is shown.

has been implicated in the organization of polymerized actin [Ridley et al., 1992b]. In fibroblasts, actin filaments are found in the cortical network, stress fiber and membrane ruffles [Small, 1988]. As we have shown here (Fig. 4), the binding of cells to fibronectin was altered by the expression of α_1-chimaerin, suggesting that the interactions among actin, integrin receptors and proteins such as vinculin, talin, and tensin were altered.

Therefore, we compared the changes in actin organization upon serum stimulation between the mock transfected and the α_1-chimaerin expressing cells. In Figure 5A, actin stress fibers are not well organized in unstimulated mock-transfected cells (a,b), but upon serum (c,d) or LPA (e,f) stimulation, the actin fibers thicken and organize along the long axis of the mock transfected cells (c,e). Focal adhesions become apparent (d,f) though LPA is a less robust stimulator (f) than serum (d) of this feature of cytoskeletal organization. In Figure 5B, even less actin organization is apparent in cells transfected with *rac-1* GAP (a,b) than in the mock transfected cells shown in Figure 5A (a,b). Serum stimulation of *rac-1* GAP transfected cells (c,d) promotes some bundling of actin fibers (c), but few or no focal adhesions are apparent (d). LPA stimulation of *rac-1* GAP transfected cells appears no better than serum in organizing actin fiber bundles (e) but seems more effective at promoting the formation of focal adhesions (f).

A further observation was an increased staining for vinculin apparently associated with nuclear membranes in *rac-1* GAP transfected cells as compared to mock transfected cells under all described treatment conditions. The reason for this change in vinculin localization is at present unknown.

DISCUSSION

The activity of the small GTP-binding protein *rac-1* is controlled by a complex regulatory cycle. Key participants in this cycle include GDS, GDI, and GAP.

The role that GDS(s) play in the activation of *rac-1* was revealed by the abolition of growth-factor induced stress fiber formation in Swiss 3T3 cells microinjected with a mutated *rac-1* protein [Ridley et al., 1992b]. The mutated protein is thought to block the interaction of GDS with the endogenous *rac-1* protein.

On the other hand, the role that GDI plays in the regulation of *rac-1* activity is unclear. The interaction between GDI and *rac-1* results in an inhibition of GAP(s)-mediated GTPase activity of *rac-1* [Hancock and Hall, 1993]. Microinjection of GDI into Swiss 3T3 cells produces changes in cell morphology and stress fiber formation [Miura et al., 1993] suggesting that the interaction of GTP-bound *rac-1* with GAP(s) is crucial for signal transmission by activated *rac-1*. One known pathway that requires an active *rac-1* protein is the *rac-1*-dependent activation of *rho*, another small GTP-binding protein (Ridley and Hall, 1992a; Ridley et al., 1992b]. However, the effect that GDI causes on cell morphology and stress fiber formation can not be attributed specifically to *rac-1*, since it is known that GDI is also able to interact with *rho* [Hiraoka et al., 1992].

Thus far, the relative contribution of GAP(s) activity to the regulatory cycle that controls *rac-1* activity has not been directly tested. In a study carried out by McGlade et al. [1993], the effect of overexpression of the SH2/SH3 domains of *ras* GAP on cell shape was attributed to an activation of p190, a *ras* GAP binding protein that exhibits *rac-1* GAP and *rho* GAP activity [Settleman et al., 1992]. Here, we have directly tested the contribution of *rac-1* to cell function by overexpressing a specific *rac-1* GAP in NIH 3T3 fibroblasts.

We have established an NIH 3T3 line cells expressing the α_1 form of *n*-chimaerin by DNA-mediated expression. The expressed protein is fully functional as determined by its ability to enhance the GTPase activity of a GST-*rac-1* fusion protein in an in vitro assay (Fig. 2B). Moreover, this activity was regulated in vitro by known modulators as it has been previously demonstrated with *n*-chimaerin expressed in bacteria [Ahmed et al., 1993] (Fig. 3).

The NIH 3T3 cells expressing α_1-chimaerin had a slightly slower rate of proliferation. However, the most prominent phenotypic changes were found in the adhesive properties of the cells and their actin stress fiber distribution upon serum stimulation. The cells expressing α_1-chimaerin clearly bound less efficiently to a fibronectin-coated surface than the mock transfected cells (Fig. 4). This result suggests that α_1-chimaerin alters the ability of the cells to organize the cytoskeleton.

This was substantiated by looking at the organization of actin and vinculin distribution following serum stimulation. As shown in Figure 5, mock transfected cells, upon serum or LPA

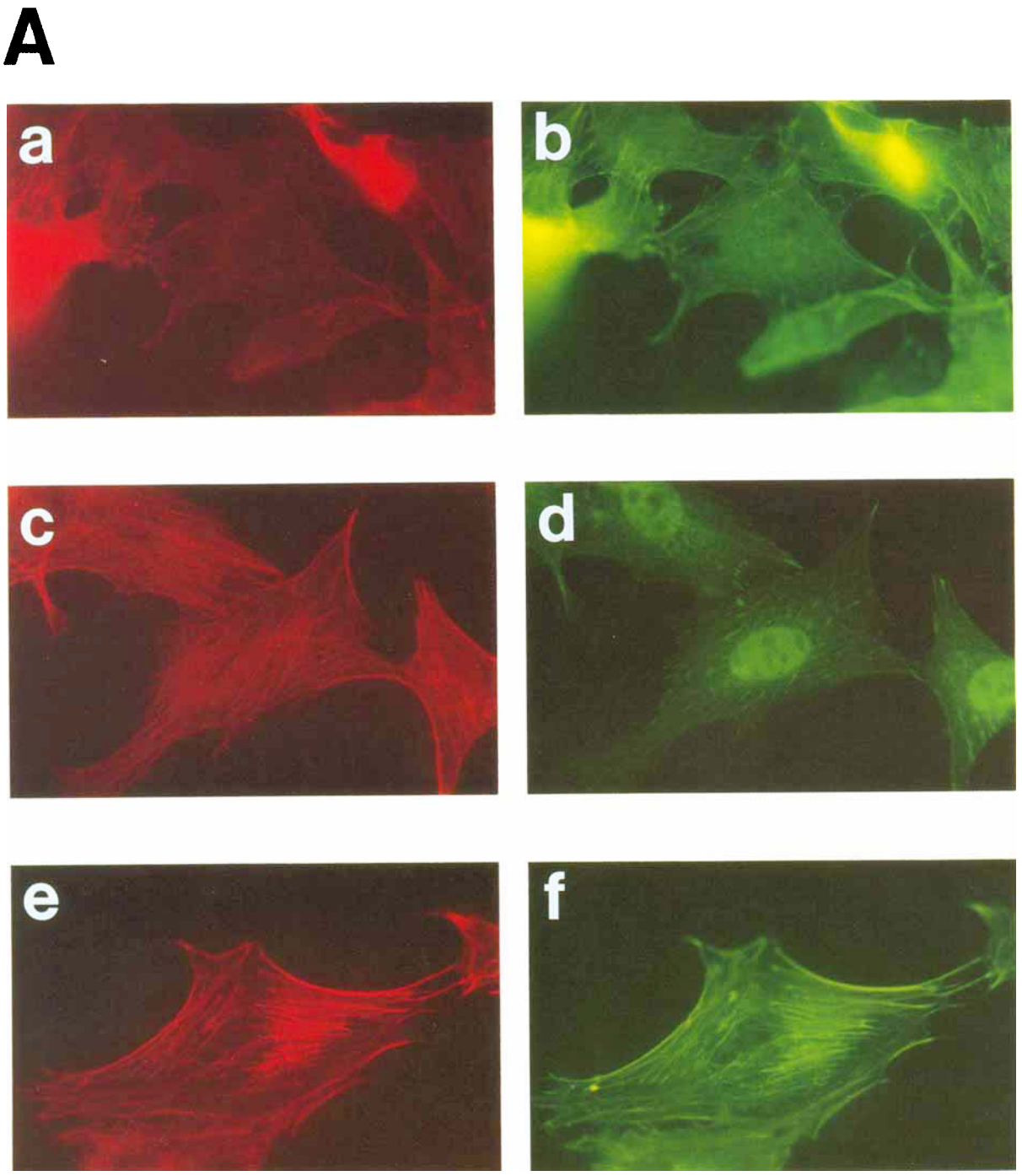

Fig. 5. Mock-transfected NIH3T3 cells **(A)** show the expected effects of serum and LPA on cytoskeletal organization while cells transfected with *rac-1* GAP **(B)** show atypical actin fiber organization. TRITC-labeled phalloidin-stained actin fibers red and antivinculin antibody, followed by FITC-labeled secondary antibody-labeled focal adhesions green. In A and B, cells were either untreated (a,b), serum stimulated (c,d), or LPA treated (e,f).

stimulation, showed a regular pattern of actin fiber distribution running the length of the cell. The vinculin staining in response to serum stimulation showed a clear clustering indicating the formation of focal adhesions. In contrast, the organization of actin fiber of the cells expressing α_1-chimaerin in response to serum stimulation was atypical. These cells showed an irregular array of actin fibers and diminished clustering of vinculin. The LPA-mediated stress fiber for-

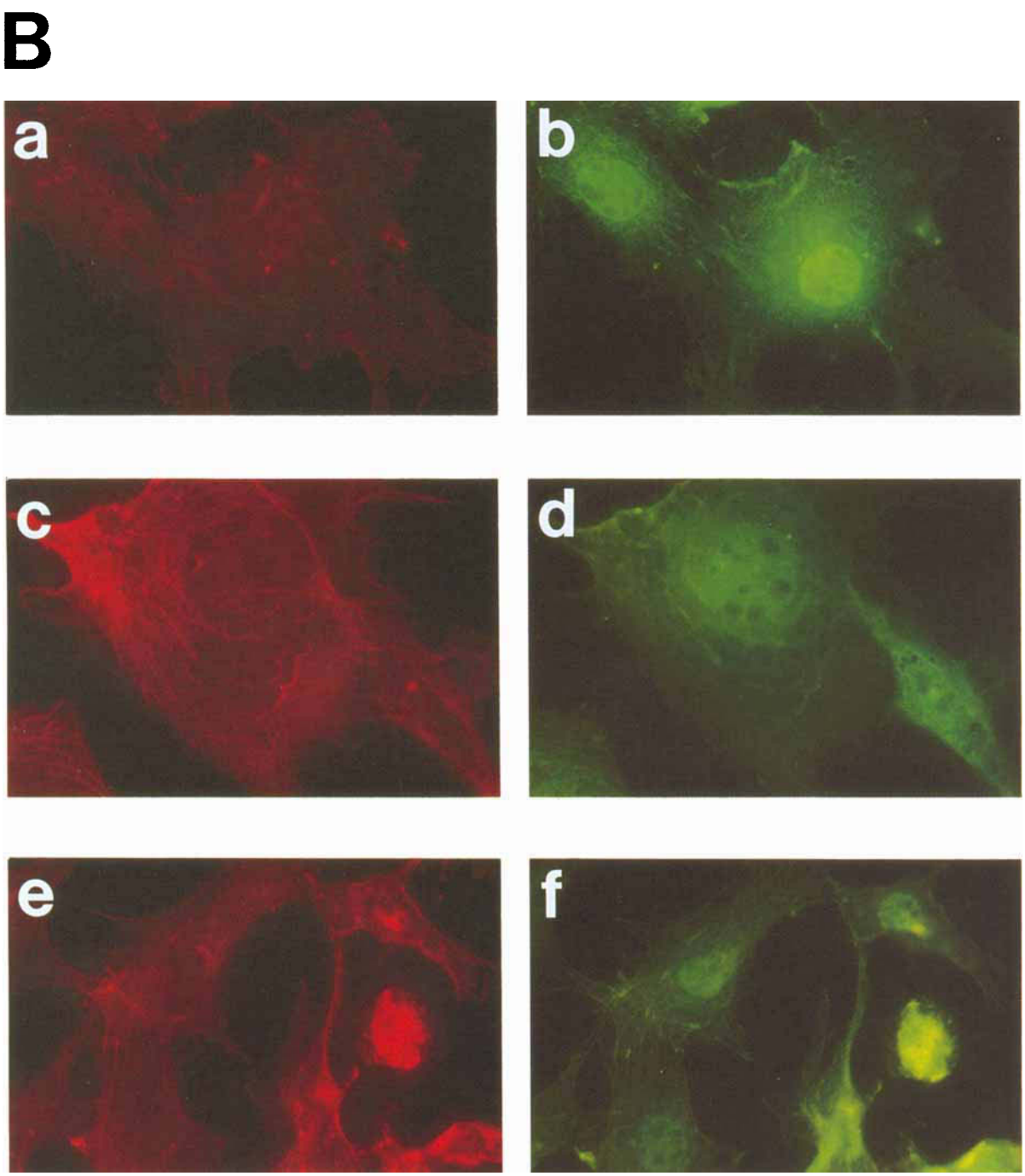

Figure 5. Continued.

mation was only partially blocked indicating that α_1-chimaerin has only a minor effect on the *rac-1* independent activity of *rho*. On the other hand, it is known that LPA is a negative modulator of the *rac-1* GAP activity associated with α_1-chimaerin [Ahmed et al., 1993]; therefore, the partial effect seen in the LPA-stimulated cells could be due to a neutralization of the effect caused by overexpression of α_1-chimaerin.

The effects of α_1-chimaerin on the cytoskeletal organization and adhesive properties in the cells described above suggest that *rac-1* GAP activity contributes to the regulation of *rac-1* as a negative modulator and highlights the role of *rac-1*

in the organization of the cytoskeleton. However, it should be borne in mind that this conclusion is based on the assumption that the observed effects are due to the catalytic activity of *rac-1* GAP per se and not due to interaction with other cellular components through a different domain.

The proposed role for α_1-chimaerin as a negative modulator of *rac-1* activity is similar to what has been proposed for other GAPs. Overexpression of full length *ras* GAP in NIH 3T3 suppresses the transforming activity of c-ras, as well as the transformation mediated by c-src [Zhang et al., 1990; DeClue et al., 1991]. Similarly, expression of the carboxyterminal catalytic domain of *ras* GAP in NIH 3T3 cells blocks the signal transduction pathway initiated by binding of CSF-1 to its receptor [Bortner et al., 1991]. It has also been shown that TPA-induced activation of p42MAPK, c-fos expression, and DNA synthesis could be blocked by overexpression of *ras* GAP [Nori et al., 1992].

Since *ras* GAP is a multidomain protein it is possible that some of these domains may participate in some signaling mechanism that are independent from down-regulation of *ras* activity. Indeed, overexpression of the N-terminal region of GAP (SH2 and SH3 domains) in Rat-2 fibroblast regulates the cytoskeletal structure of the cell, presumably by interaction with p190 a *rac-1* GAP and *rho* GAP [McGlade et al., 1993]. Similarly, the SH2-SH3 regions of GAP are responsible for the ras-dependent inhibition of the cardiac muscarinic-regulated K^+ channel [Martin et al., 1992]. Furthermore, the effect of GAP on gene expression also seems to be mediated by regions different from the C-terminal catalytic domain [Schweighoffer et al., 1992]. Similarly, the GAP-mediated enhancement of *Xenopus* oocytes germinal vesicle breakdown requires a functional SH3 domain [Duchesne et al., 1993]. Therefore, it remains to be determined whether the biological role of some of the forms of chimaerin, given their tissue specific pattern of expression, are regulated in a similar fashion.

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