

# Microinjection of Recombinant p21<sup>rho</sup> Induces Rapid Changes in Cell Morphology

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**Abstract.** The rho proteins, p21<sup>rho</sup>, are ubiquitously expressed guanine nucleotide binding proteins with ~30% amino acid homology to p21<sup>ras</sup>, but their biochemical function is unknown. We show here that microinjection of constitutively activated recombinant rho protein (Vall4rho) into subconfluent cells induces dramatic changes in cell morphology: 15–30 min after injection cells adopt a distinct and novel phenotype with a contracted cell body and finger-like processes still adherent to the substratum. Ribosylation of Vall4rho with the ADP-ribosyltransferase C3 from *clostridium botulinum*, before microinjection, renders the protein

biologically inactive, but it has no effect on either its intrinsic biochemical properties or on its interaction with the GTPase activating protein, rho GAP. Microinjection of ribosylated normal rho, on the other hand, has a similar effect to injection of C3 transferase and induces complete rounding up of cells. We also report striking biochemical changes in actin filament organization when contact-inhibited quiescent 3T3 cells are injected with Vall4rho protein. The effects induced by activation or inactivation of p21<sup>rho</sup> described here, suggest that the biological function of this protein is to control some aspect of cytoskeletal organization.

THE guanine nucleotide binding proteins, p21<sup>ras</sup> have attracted a great deal of attention since they appear to be regulatory components of the proliferative response (Barbacid, 1987; Morris et al., 1989). However, it is now evident that the three ras proteins are members of a much larger superfamily of related proteins (Chardin, 1988). Six proteins, rap1 (A and B), rap2, R-ras, and ral (A and B) have ~50% amino acid homology to ras and there is evidence that some of these may also be involved in the control of cell proliferation (Pizon et al., 1988; Lowe et al., 1987; Kitayama et al., 1989; Garrett et al., 1989). A large subgroup of ras-related proteins, rab, have less homology to ras (30%) and almost nothing is known of their function, though two *S. cerevisiae* proteins, YPT1 and SEC4, that belong to this group are involved in intracellular vesicle transport (Zahraoui et al., 1989; Segev et al., 1988; Salmien and Novick, 1987). The three mammalian rho proteins (A, B, and C) are ~30% homologous to ras and are ubiquitously expressed (Madaule and Axel, 1985; Yeramian et al., 1987; Olofsson et al., 1988). Based on the resemblance of their carboxy termini to ras, they are presumed to function at the plasma membrane (Hancock et al., 1989), though significant amounts of rho can be found in cytoplasm (Narumiya et al., 1988). The RHO1 gene of *S. cerevisiae* is closely related to mammalian rho and deletion of the yeast gene is lethal (Madaule et al., 1987). It has been shown that an ADP-ribosyltransferase from *clostridium botulinum*, C3, can ribosylate rho on asparagine 41 (Narumiya et al., 1988; Aktories et al., 1989; Sekine et al., 1989), and that introduction

of C3 into cells induces rounding up and the dissolution of actin microfilaments (Rubin et al., 1988; Chardin et al., 1989;). However, two additional C3 substrates, rac1 and rac2, have recently been identified (Didsbury et al., 1989), and the role of rho in the C3-induced effects is unclear.

We have previously reported the purification of recombinant rho protein from an *E. coli* expression system (Garrett et al., 1989). The intrinsic biochemical properties of the protein were similar to those previously described for ras; i.e., a slow, Mg<sup>2+</sup>-dependent, guanine nucleotide exchange rate and a slow intrinsic GTP hydrolysis rate. In addition, we identified a 29-kD GTPase activating protein, rho GAP, that could stimulate the intrinsic GTPase activity of rho. Mutation of glycine to valine at codon 14 of rho had similar biochemical consequences to oncogenic codon 12 changes in ras; namely, it reduced the intrinsic GTPase activity and blocked rho GAP-stimulated GTP hydrolysis (Garrett et al., 1989). We reasoned that as with ras, the biological effects of a Vall4rho protein should represent those of a constitutively activated protein. We have, therefore, made use of the microinjection technique to examine both the biological function of rho and the effect of ribosylation on its biological and biochemical activities.

## Materials and Methods

### Expression and Purification of rho Protein

Normal and mutant rhoA cDNAs were expressed under the control of the tryptophan promoter in *E. coli* as described earlier (Garrett et al., 1989).

We reported then that over 90% of the protein obtained was clipped in the carboxy terminal region. However, we serendipitously observed that protein derived from a cDNA clone with a hpa I site engineered at codon 25 (and as a consequence having a Phe→Asn amino acid substitution) was largely full length and very little clipping was observed. The recombinant protein obtained using this vector has a similar intrinsic GTPase activity to nonmutated rho protein and responds normally to rho GAP stimulation. We have used this cDNA expression vector throughout this work. Sonicated extracts were chromatographed over a DEAE-Sephacel ion-exchange column and through a G75 Sephadex gel filtration column as described previously in the purification of ras proteins (Hall and Self, 1986). The protein was judged to be ~30% pure by Coomassie blue staining after electrophoresis on polyacrylamide gels and ~0.5 mg of rho protein was obtained from 4 liters of culture. The protein was concentrated to 0.5 mg Vall4rho/ml or 5 mg normal rho/ml, sterilized by filtration, and snap-frozen in liquid nitrogen. Mutagenesis of Asn to Ile at codon 41 was carried out by site-directed mutagenesis using a mutagenesis kit (Amersham International, Amersham, UK). Mutant cDNAs were completely sequenced before being reintroduced into the *E. coli* expression plasmid.

### Microinjection of rho cDNA in a Mammalian Expression Vector

Normal and activated (Vall4) rho cDNAs were introduced into the eukaryotic expression vector pEXV (Hancock et al., 1989). These cDNAs lacked the Phe→Asn substitution used to stabilize recombinant protein in our *E. coli* expression system. DNA expression from pEXV is under the control of the SV40 early region promoter and enhancer. Plasmid DNA (0.1 µg/ml) was microinjected into the nuclei of subconfluent cells and its effects on individual cells observed over 48 h using time-lapse video recordings.

### Ribosylation of rho Proteins

C3 transferase from *Clostridium botulinum* type C3 was purified to homogeneity and concentrated to 0.3 mg/ml as described previously (Aktories et al., 1988). Titration of C3 by microinjection revealed that >3 µg/ml was required for efficient rounding up of cells. For microinjection, rho proteins were incubated with C3 transferase as follows: 10 mM Tris-HCl, pH 7.4, 0.1 mM GTP, 50 mM NaCl, rho (5 µg) C3 transferase (3 ng) in a volume of 10 µl. Ribosylation was initiated by addition of 0.5 mM NAD (in the absence of NAD no ribosylation occurred) and the mixture was left for 60 min at 37°C. The mixture was then directly injected into Swiss-3T3 cells. For gel electrophoresis, rho proteins (50 µg/ml) were ribosylated by incubation with [<sup>32</sup>P]NAD (0.5 µCi, 2 mCi/ml) and C3 transferase (0.75 µg/ml) for 1 h at 37°C in 50 mM Tris, pH 7.5, 2 mM MgCl<sub>2</sub>, 0.3 mM GTP. Protein was precipitated with 30% trichloroacetic acid and ribosylated proteins observed after PAGE and autoradiography as described previously (Aktories et al., 1988). Western blots were developed using as the first antibody a rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 65-75 of mammalian rhoA. To analyze the effects of ribosylation on the biochemical properties of rho, the protein was ribosylated as described above except nonradioactive NAD was used at 0.2 mM. The ribosylated protein was preincubated with [γ-<sup>32</sup>P]GTP and the intrinsic and rho GAP-stimulated GTPase activities were determined by measuring the loss of bound counts in a filter binding assay as described previously (Garrett et al., 1989). To test the extent of ribosylation a parallel ribosylation reaction was set up containing in addition [<sup>32</sup>P]NAD (0.7 µCi). This parallel reaction was collected onto nitrocellulose filters, washed, and the extent of labeling determined by scintillation counting. The amount of rho present in the incubation mixture was determined by a [<sup>3</sup>H]GTP binding assay (Hall and Self, 1986) and a comparison of the GTP binding assay and the amount of <sup>32</sup>P incorporated after ribosylation revealed that 40-50% of the protein was ribosylated.

### Fluorescent Labeling of Cytoskeletal Proteins

Cells cultured on marked areas of glass coverslips were microinjected cytoplasmically with 0.5 mg/ml Vall4rho. 3 h after injection cells were fixed and processed as follows: for actin, cells were fixed in 3% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with TRITC-Phalloidin (Sigma Chemical Co., St. Louis, MO) for 30 min. For tubulin and vimentin, cells were fixed in acetone/methanol and incubated with mouse anti-β-tubulin (Amersham International) or goat antivimentin (Sigma Chemical Co.) primary antibodies, followed by appropriate FITC-

conjugated second antibodies. Images were recorded using a Bio-Rad MRC500 confocal microscope.

## Results

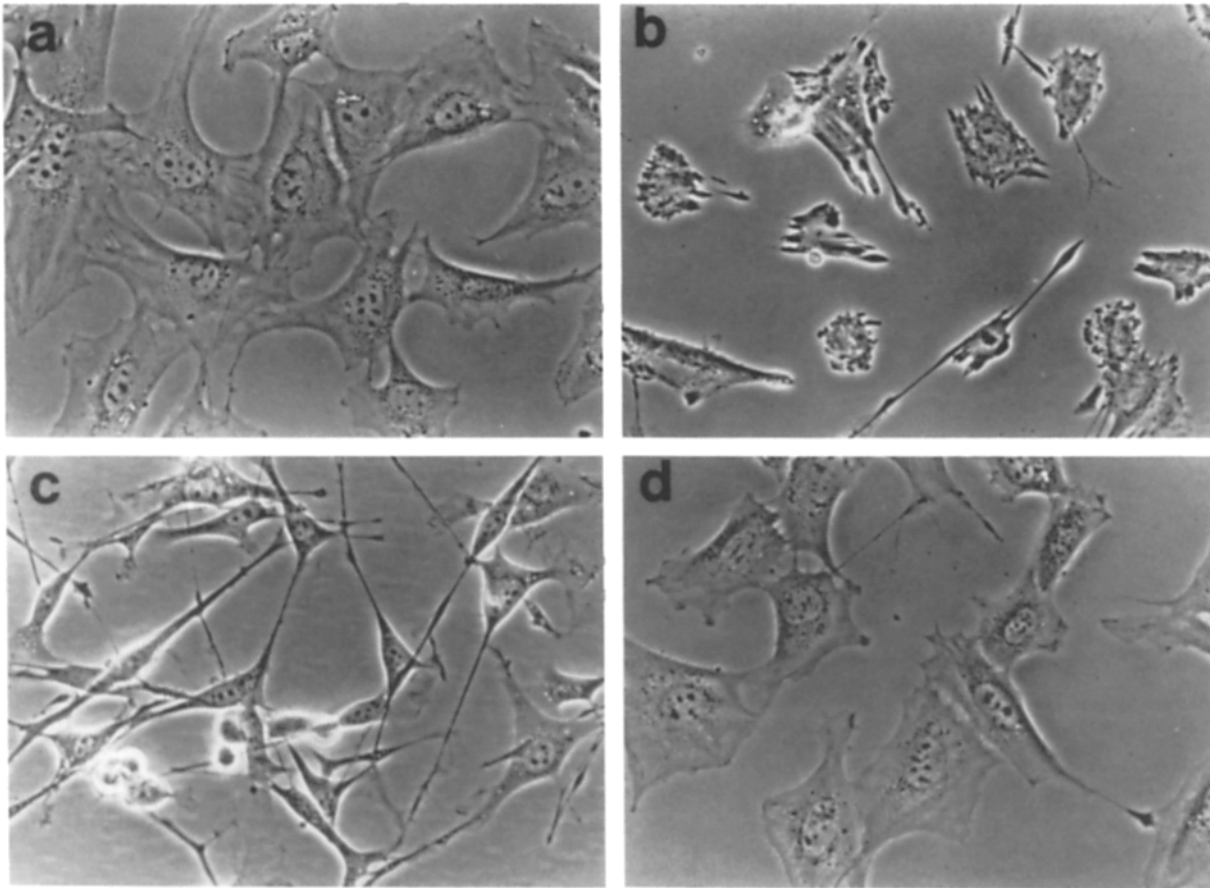
### Microinjection of Activated rho Protein

Normal and mutant rhoA cDNAs were expressed under the control of the tryptophan promoter in *E. coli* as described previously (Garrett et al., 1989). Approximately 0.5 mg of rho protein was obtained from 4 L of culture and judged to be around 30% pure. Vall4rho protein was microinjected into subconfluent Swiss 3T3 cells and its effects on individual cells were observed using time-lapse video recordings over a period of 2 d. Fig. 1, *a* and *b* shows that at concentrations of rho protein >0.3 mg/ml and beginning ~15 min after injection, dramatic changes are induced in the cells' morphology. The cells contract but leave parallel finger-like cytoplasmic processes still adherent to the substratum, resulting in the unusual but characteristic phenotype shown in Fig. 1 *b* (rho phenotype). After 10-20 h, most injected cells begin to detach and die. Any cells still attached after ~2 d regain their normal phenotype after degradation of injected protein. If confluent cells are injected, changes in cell shape are less dramatic though still apparent and the cells do not detach. After ~2 d all injected cells regain their normal cellular morphology. These effects are quite distinct from those seen after injecting Vall2 ras (at 0.5 mg/ml). Fig. 1 *c* shows the typical transformed morphology induced by ras and seen only after ~10-15 h (Stacy and Kung, 1984). As a control for any nonspecific effects due to contaminating *E. coli* proteins or the microinjection protocol itself, for example, Fig. 1 *d* shows that a Vall4rho protein containing an additional mutation (Thr37→Ala37) in a region likely to be the effector domain of ras-like proteins (Barbacid, 1987) is biologically inactive after microinjection.

It has been shown that the biological effects of a Vall2 mutation in ras can be mimicked by high level expression of the normal protein (McKay et al., 1986). Similarly, we find that injection of normal rho at >1.2 mg/ml gives the same morphological changes as injection of Vall4rho at 0.3 mg/ml (data not shown). Furthermore, if normal rho is first preincubated with the nonhydrolyzable GTP analogue, GTPγ S, before injection, its activity increases fourfold and it is now similar in potency to the Vall4rho protein (data not shown). These characteristic changes in morphology are not peculiar to Swiss 3T3 cells; we have observed similar effects in NIH-3T3, human fetal lung fibroblasts, and in NRK cells.

To confirm further that the observed phenotypic changes are in fact due to the rho protein itself, we have microinjected a eukaryotic plasmid expression vector containing rho cDNA into the nuclei of subconfluent cells. After ~5 h the characteristic rho phenotype as shown in Fig. 1 *b* is observed. This experiment also indicates that the amino acid substitution used to stabilize the *E. coli*-produced recombinant rho (Phe→Asn at codon 25) does not contribute to the observed effects.

The changes in morphology induced by microinjection of rho protein are rapid; after 30 min most injected cells have taken on the rho phenotype. Using cycloheximide at 10 µg/ml, we have shown that this effect does not require new protein synthesis. Furthermore, the changes were indepen-



**Figure 1.** Microinjection of recombinant rho proteins into Swiss-3T3 cells. Injection was into the cytoplasm of subconfluent cells grown on plastic petri dishes. The cells were visualized under phase-contrast optics. Cells were injected with the following: (a) buffer; (b) Vall4rho (0.5 mg/ml); (c) Vall2 ras (0.5 mg/ml); (d) Vall4/Ala37 rho (0.5 mg/ml); and photographed after 1 h (a, b, and d) or 10 h (c).

dent of the metabolic or growth state of the cells. Quiescent, growth factor-starved cells or actively growing cells both took on the rho phenotype 15–30 min after injection.

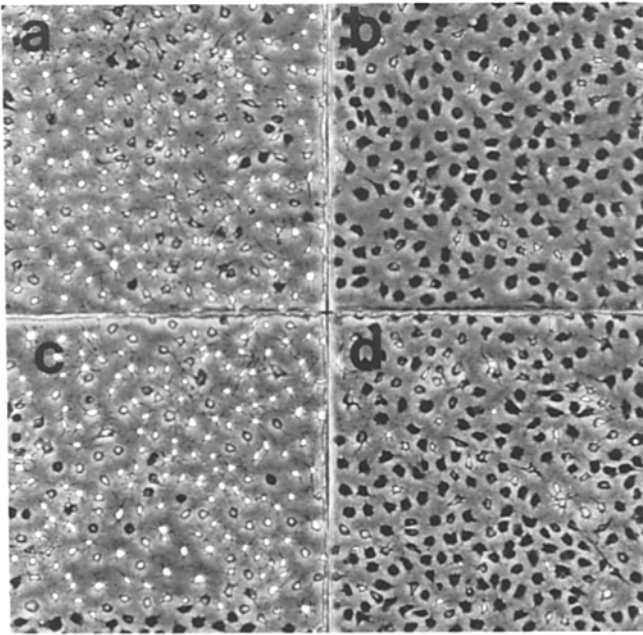
### **Biological Effects of rho Ribosylation**

Fig. 2 a shows the effects of microinjecting a pure preparation of C3 transferase (30  $\mu$ g/ml) into Swiss 3T3 cells. Cells round up completely and become refractile after  $\sim$ 1–2 h, though they still remain loosely attached to the substratum. The effects on morphology of activated rho and of C3 are, therefore, very different and distinctive. We find that concentrations  $>$ 3  $\mu$ g/ml of C3 are required for efficient rounding up of cells. Uninjected cells are shown in Fig. 2 b. A similar effect has been observed by others but since rho is not the only substrate in cells it is not clear whether rounding up is a direct consequence of ribosylation of rho (Chardin et al., 1989; Rubin et al., 1988; Didsbury et al., 1989). With the microinjection assay described above we are able to examine the effects of ribosylation on rho activity. First we incubated C3 transferase (0.3  $\mu$ g/ml) and Vall4rho (0.5 mg/ml) in vitro with NAD for 1 h. The mixture was then injected directly into cells and ribosylated Vall4rho was found to be inactivated (data not shown). The experiment was next repeated

using normal rho (also at 0.5 mg/ml) that had been preincubated with C3 transferase with or without NAD. In the absence of NAD no ribosylation occurs and very little effect could be observed since the levels of normal rho and C3 injected are below their individual thresholds (Fig. 2 d). However, Fig. 2 c shows that ribosylated normal rho had a similar effect on cells as high concentrations of C3, i.e., the injected cells rounded up.

### **Biochemical Effects of rho Ribosylation**

To understand the different biological effects observed after injection of ribosylated normal and Vall4rho, the effect of ribosylation on the biochemical properties of the protein was determined. Normal and Vall4rho were treated with C3 transferase in vitro and the extent of ribosylation determined to be  $>$ 40% (see Materials and Methods). The guanine nucleotide exchange rates were then measured in high or low  $Mg^{2+}$  as described previously (Hall and Self, 1986). No effect of ribosylation was observed (data not shown). Fig. 3 shows that ribosylation of normal rho also had no effect on either the intrinsic or the rhoGAP-stimulated GTPase activities. Ribosylation had no effect on the GTPase activity of Vall4rho (data not shown).



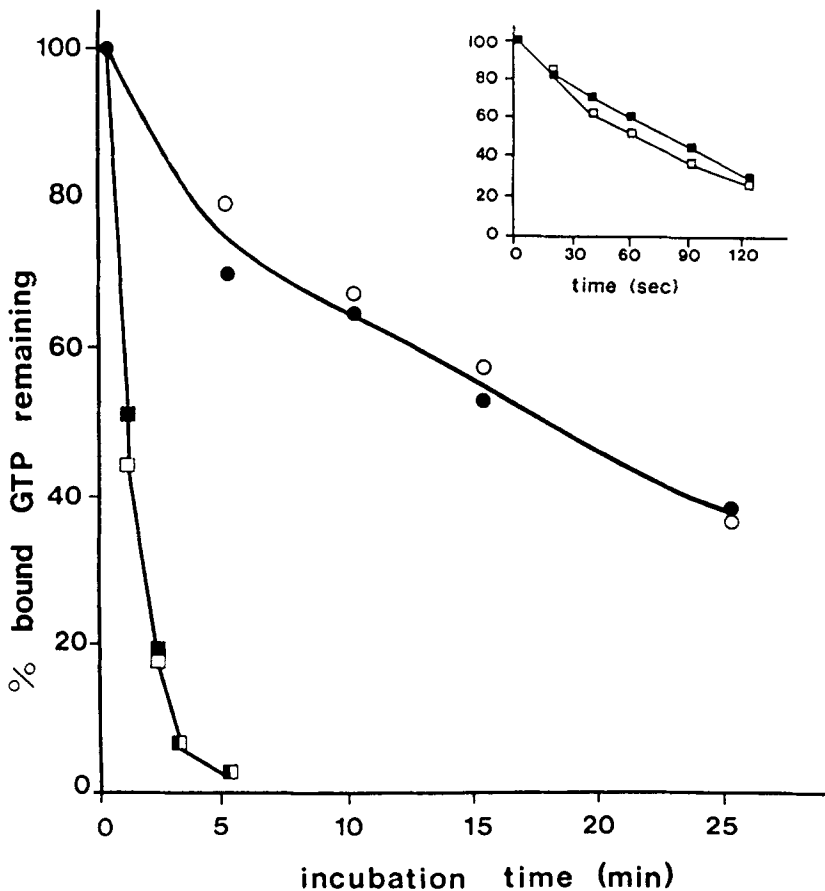
**Figure 2.** Microinjection of Swiss-3T3 cells with ribosylated rho proteins. Cells were photographed 2 h after injection. Cells in each quadrant of the petri dish were injected with the following: (a) C3 transferase (30  $\mu\text{g}/\text{ml}$ ); (b) buffer; (c) a mixture of normal rho (0.5 mg/ml) and C3 transferase (0.3  $\mu\text{g}/\text{ml}$ ) preincubated in the presence of NAD; and (d) as in c but preincubated in the absence of NAD.

### Effects of a Ribosylation Resistant rho Protein

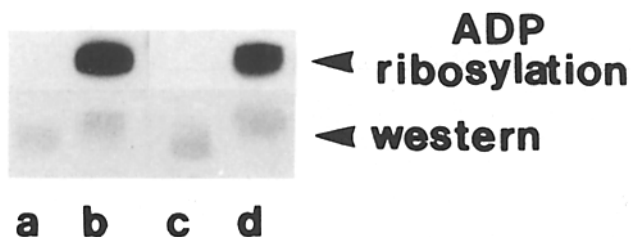
To test whether the rounded and refractile phenotype of cells treated with C3 transferase could be overcome with recombinant rho, we have introduced an Asn $\rightarrow$ Ile mutation at the ribosylation site (codon 41) of normal and Val14rho (Sekine et al., 1989). Fig. 4 shows that these proteins are, as expected, resistant to ribosylation. Injection of Val14/Ile41 rho at 0.5 mg/ml into cells has the same effect as the Val14rho protein showing that this amino acid substitution at codon 41 does not impair biological activity. When cells were coinjected with C3 transferase (at 30  $\mu\text{g}/\text{ml}$ ) and Val14/Ile41 rho, cells took on the rho phenotype (as shown in Fig. 1 b) and did not round up.

### Effects of Val14rho on the Cytoskeleton

It has been reported that C3 transferase when introduced into cells leads to a dissolution of the actin microfilament network but has no effect on microtubules (Chardin et al., 1989). We wished to look at the effect of Val14rho on subcellular structures. Fig. 5 shows the results of fluorescent staining of Val14rho-injected subconfluent Swiss 3T3 cells for three major cytoskeletal proteins. No obvious abnormalities could be discerned in the overall intracellular distribution of either actin or  $\beta$ -tubulin with extensive microfilament (Fig. 5, a and b) and microtubule (Fig. 5, c and d) networks both clearly visible. Immunofluorescent labeling with antibodies against vimentin, however, revealed that in Val14rho-inject-



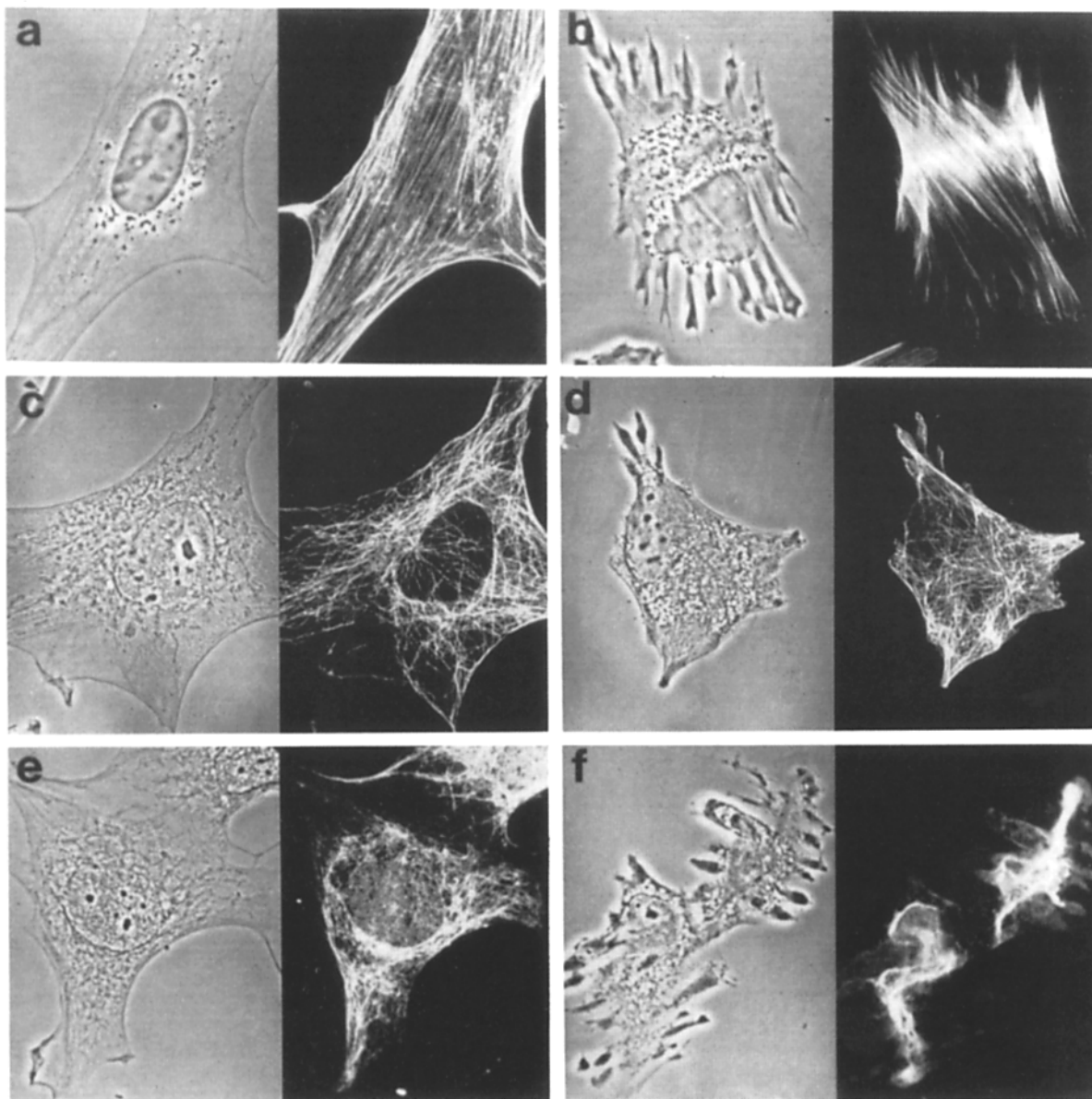
**Figure 3.** Effect of ribosylation on the intrinsic and rho GAP-stimulated GTPase activity of normal rho. Control experiments (see Materials and Methods) indicated that at least 40% of the protein was ribosylated. (○) Nonribosylated protein in the absence of GAP; (□) non-ribosylated protein in the presence of GAP; (●) ribosylated protein in the absence of GAP; and (■) ribosylated rho in the presence of GAP. The inset shows an expanded time course for the effects of ribosylation or rho GAP activity.



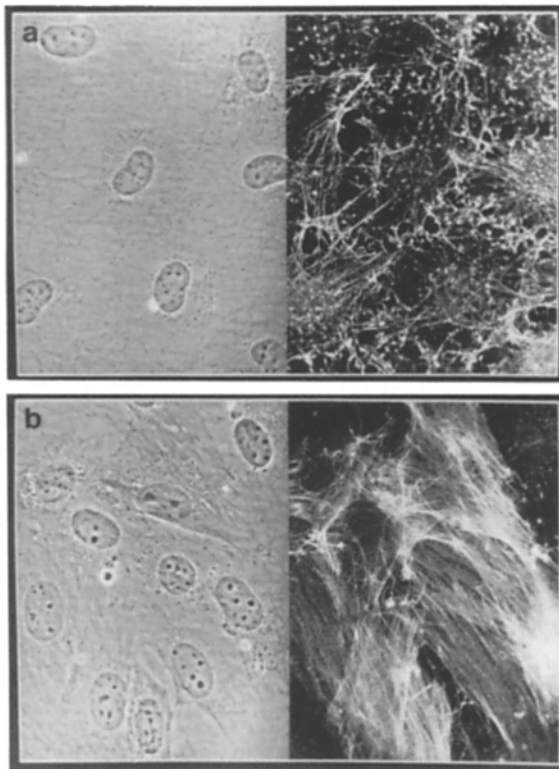
**Figure 4.** Effect of Asn41→Ile41 mutation on ribosylation of rho. Proteins purified from *E. coli* expression vectors were visualized by Western blot analysis using an anti-rho antibody and tested as substrates for C3 transferase using [<sup>32</sup>P]NAD followed by gel electrophoresis and autoradiography. (lane *a*) Normal rho with Ile41 mutation; (lane *b*) normal rho; (lane *c*) Vall4/Ile41 rho; and (lane *d*) Vall4rho.

ed cells the intermediate filaments had collapsed into irregular thick bundles within the cytoplasm (Fig. 5, *e* and *f*).

A quite different picture emerged when contact-inhibited quiescent Swiss 3T3 cells were used. It can be seen (Fig. 6 *a*) that, in contrast to subconfluent cells (Fig. 5 *a*), contact-inhibited cells do not possess well-defined actin filament networks but instead show bright punctate actin staining. Injection of Vall4rho into these cells (Fig. 6 *b*), however, rapidly (30 min) induced the reappearance of dense microfilament networks. Injection of Vall2ras or the biologically inactive Vall4A1a37rho proteins had no effect. No collapse of intermediate filaments or changes in microtubule organization were induced in contact-inhibited cells injected with Vall4rho.



**Figure 5.** Fluorescence studies on Vall4rho-injected subconfluent Swiss 3T3 cells. 3–4 h after injection cells were fixed and stained as described in Materials and Methods. Phase contrast and fluorescence are shown for uninjected (*a*, *c*, and *e*) and Vall4rho-injected (*b*, *d*, and *f*) cells using TRITC-phalloidin (microfilaments) (*a* and *b*) or antitubulin (microtubules) (*c* and *d*) and antivimentin (intermediate filaments) (*e* and *f*) antibodies.



**Figure 6.** Fluorescence studies on VAL14 injected contact-inhibited quiescent Swiss 3T3 cells. Cells were grown and left confluent for 8 d before injection. 3–4 h after injection cells were fixed and stained as described in Materials and Methods. Phase contrast and fluorescence are shown for uninjected (*a*) and Val14rho-injected (*b*) cells using TRITC-phalloidin stain.

## Discussion

The availability of full length recombinant rho protein has allowed us to examine its biological effects by using a microinjection technique. Injection of constitutively activated rho protein (Val14rho) into a variety of cell lines induces dramatic changes in cell morphology commencing within 15 min. The cell body contracts but finger-like processes remain, resulting in a very distinctive phenotype (rho phenotype). The rho phenotype is also induced after microinjection of normal rho protein but higher concentrations are required. Prebinding of GTP $\gamma$ S to normal rho, however, activates the protein and its effects are now similar to Val14rho. The changes in morphology are less pronounced when confluent cells are injected: the contacts between neighboring cells seem to prevent the extreme changes in cell shape.

It has been reported that treatment of cells with the ADP-ribosyltransferase C3 leads to a very different morphological change; cells round-up and this is accompanied by dissolution of the actin microfilaments (Chardin et al., 1989). However, since rho is not the only substrate for C3 and since the biochemical effect of ribosylation of rho is unknown, it is not clear if rounding up is due to rho ribosylation (Chardin et al., 1989; Didsbury et al., 1989). We show here that ribosylation of recombinant rho protein has no effect on its intrinsic biochemical properties or on its interaction with the rho GTPase activating protein rho GAP. However, ribosylation

of Val14rho renders the protein biologically inactive. Since Val14rho is expected to be constitutively in the active GTP-bound conformation (Garrett et al., 1989), we conclude that ribosylation blocks its interaction with its target. This is analogous to mutations described in the effector region of ras (amino acid 30–40) which also has little effect on intrinsic biochemical properties but block its biological activity (Sigal et al., 1986; Willumsen et al., 1986). Indeed the site for ribosylation in rho has been shown to be Asn 41 (equivalent to codon 39 in ras), which is consistent with this being the region of rho interacting with its target (Sekine et al., 1989). Interestingly, effector mutations in ras block interaction with ras GAP, leading to the speculation that ras GAP may be the target for regulation by ras (Calés et al., 1988; Adari et al., 1988). Since ribosylation of rho does not affect its interaction with the 29-kD rho GAP (Fig. 3), we conclude that this protein cannot be the sole target for regulation by rho, though it is still possible it is part of a larger regulatory complex.

An unexpected result was obtained after injection of cells with ribosylated normal rho: cells rounded up in an analogous fashion to that observed after injecting C3 transferase. We can conclude from this experiment that the rounding up of cells induced by injection of C3 transferase can be totally accounted for by ribosylation and inactivation of endogenous rho. However, we also have to account for why injection of ribosylated Val14rho has no effect on cells, whereas injection of ribosylated normal rho leads to rounding up; in both cases endogenous rho proteins are present. The best explanation we can offer is that injected ribosylated normal rho, which is in the GDP form, blocks endogenous rho proteins by competing for an upstream factor perhaps an exchange factor (Ohga et al., 1989) but cannot itself produce an effect since it is ribosylated; ribosylated Val14rho cannot bind to this putative factor since it is permanently in the GTP form. Since ribosylated normal rho still interacts with rho GAP it will be maintained in the GDP form.

We have obtained further confirmation that ribosylation of endogenous rho is responsible for C3-induced effects using a ribosylation resistant Val14/Ile41 rho protein. When cells are coinjected with high levels of C3 transferase and Val14/Ile41 rho, the cells take on the distinctive Val14rho phenotype and do not round up. This indicates that the rounding up of cells and the dissolution of actin microfilaments caused by C3 (Chardin et al., 1989) can be reversed by exogenous activated rho. We have not, however, been able to prevent C3-induced rounding up of cells using ribosylation resistant normal rho. It is possible that the normal cellular phenotype requires the activity of all three endogenous rho proteins, and we are only supplying rhoA; alternatively, it is possible that other non-rho C3 substrates, perhaps rac1 or 2, are required in addition to rho for maintenance of normal cell morphology.

The biochemical basis of the rho effects is not known. It has been shown that introducing C3 transferase into cells is followed by dissolution of actin filaments (Chardin et al., 1989) without any major effect on microtubules. We find no obvious changes in actin filaments or microtubules after injection of Val14rho into subconfluent cells but we have found collapse of the intermediate filament (IF)<sup>1</sup> network as judged by immunofluorescence with antivimentin. Collapse of IFs

1. Abbreviation used in this paper: IF, intermediate filament.

has been observed by others after a variety of treatments including injection of antivimentin antibodies or stress shock (Klymkowsky, 1981; Thomas et al., 1982), but in both cases the collapse of the IF network was not accompanied by any changes in cell shape. It seems likely, therefore, that the collapse of IF shown in Fig. 5 *e* is a secondary effect and not the cause of the Vall4rho-induced shape changes. Interestingly, the morphological changes elicited by Vall4rho in Swiss 3T3 cells can be mimicked by treatment with colchicine (H. F. Paterson, unpublished data). However, unlike Vall4rho-treated cells, colchicine treatment is accompanied by complete collapse of microtubules and IF. For this reason we think that the observed morphological effects are not due to changes in the microtubule network.

We have shown here that the inactivation of endogenous rho by C3 directly leads to rounding up of cells and the dissolution of microfilament network. An identical phenotype is observed after introducing another *Clostridium* ADP-ribosyltransferase, C2, into cells, and C2 is known to ribosylate actin monomers preventing their polymerization (Aktories et al., 1986). Although there are no obvious effects on actin microfilaments after introducing Vall4rho into subconfluent cells, the fluorescence studies would not rule out subtle effects on actin polymerization or cross-linking or on the attachment of microfilaments to the plasma membrane. We have obtained more direct experimental evidence that rho does indeed affect actin filament organization, by looking at contact-inhibited quiescent cells. When Swiss 3T3 cells reach confluence and become quiescent, we have observed that they lose their actin filament network and show disorganized punctate actin staining (Fig. 6 *a*). If Vall4rho is injected into these cells, a dense actin filament network appears after ~30 min (Fig. 6 *b*). Furthermore, the collapse of intermediate filaments observed in subconfluent cells injected with Vall4rho no longer occurs in these quiescent cells, consistent with the idea that the collapse seen in Fig. 5 *f* is a secondary effect.

From the data presented in this paper, it seems clear that rho has a critical role in maintaining some aspect of the organization of the cytoskeletal network that affects cell shape. A more detailed biochemical analysis of the cytoskeleton and, in particular, actin microfilaments in Vall4rho containing cells is underway in an attempt to define further the function of this protein.

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