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Human RhoA/RhoGDI complex expressed in yeast: GTP exchange is sufficient for translocation of RhoA to liposomes

PAUL W. READ, XIAOPU LIU, KENTON LONGENECKER, CHARLES G. DIPIERRO, LORI A. WALKER, AVRIL V. SOMLYO, ANDREW P. SOMLYO, AND ROBERT K. NAKAMOTO

Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22906-0011

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Abstract

The human small GTPase, RhoA, expressed in *Saccharomyces cerevisiae* is post-translationally processed and, when co-expressed with its cytosolic inhibitory protein, RhoGDI, spontaneously forms a heterodimer in vivo. The RhoA/RhoGDI complex, purified to greater than 98% at high yield from the yeast cytosolic fraction, could be stoichiometrically ADP-ribosylated by *Clostridium botulinum* C3 exoenzyme, contained stoichiometric GDP, and could be nucleotide exchanged fully with [^{3}H]GDP or partially with GTP in the presence of submicromolar Mg^{2+}. The GTP-RhoA/RhoGDI complex hydrolyzed GTP with a rate constant of 4.5×10^{-5} s^{-1}, considerably slower than free RhoA. Hydrolysis followed pseudo-first-order kinetics indicating that the RhoA hydrolyzing GTP was RhoGDI associated. The constitutively active G14V-RhoA mutant expressed as a complex with RhoGDI and purified without added nucleotide also bound stoichiometric guanine nucleotide: 95% contained GDP and 5% GTP. Microinjection of the GTP-bound G14V-RhoA/RhoGDI complex (but not the GDP form) into serum-starved Swiss 3T3 cells elicited formation of stress fibers and focal adhesions. In vitro, GTP-bound-RhoA spontaneously translocated from its complex with RhoGDI to liposomes, whereas GDP-RhoA did not. These results show that GTP-triggered translocation of RhoA from RhoGDI to a membrane, where it carries out its signaling function, is an intrinsic property of the RhoA/RhoGDI complex that does not require other protein factors or membrane receptors.

Keywords: GTP hydrolysis; membrane translocation nucleotide exchange; RhoA; RhoGDI; small G-proteins

RhoA is a member of the Ras superfamily of small G-proteins that has been implicated in various actomyosin-dependent cell functions: cell adhesion and motility (Abedi & Zachary, 1995; Nobes & Hall, 1995; Hotchin & Hall, 1996; Amano et al., 1997; Bobak et al., 1997; Santos et al., 1997; Hall, 1998; Seasholtz et al., 1998), enhancement of vascular smooth muscle contractile responses (calcium sensitization) (Gong et al., 1996, 1997a, 1997b; Otto et al., 1996; Fujihara et al., 1997; Kureishi et al., 1997; Uehata et al., 1997; Fu et al., 1998; Van Eyk et al., 1998), tumor cell migration (Yoshioka et al., 1998; Itoh et al., 1999; Somlyo, 1999; Somlyo et al., 1999), and cytokinesis (Aepfelbacher et al., 1995; Goto et al., 1998), as well as transcriptional regulation (Hill et al., 1995; Alberts et al., 1998; Seasholtz et al., 1998) and cell cycle progression (Olson et al., 1995; Hirai et al., 1997; Noguchi et al., 1998). Rho-kinase, one of the main RhoA downstream signal transduction effectors (Leung et al., 1996; Matsui et al., 1996), has been shown, through the use of the Rho-kinase inhibitor, Y-27632, to be directly involved in some forms of hypertension (Uehata et al., 1997) and metastatic progression (Itoh et al., 1999).

Rho family proteins undergo three carboxyl-terminal, post-translational modifications that are required for full functionality (Hori et al., 1991; Ando et al., 1992; Gong et al., 1996). These modifications, which do not occur in prokaryotic cells, include geranylgeranylation of the cysteine in the carboxyl terminal "CAAX" box via a thioester linkage, proteolytic cleavage of the final three amino acids, and carboxymethylation of the cysteinal carboxyl group (Adamson et al., 1992; Backlund, 1997). The most important modification, geranylgeranylation, is required for RhoA to bind to RhoGDI (GDP dissociation inhibitor; Hori et al., 1991; Hancock & Hall, 1993), as well as for its association with the cell membrane upon agonist stimulation (Hori et al., 1991; Ando et al., 1992; Fujihara et al., 1997; Gong et al., 1997a).

Reprint requests to: Robert K. Nakamoto, Department of Molecular Physiology and Biological Physics, University of Virginia, P.O. Box 10011, Charlottesville, Virginia 22906-0011; e-mail: rkn3c@virginia.edu.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; G14V-RhoA, RhoA with the Gly14 to Val mutation; GTPγS, guanosine 5′-O-(3-thiotriphosphate); GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; RhoGDI, GDP dissociation inhibitor; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

The association of RhoA with RhoGDI also depends upon specific protein–protein interactions. The crystal structure at 4.0 Å (Longenecker et al., 1999) indicates that, although the protein contacts do not contribute as much to binding energy as the hydrophobic interactions with the geranylgeranyl moiety, the amino terminus of RhoGDI interacts with the Switch I and II regions of RhoA. In particular, RhoGDI makes close contact with Thr37 of RhoA, a residue that is directly involved in coordination of Mg^{2+} (Wei et al., 1997; Ihara et al., 1998; Longenecker et al., 1999), and plays a key role in the nucleotide exchange mechanism of Dbl-like guanine nucleotide exchange factors or GEFs (Li & Zheng, 1997). RhoGDI likely inhibits GEF function by shielding the side chain of Thr37 in its complex with Rho GTPases (Longenecker et al., 1999). Moreover, the crystal structure shows that the RhoA insert helix (amino acids 124–136) is distal to the RhoGDI binding regions and does not participate in interactions with RhoGDI. Purified native and recombinant RhoA/RhoGDI complex can be immunoprecipitated with an antibody to the insert loop (Longenecker et al., 1999). This finding suggests that the lack of immunoprecipitability of the nonpurified native complex from the cytosolic fraction of tissue (Fujihara et al., 1997) is due to the presence of another, as yet unidentified protein(s).

Prenylated RhoA is hydrophobic, but is solubilized when bound to RhoGDI with the geranylgeranyl moiety inserted into the hydrophobic pocket of RhoGDI (Gosser et al., 1997; Keep et al., 1997; Longenecker et al., 1999); in fact, in unstimulated cells most endogenous RhoA exists as a cytosolic GDP-RhoA/RhoGDI complex (Fukumoto et al., 1990; Gong et al., 1997a). Agonist binding to a membrane receptor causes stimulation of hetero-trimeric G-protein signaling and activates GEF function to exchange GTP for GDP on RhoA (Kozasa et al., 1998; Klages et al., 1999). GTP-RhoA is translocated to the plasma membrane and activates its downstream effectors, while RhoGDI remains cytosolic (Gong et al., 1996; Fujihara et al., 1997). Thus, critical steps in RhoA and, probably also, Rac (Bokoch et al., 1994) activation include nucleotide exchange, dissociation of the RhoA/RhoGDI complex and translocation of GTP-RhoA (GTP-Rac) to the cell membrane. Because RhoGDI can extract RhoA from membranes (Araki et al., 1990; Leonard et al., 1992; Lang et al., 1996), it is thought that completion of the Rho activation cycle includes conversion of active GTP-Rho to GDP-Rho through intrinsic GTPase activity facilitated by GTPase-activating proteins (GAPs) and extraction of membrane-bound GDP-Rho by RhoGDI.

Nonprenylated RhoA, expressed as a recombinant protein in *Escherichia coli*, is unable to complex with RhoGDI (Hancock & Hall, 1993; Santos et al., 1997) and, more important, does not activate at least some of the Rho effectors, such as Rho-Kinase, as indicated by its inability to induce Ca^{2+}-sensitization in smooth muscle (Gong et al., 1996). Previous efforts to produce recombinant post-translationally modified RhoA have relied on the use of a baculovirus/SF9 system (e.g., Gong et al., 1996; Fauré et al., 1999); however, production in the baculovirus/SF9 system is costly. In contrast, prokaryotic-derived RhoGDI is physiologically active (e.g., Matsui et al., 1990; Chuang et al., 1992; Leonard et al., 1992; Sheffield et al., 1999).

The purpose of the present study was to provide stoichiometric complexes of RhoA with RhoGDI in quantities sufficient for exploring its mechanism in signal transduction in eukaryotic cells. We describe a co-expression system for differentially tagged human RhoA and RhoGDI in *Saccharomyces cerevisiae* yielding milligram quantities of highly purified, post-translationally modified RhoA/RhoGDI complex in a 1:1 stoichiometry and uncontaminated by yeast homologues. This preparation was used in the crystallization and X-ray structure determination of the complex to 4 Å resolution (Longenecker et al., 1999). We also show that, whereas the wild-type RhoA in the complex exists as the GDP form, expression of the G14V, constitutively active mutant yields a complex that contains a small proportion of bound GTP. We further show that the GTP-bound RhoA/RhoGDI complex is biologically active and, unlike the GDP-RhoA/RhoGDI complex, allows translocation of RhoA to liposomes in vitro. Our results suggest that nucleotide exchange may precede dissociation of RhoA from RhoGDI, and that following nucleotide (GTP for GDP) exchange, no other protein is required for RhoA to dissociate and translocate to a membrane.

Results

Expression of human RhoA/RhoGDI complex in yeast

The extent of purification, estimated to be greater than 98%, of FLAG-RhoA/His_6-RhoGDI complex with sequential metal chelate (lane 6) and anti-FLAG M2 antibody (lane 9) chromatographies is shown in Figure 1. There was no evidence of proteolysis. Additionally, silver staining or immunoblots (Longenecker et al., 1999) also revealed only the single bands of RhoA and RhoGDI. The metal chelate chromatography provided the largest degree of purification, but the subsequent anti-FLAG M2 chromatography resulted in the high level of purity. The final yield was routinely 4–5 mg of complex per 18 L culture, which represented 0.2% of

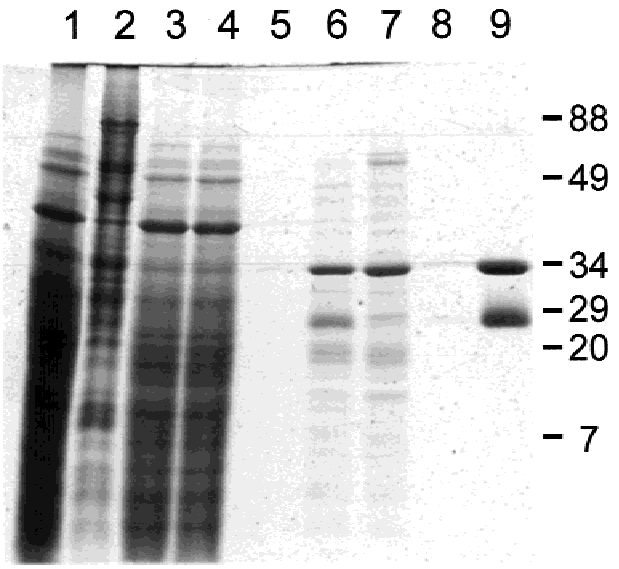

Fig. 1. Purification of FLAG-RhoA/His6-RhoGDI complex from yeast. Fifteen percent Coomassie Blue stained SDS-PAGE analysis of the FLAG-RhoA/His_6-RhoGDI purification: lane 1, whole cell lysate of yeast that have been galactose induced to express the human complex; lane 2, membrane fraction; lane 3, cytosolic fraction; lane 4, flow through from the metal affinity resin (Talon, Clonetech) column; lane 5, 5 mM imidazole wash of Talon column; lane 6, 100 mM imidazole elution from the Talon column; lane 7, flow through from the anti-FLAG M2 antibody column; lane 8, wash of the M2 column; lane 9, 15 μg of eluant from the M2 column with the FLAG peptide. Protein was prepared by addition to SDS sample buffer and boiling for 5 min.

the total cytosolic protein. We were also able to purify the Gly14 to Val RhoA mutant, some in the GTP form, in complex with RhoGDI, providing the first evidence that the constitutively active, GTPase-deficient mutant binds to RhoGDI in vivo.

The procedure used to isolate the complex assured that the purified RhoA was prenylated. Binding to RhoGDI requires the addition of a geranylgeranyl group to the carboxyl terminal CAAX motif (Hori et al., 1991; Hancock & Hall, 1993), whereas proteolytic removal of the final three amino acids (-LVL in human RhoA) is of only minor importance (Hancock & Hall, 1993). Carboxymethylation of the new carboxyl terminal cysteine is also not required for complex formation with RhoGDI, but the activity is present in *S. cerevisiae* (Sapperstein et al., 1994). Also consistent with its prenylation, a considerable portion of RhoA was found membrane associated. When RhoA was expressed without RhoGDI, 30–40% was found membrane associated. Of the RhoA in the cytosolic fraction, most was believed not prenylated because it was not associated with endogenous yeast RhoGDI (data not shown). The membrane-associated fraction was significantly decreased when RhoA was co-expressed with RhoGDI to a level similar (5–10%) to that observed in mammalian cells in vivo (Gong et al., 1996).

Purified RhoA/RhoGDI complex is a substrate for ADP ribosylation

The purified human complex expressed in yeast was an excellent substrate for ADP ribosylation and incorporated nearly stoichiometric amounts of [^{32}P]ADP from [^{32}P]-labeled NAD^{+} in the presence of the C3 ADP-ribosyl transferase (Fig. 2). The results were not affected by the presence of the His_6 or FLAG affinity tags on either protein. A previous paper presented evidence that RhoA/RhoGDI complex expressed and isolated from the cytosolic fraction of SF9 insect cells was not a good substrate for the C3 exoenzyme (Fauré et al., 1999). We also found this to be true for native complex in the cytosol of rabbit ileum smooth muscle (Fig. 2, lane 1). Upon partial purification, however, RhoA in complex with RhoGDI became a substrate for ADP ribosylation (lane 2), behavior reminiscent to the partially purified complex from bovine neutrophil cytosol (Bourmeyster et al., 1992). Immunoblot analysis using anti-RhoA and anti-RhoGDI antibodies (Fig. 2, middle and lower panels, respectively) showed that both RhoA and RhoGDI were present in similar amounts on the gel. These results suggested that the native complex interacts with another factor that blocks access to Asn41 from the C3 exoenzyme.

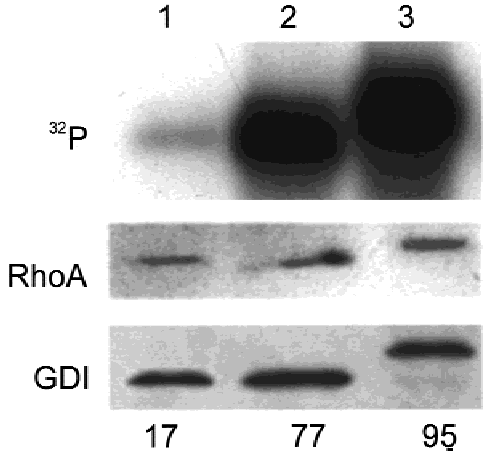

Fig. 2. ADP-ribosylation of RhoA/RhoGDI complex. RhoA was ADP-ribosylated by C3 exoenzyme with [^{32}P]NAD as described in Materials and methods, separated by 15% SDS-PAGE and transferred to PVDF membranes. Top panel: autoradiogram of [^{32}P]-labeled protein. The middle and lower panels: Western blots using anti-RhoA monoclonal antibody and anti-RhoGDI polyclonal antibody, respectively. Immunoreactive proteins were visualized using the ECL system (Amersham Pharmacia Biotech). Lane 1, native RhoA in the cytosolic fraction from rabbit ileum smooth muscle; lane 2, partially purified RhoA/RhoGDI complex from the sample in lane 1; and lane 3, pure RhoA/RhoGDI complex from yeast. Note that the proteins from yeast are slightly larger due to the affinity tags. See Materials and methods for purification procedures. The numbers below each lane indicate the percentage of RhoA that incorporated [^{32}P]ADP-ribose (average of two trials).

Human RhoA co-expressed with RhoGDI in yeast is stoichiometrically the GDP form

The only nucleotide associated with the wild-type FLAG-RhoA/His_6-RhoGDI complex purified in the absence of exogenous nucleotide was GDP (Fig. 3A) and, by comparison with internal standards, stoichiometric (1:1) within experimental error (±10%). The GDP-RhoA/RhoGDI complex was extremely stable in 5 mM Mg^{2+}: during incubation at 22 °C in the presence of 5 mM Mg^{2+} more than 97% of the complex retained nucleotide over a period of several days. There was little difference in stability if the tags were switched (i.e., His_6-RhoA/FLAG-RhoGDI). In contrast, one-third of the purified free FLAG-RhoA (not in complex with RhoGDI) was nucleotide-free apoprotein while the remainder was also only GDP bound (Fig. 3A). It is probable that a portion of this RhoA contained GTP at the time of cell lysis, and hydrolysis and release of nucleotide occurred during purification.

The bound GDP in the RhoA/RhoGDI complex could be exchanged with [^{3}H]GDP in the presence of submicromolar Mg^{2+} (Fig. 3B), with a rate constant of 3.3×10^{-4} s^{-1}, which is two orders of magnitude slower than that for free RhoA (Table 1). The rate of GDP exchange in free RhoA expressed in yeast was similar to that for RhoA expressed in *E. coli*, and both were similar to values previously reported (Self & Hall, 1995). These results indicated that all of the purified complex was competent to exchange nucleotide, and that little or none of the protein was inactive. Similar results were obtained with the affinity tags switched or with the tags removed (data not shown), which indicated that the amino terminal tags did not interfere with nucleotide exchange.

G14V-RhoA/RhoGDI has some GTP bound

The constitutively active FLAG-G14V-RhoA mutant purified in complex with His_6-RhoGDI also contained stoichiometrically bound guanine nucleotide. However, in contrast to the wild-type, we reproducibly found that 5 ± 2% of bound nucleotide was GTP with the remainder GDP (Fig. 3A). The GTP-bound RhoA remained in complex with RhoGDI, as similar results were obtained with the affinity tags switched. In this case, the last step of purification was via the FLAG tag on RhoGDI. The GTP bound G14V-RhoA was purified via the RhoGDI tag and was, therefore, prenylated and associated. Furthermore, anion exchange chromatography did not resolve any free RhoGDI mixed with the purified complex (see

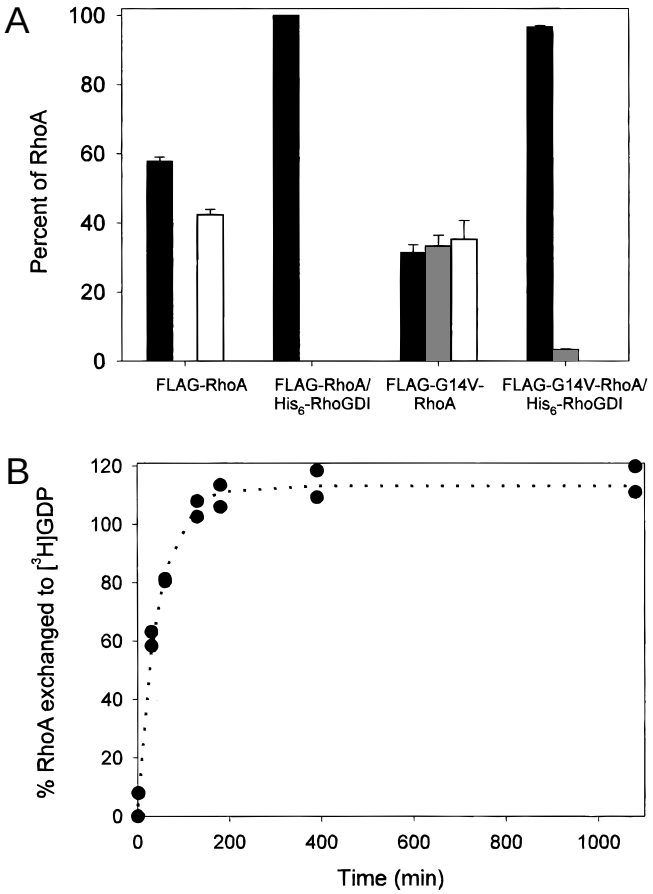

Fig. 3. Properties of yeast expressed RhoA/RhoGDI complex. **A:** Nucleotide content of cytosolic free RhoA and RhoA/RhoGDI complex. Nucleotides were extracted, identified, and quantified as described in Materials and methods. Values are the percent of complex occupied by GDP (black) or GTP (gray), or unbound (unfilled). Error bars indicate the standard deviation from a minimum of three determinations. **B:** Exchange of [^{3}H]GDP/GDP. At time 0, 1 mM [^{3}H]GDP was added to 20 μM FLAG-RhoA/His$_6$-RhoGDI in Exchange Buffer (62 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM EDTA, pH 7.6) and incubated at 22 °C. Aliquots were taken at the specified times and $MgCl_2$ added to 13 mM final concentration to stop the exchange reaction. The amount of protein-associated [^{3}H]GDP was determined by removing unbound nucleotide with a centrifuge desalting column (Penefsky, 1979) equilibrated with Buffer A (25 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 8.0) and measuring the radioactivity remaining in the filtrate. Control experiments showed that free nucleotide was completely removed. The percent [^{3}H]GDP/GDP exchange was calculated at each time point. The dotted line is a fit to a single exponential rise to a maximum.

below). Free FLAG-G14V-RhoA, purified from yeast cytosolic fraction without co-expression of RhoGDI, also contained a mixture of bound nucleotides: 40% by GTP, 36% by GDP, while the remaining protein was devoid of nucleotide (Fig. 3A).

Exchange of GTP and GTPγS into the complex

GTP or GTPγS was exchanged onto FLAG-RhoA/His$_6$-RhoGDI complex in the same manner as the [^{3}H]GDP-GDP exchange described above, at submicromolar concentrations of free Mg^{2+}. No detectable nucleotide exchange occurred in buffer with 5 mM Mg^{2+}. Only half of the GDP on the complex exchanged to GTPγS, due to 10% contamination of the GTPγS stock with GDP and a relatively low GTPγS to protein ratio of 18:1. Between the contaminating GDP and the GDP released from RhoA, we estimated that 15% of total nucleotide in the exchange solution was GDP, sufficient to prevent complete exchange to GTPγS.

As reported previously (Longenecker et al., 1999), GTP could also be exchanged onto the complex to a content of 60–70%, and bound GTP was hydrolyzed at rates approximately seven times slower than free FLAG-RhoA expressed in yeast cytosol (Table 1). Free FLAG-RhoA expressed in yeast had a similar GTP hydrolysis rate as RhoA expressed in *E. coli* (Table 1; Self & Hall, 1995). The slow hydrolysis rate by the complex was not due to GTP dissociating during the course of the measurement (out to 36 h) because, as demonstrated above, the complex does not release nucleotide in the presence of 5 mM Mg^{2+}. Again, switching or removing the affinity tags did not affect the rate of hydrolysis. GTP was hydrolyzed with pseudo-first-order kinetics (data not shown). This result strongly indicated that RhoA remained bound to RhoGDI while hydrolysis occurred. Had some RhoA been dissociated from RhoGDI, hydrolysis would have occurred with kinetics characterized by fast and slow components. This was clearly not seen over the complete time course of GTP hydrolysis. In addition, GTP was not released from the complex because hydrolysis occurred at a constant rate until no GTP remained (up to 36 h), and no free GTP was detected in the filtrates.

The hydrolysis and exchange rate constants of various complexes, FLAG-RhoA/His$_6$-RhoGDI, His$_6$-RhoA/FLAG-RhoGDI, and RhoA/RhoGDI with the tags removed were very similar, indicating that the amino-terminal tags did not interfere with either the ability of RhoA to hydrolyze GTP or the GTPase inhibitory function of RhoGDI. We point out that GTP hydrolysis by His$_6$-G14V-RhoA/FLAG-RhoGDI at 4 °C was extremely slow, with a $t_{1/2}$ of approximately seven days. Therefore, we expect that very little GTP hydrolysis occurred during the purification and concentration protocol, most of which was performed at 4 °C over the course of 12 h.

With respect to the exchange of GTPγS or GTP onto the complex, an important question is whether GDP-RhoA remains bound to RhoGDI in the presence of low Mg^{2+} or after GTP has been exchanged for GDP. We tested the stability of the heterodimer by observing the behavior of the complex over an analytical anion exchange column. FLAG-RhoA/His$_6$-RhoGDI consistently eluted between 145 and 180 mM NaCl in the presence of 5 mM free Mg^{2+}. SDS-PAGE analysis showed that the peak represented FLAG-RhoA and His$_6$-RhoGDI at a constant 1:1 ratio (Fig. 4BI). After the 3 h exchange reaction to GDP, GTP, or no added nucleotide, a small amount of dissociated RhoA (never more than 5% of the total) appeared as a peak at 95 mM NaCl just prior to the major heterodimer peak (Fig. 4A,BII–V). The corresponding free His$_6$-RhoGDI peak eluted later at 210 mM NaCl. This small amount of dissociated RhoA appeared only after incubation at room temperature in the exchange buffer even without EDTA, and was not related to the type of nucleotide exchanged. We note that separation of dissociated RhoA from the RhoA/RhoGDI complex required the use of the analytical Q anion exchange column, and a clean separation was not obtained by other chromatography methods. The ability to resolve the complex from the dissociated proteins provided additional evidence that the associated proteins are in a stable 1:1 complex.

Table 1. *Rate constants for nucleotide exchange and GTP hydrolysis at 22°C by RhoA/RhoGDI complex and free RhoA*[a]

	$k_{Exchange}$ with [^{3}H]GDP[b] (s^{-1})	$k_{GTP\ hydrolysis}$[b] (s^{-1})
FLAG-RhoA/His_6-RhoGDI	$3.3 \times 10^{-4} \pm 0.3 \times 10^{-4}$	$0.45 \times 10^{-4} \pm 0.021 \times 10^{-4}$
RhoA from *E. coli*	>0.040[c]	$2.1 \times 10^{-4} \pm 0.13 \times 10^{-4}$
FLAG-RhoA from yeast cytosol	>0.040[c]	$3.2 \times 10^{-4} \pm 0.089 \times 10^{-4}$

[a] Values are reported ± one standard deviation from at least three trials.
[b] See Figure 3 caption for experimental conditions.
[c] This rate constant is a minimum value because the reaction was nearly finished at the first time point (15 s).

Dissociation and translocation of GTP-RhoA from the complex to liposomes

The highly purified RhoA/RhoGDI complex allowed us to assess the mechanism of translocation of the prenylated RhoA from its complex with RhoGDI to membranes. The purified complex was incubated in the presence of liposomes made from purified *E. coli* phospholipids. Under conditions of submicromolar Mg^{2+}, the GTPγS (or GTP)-FLAG-RhoA dissociated from its complex with His_6-RhoGDI and associated with liposomes (Fig. 5). An average of 54 ± 5% (from four determinations) of the RhoA dissociated from RhoGDI and translocated to the liposomes. The translocation also occurred when the complex was first loaded with GTPγS, exchanged into a buffer containing 1 mM free Mg^{2+}, then incubated with liposomes (data not shown). In contrast, little or no translocation occurred when (1) the complex was occupied by GDP; or (2) when GTPγS was incubated with complex in the presence of 5 mM free Mg^{2+} (left side of Fig. 5), which did not allow nucleotide exchange. Immunoblot analysis with anti-RhoGDI antibody revealed that there was no significant amount of RhoGDI associated with the liposomes (data not shown), confirming that RhoA had dissociated from RhoGDI before becoming membrane associated. Consistent with the findings on RacI of Bokoch et al. (1994), the addition of fluoroaluminate, AlF_4^-, did not stimulate translocation to the membrane (data not shown). The absence of any other proteins in this assay that employed *E. coli* liposomes precluded any chance of contamination with specific protein prenyl receptors or GEFs. These results strongly suggested that the GTP-bound form of RhoA is required and sufficient for translocation from RhoGDI to the membranes.

The RhoA/RhoGDI complex expressed in yeast is signaling competent

The physiological function of the yeast expressed FLAG-RhoA/His_6-RhoGDI was tested by microinjection of nucleotide exchanged complex (GTP-G14V-RhoA/RhoGDI) into Swiss 3T3 cells. Only complex that contained GTP-RhoA (Figs. 6A,B), but not the GDP-containing complex (Fig. 6C), stimulated the formation of stress fibers and focal adhesions. Based on the translocation of GTP-bound RhoA to membranes shown above and the notion that RhoA must be membrane associated to carry outs it signaling function, it is likely that after injection into the cells, the complex dissociated and the RhoA translocated to the cell membranes where it stimulated stress fiber and focal adhesion formation. This response was similar to that described in published reports for free, unmodified RhoA, which when injected probably became prenylated by the endogenous geranylgeranyl transferase (A. Hall, pers. comm.; Paterson et al., 1990; Ridley, 1994).

Previously, we have shown that the GTP-loaded complex will elicit Ca^{2+}-sensitization of rabbit ileum smooth muscle (Longenecker et al., 1999). Similar to the stimulation of stress fiber and focal adhesion formation described here, the GDP form of the complex did not cause Ca^{2+}-sensitization, nor did unprenylated RhoA expressed in *E. coli* (Gong et al., 1996).

Discussion

The functionality of the purified RhoA/RhoGDI complex, with or without the amino-terminal affinity tags, was indicated by several observations. First, all of the RhoA in the complex was stably nucleotide bound in the presence of 5 mM Mg^{2+}, whereas all of the bound nucleotide could be exchanged at low Mg^{2+}. Second, bound GTP was hydrolyzed to completion at a rate considerably slower than that of free RhoA (0.45×10^{-4} vs. $2–3 \times 10^{-4}$ s^{-1}; also see Self & Hall, 1995). Third, we showed for the first time that the GTP-RhoA/RhoGDI complex was competent in downstream signaling in eliciting the well-documented RhoA physiological response of formation of stress fibers and focal adhesions. Presumably, the GTP-RhoA dissociated from RhoGDI upon injection into cells and membrane associated to carry out its signaling function. Switching or removal of the affinity tags did not significantly change any of the characteristics or activities.

A recent paper utilizing a purified RhoA/RhoGDI complex expressed and purified from insect cells (Fauré et al., 1999) presented evidence suggesting that, unless incubated with high concentrations of phosphoinositides, the purified complex was not accessible to modification by the *Clostridium botulinum* C3 ADP-ribosyl transferase or significant nucleotide exchange. In contrast, we found that the purified complex was an excellent substrate for ADP-ribosylation, having achieved stoichiometric labeling of the yeast expressed complex. Our finding is consistent with the crystal structure in which the ADP-ribosylation site, Asn41 of RhoA, is not obscured by RhoGDI in the complex (Longenecker et al., 1999) and with the ability to ADP-ribosylate partially purified complex isolated from neutrophil cytosol (Bourmeyster et al., 1992) or smooth muscle that was dependent upon the purity of the complex (Fig. 2). These findings suggested that a cytosolic factor removed by extensive purification may obscure the ADP-ribosylation site.

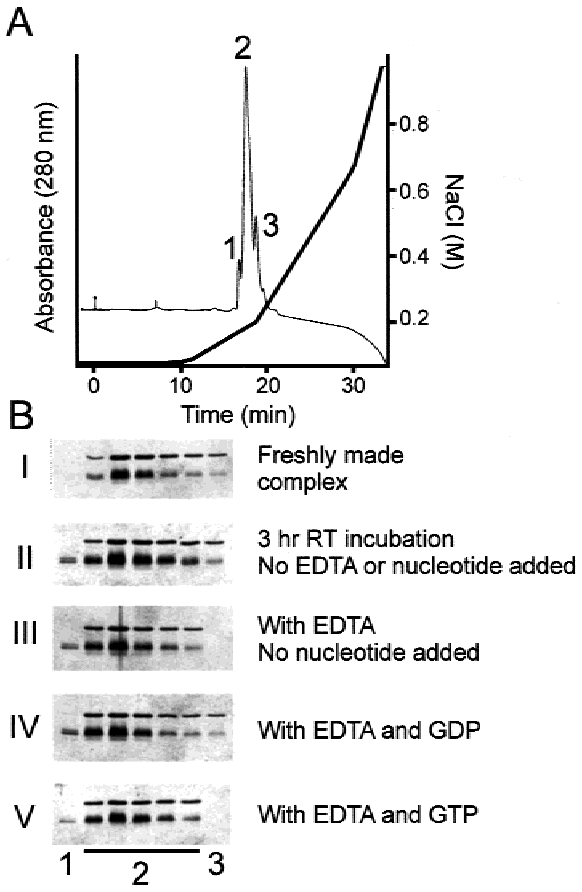

Fig. 4. Stability of the FLAG-RhoA/His_6-RhoGDI complex in exchange buffer or after storage. Purified complex was incubated for 3 h at room temperature in exchange buffer (see legend to Fig. 3) in the following conditions: with or without 10 mM EDTA, and 10 mM EDTA plus 7.5 mM GDP, or GTP. The samples were passed over desalting columns into 25 mM Tris-HCl plus 5 mM $MgCl_2$. Fifty microgram samples were then passed over a SMART mini-Q PC anion exchange column (Amersham Pharmacia Biotech) and eluted by a NaCl gradient. Fifty microliter fractions were collected, the samples subjected to SDS-PAGE analysis, and visualized by silver staining. **A:** Chromatogram (following absorbance at 280 nm) of sample (II) to indicate the peaks of free RhoA (1), RhoA/RhoGDI (2) and free RhoGDI (3). **B:** (I) SDS-PAGE analysis of fractions from chromatography from freshly prepared RhoA/RhoGDI complex. Note that the relative staining intensities of RhoGDI (upper band) and RhoA (lower band) remain constant, indicating that the proteins were stably bound to each other. Fractions from chromatographies of complex incubated 3 h at room temperature (II) without EDTA or added nucleotide, (III) with EDTA and no added nucleotide, (IV) with EDTA and GDP, or (V) with EDTA and GTP. The numbers below the gels indicate the fractions containing RhoA, RhoA/RhoGDI complex, or RhoGDI, and correspond to the numbered peaks in **A**.

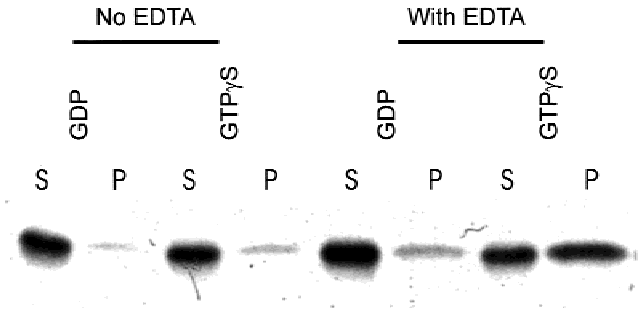

Fig. 5. GTP stimulation of RhoA translocation from RhoGDI to liposomes. Translocation of prenylated RhoA to liposomes made from purified *E. coli* lipids was performed as described in the Material and methods. Samples in which no EDTA was added had 5 mM $MgCl_2$ present during the exchange with the indicated nucleotide, while samples with EDTA had 10 mM EDTA present. This representative immunoblot shows the amount of RhoA remaining soluble (S) and associated with the lipid pellet (P). Immunoblot analysis detected a very small and consistent amount of RhoGDI carried over with the liposome pellet.

The highly purified 1:1 complex of RhoA with RhoGDI allowed accurate determination of the kinetic constants of nucleotide exchange and GTP hydrolysis and provided some new observations about properties of RhoA: (1) The constitutively active G14V-RhoA can form a stable complex in vivo with RhoGDI, even in the GTP bound form. (2) GTP can be exchanged onto RhoA while associated with RhoGDI. (3) GTP binding can precede translocation of RhoA from RhoGDI and binding to membranes (see below).

Interestingly, a small proportion (5%) of the purified G14V-RhoA/RhoGDI complex retained GTP, rather than GDP, whereas a much larger proportion (40%) of purified free cytosolic G14V-RhoA was GTP bound. According to a recent review of the literature (Olofsson, 1999), ours appears to be the first observation of cytosolic GTP-RhoA complexed with RhoGDI. Bound GTP likely did not hydrolyze during the course of preparing G14V-RhoA/RhoGDI complex, because most of the purification occurs at 4 °C where the GTP hydrolysis is extremely slow. It is interesting that, despite the high GTP:GDP ratio in the cell, there was no detectable GTP bound to the wild-type free RhoA or RhoA/RhoGDI, consistent with prior findings (reviewed in Olofsson, 1999). Our data suggest that the GTP-RhoA species is relatively short lived in vivo and that, in the presence of membranes, the GTP-RhoA/RhoGDI complex is less stable than the GDP bound complex. The GTP-facilitated dissociation of the RhoA/RhoGDI complex may be due to the known conformational change in the Switch I and II regions of RhoA between the GDP and GTP-bound states (Ihara et al., 1998) that may result in a less favorable interaction with the amino terminus of RhoGDI (amino acids 31–55) (Longenecker et al., 1999). Effector proteins that bind to this region may also compete with RhoGDI for binding GTP-RhoA. Clearly, the lipid bilayer provides a thermodynamically more favorable environment than RhoGDI for GTP-RhoA.

The GTP-induced dissociation of the complex may play a key role in RhoA function. GTP and GTPγS stimulate translocation of RhoA from RhoGDI to protein-free liposomes leaving RhoGDI behind, consistent with the translocation observed during physiological activation of the RhoA-RhoKinase-induced Ca^{2+} sensitization (myosin phosphatase inhibition) by GTP or GTPγS (Gong et al., 1997a; Fujihara et al., 1997). Similarly, microinjection of GTP (but not GDP)-loaded complex elicited formation of stress fibers and focal adhesions in serum starved Swiss 3T3 cells. It is tempting to speculate that if GEF-induced RhoA nucleotide exchange can occur while RhoA is bound to RhoGDI, then the subsequent dissociation of the RhoA/RhoGDI complex and translocation of RhoA to the membrane may be thermodynamically driven. Our results obtained with the highly purified, functional complex (Fig. 5) support this simple model of RhoA activation.

The RhoA/RhoGDI complex expressed in *Saccharomyces cerevisiae* and assembled in vivo allows the facile purification

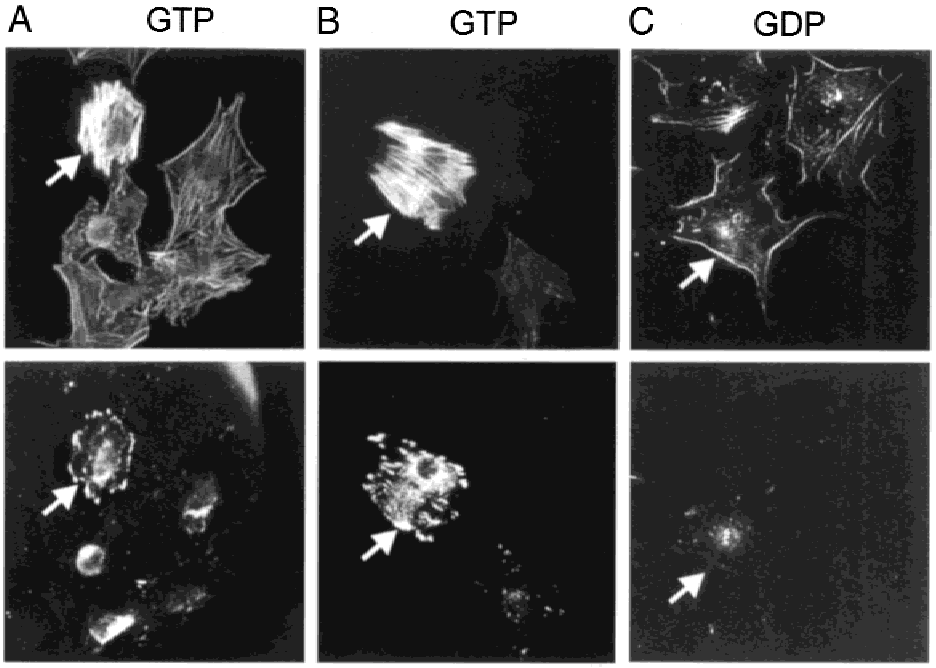

Fig. 6. The GTP-RhoA/RhoGDI complex elicits formation of stress fibers and focal adhesions. Swiss 3T3 cells were microinjected as described in the Materials and methods. Stress fibers (top row) were visualized by reacting with FITC-conjugated phallicidin, and focal adhesions (bottom row) with anti-vinculin IgG. Representative samples are shown in these frames. (**A,B**) Two examples of cells injected with RhoA/RhoGDI that had been exchanged to GTP (~70% GTP); or (**C**) exchanged to GDP. The injected cells are indicated by arrows. Note that cells injected with GTP-RhoA/RhoGDI have considerably more stress fibers and focal adhesions than the surrounding cells, whereas the cells injected with GDP-bound complex have similar numbers of both features.

and manipulation of prenylated RhoA. Without association with RhoGDI, handling prenylated RhoA is difficult due to solubility problems, nucleotide loss, and hydrolysis, and relatively low yields obtained from membrane fractions of eukaryotic expression systems. For this reason, much of the experimental work to date has been conducted with RhoA overexpressed in prokaryotic expression systems, which lack the ability to carry out the post-translational modifications. Although these systems provide high yields of soluble protein, the lack of prenylation required for membrane translocation and functionality limits their use in signal transduction studies. GTP-RhoA/RhoGDI was capable of activating RhoA pathways when introduced into mammalian systems, whereas GDP-RhoA/RhoGDI was not, confirming that dissociation and translocation of RhoA and subsequent activation of downstream effectors is dependent on GTP binding. The nucleotide (GTP replacing GDP) exchanged complex provides a novel method for introducing GTP loaded, post-translationally modified RhoA into mammalian systems at high concentrations in a soluble form, overcoming the difficulties of introducing the hydrophobic, free prenylated RhoA, that has higher GTP hydrolytic activity, or the unmodified RhoA, that requires in situ prenylation for activity. The nucleotide is easily exchanged in vitro, while the complex remains intact and RhoGDI renders the nucleotide state stable.

Materials and methods

Construction of the YEpP_{Gal}/t_{PMA1} expression vector

The *Gal1–10* promoter region (Johnston & Davis, 1984) was inserted between the *Eco*RI and *Bam*HI sites of the yeast 2 μm shuttle vectors YEplac181 (*LEU2*) and YEplac195 (*URA3*) (Gietz & Sugino, 1988). In addition, the 3′ half of the yeast *PMA1* gene including its transcription termination signal was inserted between *Bam*HI and *Xba*I (Nakamoto et al., 1991). The *PMA1* segment included a *Xho*I site that was created by ligation of a linker in the *Sal*I site 260 bases upstream of the *PMA1* termination codon. This created plasmid $YEpP_{GAL}/t_{PMA1}$.

Expression of human RhoA and RhoGDI cDNA

The RhoA cDNA in an *E. coli* expression plasmid, generously provided by Dr. Alan Hall of the Institute of Cancer Research, London, contained the Phe25 to Asn mutation (Paterson et al., 1990), which we restored to the wild-type sequence by site-directed mutagenesis. A cDNA clone of human RhoGDI was generously provided by Dr. Gary Bokoch of Scripps Institute (San Diego, California). Modifications to the ends of the open reading frames of both cDNA were introduced on oligonucleotide primers that were used in PCR amplifications (Mullis et al., 1994) with *Pfu* polymerase (Stratagene, Ja Jolla, California). To facilitate switching the amino-terminal affinity tags, an *Eag*I restriction site was added just prior to the second codon of RhoA and RhoGDI. For this PCR amplification, the 5′ oligonucleotide primer contained the *Eag*I site and the overlap sequence of the cDNA. The 3′ primer was extended to incorporate a *Xho*I site downstream of the termination codon and a *Sac*I site for cloning. The PCR products were digested with *Sac*I and *Eag*I and ligated into pBluescript IIKS(+) (Stratagene). In all cases, the sequences of the entire DNA inserts were confirmed by automated DNA sequencing of both strands performed at the University of Virginia Biomolecular Core Facility.

For optimized expression in yeast, we used the sequences immediately upstream of the initiation codon and the first five codons from the highly expressed yeast *PMA1* gene. This sequence was immediately followed by the His_6 or FLAG affinity tags and the *Eag*I site. The *Eag*I site added Arg-Pro between the tags and RhoA or RhoGDI. These sequences were added by ligation of double-stranded oligonucleotide cassettes between the *Bam*HI and *Eag*I sites in the RhoA or RhoGDI pBluescript clones described in the previous paragraph. The *Bam*HI and *Xho*I expression insert was isolated and ligated into $YEpP_{GAL}/t_{PMA1}$.

When a protease site was desired for removal of the affinity tags, the recognition sequence for the rTEV protease (-DYDIPTT ENLYFQG-; GIBCO-BRL, Rockville, Maryland) was added to the RhoA and RhoGDI cassettes, 3′ of the *Eag*I site, by PCR with extended primers.

Complete DNA sequences and restriction maps are available from the authors upon request.

Growth and expression of prenylated human RhoA and RhoGDI in yeast

Yeast strain SY1 (*MATa, ura3–52, leu2–3,112, his4–619, sec6–4, GAL*; Nakamoto et al., 1991) was transformed to leucine and/or uracil independence with the desired expression plasmid by the lithium acetate method (Ito et al., 1983) and grown on minimal SC medium without leucine and/or uracil (Sherman, 1991). Two percent glucose or raffinose was used as the carbon source to repress expression of the plasmid-borne RhoA and RhoGDI cDNA under control of the *GAL1* promoter. Expression of RhoGDI is toxic in yeast (Masuda et al., 1994) and yeast transformed with $YEpP_{GAL}$-RhoGDI-t_{PMA1}, with or without $YEpP_{GAL}$-RhoA-t_{PMA1}, did not form colonies on a galactose plate. In contrast, yeast transformed with the RhoA expression plasmid formed colonies on galactose medium although expression of the constitutively active Gly14 to Val mutant (G14V) RhoA grew poorly (data not shown). The toxicity of RhoGDI probably explains the slightly lower total yields of RhoA when co-expressed with RhoGDI than when expressed alone.

For production of recombinant protein, a 50 mL 2% glucose SC medium culture was used to inoculate two 1 L cultures of 2% raffinose SC medium, which in turn were used to inoculate 15 L of the same medium. At optical density $OD_{650nm} = 0.8–1.0$, galactose was added to 2% final concentration to induce expression. After 8 h, the cells were harvested by centrifugation, resuspended in 4 mL of 10 mM sodium azide per gram of cells, and stored at −80 °C. Sixty-five to 80 g wet weight cell pellets were routinely obtained.

Fractionation of yeast

Cell suspensions from an 18 L culture were thawed at room temperature and added to an equal volume of 0.6 mg/mL of activated Zymolyase 20T (ICN Biochemicals, Costa Mesa, California) in 2.8 M sorbitol, 0.1 M potassium phosphate, 0.1 M β-mercaptoethanol, and 10 mM sodium azide at pH 7.4. The suspension was incubated at room temperature for 45 min with gentle shaking. The cells were sedimented at 2,800 *g* at 4 °C for 10 min, and resuspended in the same volume in 25 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$ at pH 8.0 (Buffer A) plus 10% glycerol. A protease cocktail was added consisting of 1 μg/mL leupeptin, 2 μM pepstatin, 2 μg/mL aprotinin, 40 μg/mL benzamidine, and 1 mM freshly diluted diisopropyl fluorophosphate (final concentrations). The suspension was subjected to two passes through a cooled French press cell at 25,000 psi. After a 5-min centrifugation at 13,000 *g* at 4 °C, the supernatant was subjected to a 60 min centrifugation at 240,000 *g* at 4 °C. The supernatant (cytosolic fraction) was kept for isolation of the RhoA/RhoGDI complex. The pellets were resuspended in Buffer A at ~5 mg of protein/mL and stored at −80 °C.

Purification of RhoA/RhoGDI complex

Purification of His_6-RhoA/FLAG-RhoGDI or FLAG-RhoA/His_6-RhoGDI followed the same procedure of passing the yeast cytosolic fraction over a metal chelate column followed by passage over the anti-FLAG antibody column (Fig. 1).

Ten milliliters of Talon Metal Affinity resin (Clonetech, Palo Alto, California), pre-equilibrated with Buffer A, was incubated with the cytosolic fraction at room temperature for 30 min with gentle agitation. After packing in a column, the resin was washed with 10 bed volumes of Buffer A followed by 2 volumes of Buffer A plus 5 mM imidazole. Bound protein was eluted with Buffer A containing 100 mM imidazole. The fractions with the highest amounts of protein were pooled and the buffer adjusted to 50 mm Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, pH 7.4 (M2 buffer). The protein suspension was passed over a 7.5 mL anti-FLAG M2 monoclonal antibody resin column (Sigma Chemicals, St. Louis, Missouri), which was pre-equilibrated in M2 buffer. After washing with 10 bed volumes of M2 buffer, bound protein was eluted with M2 buffer plus 0.1 mg/mL FLAG peptide (sequence DYKD-DDDK). Fractions containing complex were pooled and concentrated in a 15 mL Amicon Centriplus (MWCO 10) concentrator (Millipore, Bedford, Massachusetts). When the affinity tags were removed from a preparation of complex, 150 units of rTEV protease (GIBCO-BRL, Gaithersburg, Maryland) was added and incubated for 4 h at room temperature. After proteolysis, the protein was passed over the metal chelate column to separate purified complex from uncleaved material and rTEV protease, which also contained a His_6 tag.

Purification of human free RhoA from the yeast cytosolic fraction

RhoA was expressed without RhoGDI to obtain primarily unprenylated protein from the cytosolic fraction. This expression was similar to that done in SF9 cells (Mizuno et al., 1991; Gong et al., 1996). Wild-type and FLAG-G14V-RhoA were purified from the yeast cytosolic fraction using the appropriate affinity chromatography as described above. SDS-PAGE analysis showed that no detectable endogenous yeast RhoGDI was associated with this RhoA.

Partial purification of native RhoA/RhoGDI complex from rabbit ileum

Twenty-five grams of tissue were homogenized in 100 mL of a buffer containing 50 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, pH 8.0, and a protease inhibitor cocktail, consisting of 2 μg/mL leupeptin, 4 μM pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, and 40 μg/mL benzamidine. The cytosolic fraction was isolated by differential centrifugation and dialyzed against Low Salt Buffer (25 mM Tris-HCl and 5 mM $MgCl_2$, pH 8.0). The dialysate was passed over a DEAE Sephacel

(Amersham Pharmacia Biotech, Piscataway, New Jersey) column (20 mL bed volume) equilibrated with Low Salt Buffer and eluted with a linear NaCl gradient up to 1 M in the same buffer. The RhoA-containing fractions were pooled, dialyzed against Low Salt Buffer, and the sample passed over a Waters Q8HR strong anion exchange column (8 mL bed volume) and again eluted with a NaCl gradient. The RhoA-containing fractions were pooled, concentrated to less than 1 mL, and the sample passed over a Superdex-75 Hi-Load 16/60 size exclusion column (Amersham Pharmacia Biotech). Fractions containing RhoA and RhoGDI were pooled and concentrated. The complex was approximately 5–10% of total protein as estimated from Coomassie stained gels, protein assay, and quantitative immunoblots.

Protein determinations

Protein concentrations were determined using the Biorad (Hercules, California) Protein Assay with bovine serum albumin as a standard. Fifteen percent or 4–20% acrylamide SDS-PAGE was performed as previously described (Gong et al., 1997b). For immunoblot analysis, an anti-RhoA mouse monoclonal IgG (Santa Cruz 26-C4, Santa Cruz Biotech, Santa Cruz, California) or an anti-RhoGDI rabbit polyclonal IgG (Santa Cruz A-20) were used. Immunoreactivity was visualized with a peroxidase-conjugated goat anti-mouse (Goldmark Biologicals, Phillipsburg, New Jersey) or donkey anti-rabbit (Amersham Pharmacia Biotech) secondary antibody, respectively. For RhoA or RhoGDI immunoblot quantification, internal standards were created on the gels by running a titration of known amounts of purified RhoA or RhoGDI on the same gels. The amount of protein in the samples was quantified by comparing densitometry values to the standards; 47,750 DA (23,060 DA for FLAG-RhoA and 24,690 DA for His_6-RhoGDI) was used as the molecular weight for the complex to calculate molar concentrations.

ADP-ribosylation of RhoA proteins

ADP-ribosylation of the RhoA/RhoGDI complex was carried out as previously described (Gong et al., 1996). A volume of protein sample containing 100 ng of RhoA was incubated with 1 μg of C3 exoenzyme from *C. botulinum* and 100 μM [^{32}P]NAD^+ (25 μCi from Amersham Pharmacia Biotech) for 30 min at room temperature. The protein associated radioactivity was determined by liquid scintillation counting to quantify the ribosylation reaction.

Determination of bound nucleotide

Fifteen microliters of protein sample was added to 7.5 μL of 1% perchloric acid followed by 7.5 μL of 0.28 M sodium acetate to precipitate the protein and release the nucleotide. After adding 30 μL of 1.4 M ammonium phosphate, pH 4.0, the sample was centrifuged at 2,000 *g* for 5 min at 4 °C. Liberated nucleotides in a 50 μL aliquot were identified by isopycnic separation over a Waters (Milford, Massachusetts) 8PSAX-10μ column equilibrated with 0.7 M ammonium phosphate, pH 4.0. The amounts of each nucleotide were quantified by integration of the peaks and comparing to standards of GDP, GTP, and GTPγS eluted from the same column.

Exchange of GTP or GTPγS into complex

Complex was exchanged with 1 mM GTPγS final concentration. Aliquots were taken at the specified times and the reaction stopped by the addition of $MgCl_2$ to 13 mM final concentration. The samples were passed over a desalting column equilibrated with Buffer A to remove unbound nucleotides and concentrated in a 3.5 mL Palfiltron MWCO 10 concentrator. Bound nucleotide was determined as described in Materials and methods. No nucleotide was detected in the concentrator filtrate, indicating that all measured nucleotide was bound to RhoA. Circles: exchange in the presence of EDTA. Inverted triangles: exchange in the absence of EDTA.

GTP hydrolysis

Exchange of GTP into RhoA/RhoGDI complex and removal of free GTP were performed as described above using 2.5 mM GTP incubated with 50 μM protein for 3 h at 22 °C. After exchanging to Buffer A (containing 5 mM $MgCl_2$ which stops the exchange of nucleotide), the GTP-loaded complex was incubated at 22 °C, aliquots taken at specified time points, and the reaction quenched and the protein precipitated by addition of perchloric acid. Nucleotide bound was determined as described above.

Stability of GDP bound to FLAG-RhoA/His_6-RhoGDI

The amount of GDP bound to RhoA/RhoGDI complex was determined by incubating 100 μM of complex at 22 °C in Buffer A. At 24 h intervals, 20 μL aliquots were taken and diluted into 400 μL of the same buffer. The sample was concentrated in a 500 μL Microcon (Millipore) MWCO 10 concentrator (which effectively removes free nucleotide), and the amount of nucleotide and protein were determined as described above.

Translocation of RhoA from RhoGDI to liposomes

FLAG-RhoA/His_6-RhoGDI complex (0.5 μg) was incubated with 100 μM final concentration of GTPγS or GDP in Buffer A, with or without 10 mM EDTA in a reaction volume of 40 μL for 2 h at 22 °C. In conditions with fluoroaluminate, the complex was incubated in the same buffer with or without EDTA or added nucleotides, and with 60 μM $AlCl_3$ plus 25 mM NaF for 2 h. One hundred micrograms of purified liposomes made from purified *E. coli* lipids (Perozo et al., 1998) were added and incubated for an additional 30 min. The samples were then centrifuged at 290,000 *g* for 30 min at 4 °C to pellet the liposomes. The pellets were washed twice with Buffer A, resuspended in 100 μL of SDS sample buffer, separated by 15% acrylamide SDS-PAGE, and the proteins detected by immunoblot for RhoA and RhoGDI.

Microinjection of complex into Swiss 3T3 cells

GTP-exchanged FLAG-RhoA/His_6-RhoGDI or GDP-FLAG-RhoA/His_6-RhoGDI (1.2 mg/mL) along with Texas Red dextran marker (Molecular Probes, Eugene, Oregon) were injected into Swiss 3T3 cells (10% cell volume) as previously described (Nobes & Hall, 1995). After 1 h, the cells were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, washed, permeabilized with 0.5% Triton X-100 in PBS for 2 min, and washed again. The cells were then incubated with mouse anti-vinculin IgG (Sigma) followed by TRITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania) for staining of focal adhesions, or with FITC-conjugated phallicidin (Molecular Probes) for staining of stress

fibers. After washing and drying, the cells were observed in a Zeiss Axiopan fluorescence microscope.

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