

# Mechanism of Digeranylgeranylation of Rab Proteins

## FORMATION OF A COMPLEX BETWEEN MONOGERANYLGERANYL-Rab AND Rab ESCORT PROTEIN\*

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**Rab proteins are Ras-related small GTPases that are digeranylgeranylated at carboxyl-terminal cysteines, a modification essential for their action as molecular switches regulating intracellular vesicular transport. Geranylgeranylation of Rabs is a complex reaction that requires a catalytic Rab geranylgeranyl transferase (GGTase) and a Rab escort protein (REP). REP binds unprenylated Rab and presents it to Rab GGTase. After GG transfer, REP remains associated with diGG-Rab, which leads to insertion of the Rab into a specific membrane. We used recombinant Rab1a single cysteine mutants that accept only one GG group to study the mechanism of the digeranylgeranylation reaction. Using the prenylation assay, gel filtration chromatography, and density ultracentrifugation, we show that REP, but not Rab GGTase, forms a stable complex with unprenylated, monoGG- and diGG-Rab1a. The REP-monoGG-Rab1a complex is stable in the presence of detergents or phospholipids, whereas the REP-diGG-Rab1a complex partially dissociates under these conditions. The stoichiometry of the REP-Rab complex appears to be 1:1 before prenylation. Prenylation induces a change in complex stoichiometry, with the formation of a 2:2 or 2:1 REP-Rab complex. A possible mechanism by which Rab proteins are digeranylgeranylated is suggested by the current studies. We propose that each geranylgeranyl addition is an independent reaction that leads to the production of monoGG-Rab and diGG-Rab, respectively. The stability of the REP-monoGG-Rab complex prevents monoGG-Rab from dissociating from REP prior to the second geranylgeranylation reaction, ensuring efficient digeranylgeranylation of Rab substrates.**

Many eukaryotic proteins contain prenyl groups, either the C<sub>15</sub> farnesyl or the C<sub>20</sub> geranylgeranyl (GG),<sup>1</sup> attached via thioether linkage to cysteines at or near the carboxyl terminus (1–4). Prenyl modification is essential for function of the modified protein, since it is required for membrane association and formation of specific protein-protein interactions.

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<sup>1</sup> The abbreviations used are: GG, geranylgeranyl; GGPP, geranylgeranyl pyrophosphate; GGTase, geranylgeranyl transferase; REP, Rab escort protein; PC, phosphatidylcholine; Sf9, *Spodoptera frugiperda*-9.

Rab proteins, Ras-related small GTPases involved in the regulation of intracellular vesicular traffic in exocytic and endocytic pathways (5–8), are among the prenylated proteins present in cells (9–11). Rabs contain two cysteine residues at or near the carboxyl terminus arranged in various motifs such as XXCC, XCXC, or CCXX, where C is cysteine and X is any amino acid. Both cysteine residues present in the motif are modified by the attachment of GG groups via thioether bonds in a complex reaction mechanism that requires two components (12, 13). One component is catalytic and designated Rab GGTase (previously called Component B) or GGTase-II. It is a tightly coupled heterodimer composed of a 60-kDa  $\alpha$ -subunit and a 38-kDa  $\beta$ -subunit (14), both related to the  $\alpha$ - and  $\beta$ -subunits of the other known prenyl transferases, farnesyl transferase and CAAX GGTase (or GGTase-I).

Rab GGTase is unique among known prenyl transferases, since it is unable to catalyze the reaction on its own, but requires the presence of an additional component, designated Rab escort protein or REP (previously designated Component A, also known as choroideremia protein) (13, 15). REP binds to unprenylated Rab, presents it to Rab GGTase, and thereby facilitates GG transfer. After geranylgeranylation, REP remains bound to Rab in a stable complex that can be released *in vitro* by detergents (15). *In vivo*, REP delivers geranylgeranylated Rab to its target donor membrane (16).

Two related REPs, REP-1 and REP-2, have been identified (17, 18). Mutations in REP-1 give rise to choroideremia, a retinal degeneration disease (13, 19, 20). *In vitro*, REP-2 can assist in the prenylation of most Rab proteins as efficiently as REP-1. A notable exception is Rab3a which displays a lower  $V_{\max}$  with REP-2 than with REP-1 (18). Another exception is Rab27, which is prenylated with 3-fold higher affinity in the presence of REP-1 as compared with REP-2 (20). *In vivo*, choroideremia lymphoblasts that contain only REP-2 can efficiently prenylate all endogenous Rab substrates, except for Rab27 (20). Rab27 is the first example of, possibly, a family of Rabs that require preferentially either REP-1 or REP-2 for prenylation and that might explain the retina-restricted phenotype observed in choroideremia (20).

Different Rabs regulate different steps of intracellular vesicular transport. For example, Rab1a (and Rab1b) regulate endoplasmic reticulum to Golgi transport, while Rab5a functions in plasma membrane to endosome transport. A current view of the cyclic function of Rab proteins in vesicular transport is as follows (5–8, 15). Newly synthesized Rabs bind REP, the REP-Rab complex associates with Rab GGTase and geranylgeranylation of both carboxyl-terminal cysteines occurs. After prenylation, REP delivers diGG-Rab (presumably in the inactive GDP-bound form) to its target donor organelle membrane. Upon membrane association, diGG-Rab is activated by exchange of GDP for GTP and remains associated with the transport vesicle until the transport vesicle and the target acceptor membrane fuse. GTP is then hydrolyzed into GDP and diGG-Rab is extracted from the acceptor organelle membrane

by Rab GDP dissociation inhibitor, which can deliver diGG-Rab back to the donor organelle membrane and complete the cycle. Rab GDP dissociation inhibitor and REP share structural and functional homology, which suggests that both use similar mechanisms to associate with diGG-Rab and deliver it to intracellular membranes. However, details about the mechanism responsible for the partition of diGG-Rabs to membranes is unknown at present.

Farnsworth *et al.* (21) have established that Rab GGTase/REP can digeranylgeranilate Rab substrates, whether they contain a XXCC, a XCXC, or a CCXX double cysteine motif. It has also been shown that very little, if any, monoGG-Rab accumulates in *in vitro* reactions (21). This observation suggested that the Rab GGTase/REP enzyme system catalyzes efficiently the digeranylgeranylation reaction either because the  $K_{cat}/K_m$  of the second GG addition is much larger than that of the first GG addition or because dissociation of monoGG-Rab from the enzyme is slower than the transfer of the second GG group. In this study, we used recombinant mutated forms of Rab1a that could only accept one GG group to probe the mechanism of the prenylation reaction, and we present evidence to suggest that the latter hypothesis is more likely. Our results suggest that REP forms a stable complex with monoGG-Rab1a in order to ensure double geranylgeranylation of Rabs prior to delivery to intracellular membranes.

#### EXPERIMENTAL PROCEDURES

**Materials**—All-*trans*-[1-<sup>3</sup>H]GGPP (15 Ci/mmol) and unlabeled all-*trans* GGPP were purchased from American Radiolabeled Chemicals, St. Louis, MO. Phosphatidylcholine vesicles were prepared as described previously (22). The anti-Ha-Ras monoclonal antibody was purchased from Transduction Laboratories. J905 is a polyclonal antibody directed against recombinant rat REP-1 produced as described below and affinity-purified on a REP-1-agarose column using the AminoLink Coupling Gel (Pierce) kit. H492 is a polyclonal antiserum directed against recombinant Rab GGTase ( $\alpha$ - and  $\beta$ -subunits) prepared as described below and D576 is a polyclonal antibody directed against recombinant canine Rab1a produced as described below and IgG was purified on a protein A-Sepharose column (Pharmacia Biotech Inc.) as described (23).

**Assay for Rab GG Transferase Activity**—Rab GGTase activity was determined by measuring the amount of [<sup>3</sup>H]geranylgeranyl transferred from [<sup>3</sup>H]geranylgeranyl pyrophosphate to Rab proteins (12, 19). Unless otherwise indicated, the standard reaction mixture contained the following concentrations of components in a final volume of 50  $\mu$ l: 50 mM sodium Hepes (pH 7.2), 5 mM MgCl<sub>2</sub>, 1 mM Nonidet P-40, 1 mM dithiothreitol, 5.5  $\mu$ M [<sup>3</sup>H]GGPP (3,000 dpm/pmol), and the indicated amounts of Rab proteins, REP-1, and Rab GGTase. After incubation for the indicated time at 37 °C, the amount of ethanol/HCl-precipitable radioactivity was measured by filtration on a glass fiber filter (19). Recombinant histidine-tagged Rab proteins, recombinant histidine-tagged REP-1, and recombinant Rab GGTase were used in all *in vitro* assays.

**Recombinant Rab, Ras, Rab GGTase, and REP-1 Proteins**—Rab fusion proteins containing six histidine residues (His-tagged) at the NH<sub>2</sub> terminus of wild-type and mutant Rab1a proteins were prepared as described previously (18, 22). Briefly, transformed BL21 (DE3) *Escherichia coli* cells containing pET14b-Rab1a-CC, -CS, -SC, or -SS, were grown, lysed, and the supernatant from a 30,000  $\times$  g spin (1 h at 4 °C) was subjected to Ni<sup>2+</sup>-Sepharose affinity chromatography (Pharmacia Biotech Inc.) under the conditions recommended by the manufacturer (Novagen). The eluted His-tagged Rab1a proteins (>90% pure as judged by SDS-polyacrylamide gel electrophoresis) were each dialyzed against buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 3 mM MgCl<sub>2</sub>, 1 mM EDTA, 5 mM dithiothreitol, and 0.1 mM GDP and stored in multiple aliquots at -70 °C. Recombinant His-tagged Ha-Ras was a gift from Dr. Guy James, University of Texas Southwestern Medical Center and was produced as described above for His-tagged Rab proteins (24). Recombinant His-tagged REP-1 was prepared in Sf9 insect cells as described previously (18, 25). Briefly, Sf9 cells were infected with recombinant baculovirus encoding REP-1, grown, lysed, and a 10<sup>5</sup> g of supernatant was subjected to Ni<sup>2+</sup>-Sepharose affinity chromatography as described above for the His-tagged Rab proteins. The His-tagged REP-1 (>90% pure as judged by SDS-polyacrylamide gel electrophoresis) was dialyzed against two changes of buffer containing 50 mM

sodium Hepes (pH 7.2), 10 mM NaCl, 0.1 mM Nonidet P-40, and 1 mM dithiothreitol and stored in multiple aliquots at -70 °C. Recombinant Rab GGTase was prepared as described previously (18, 25). Briefly, Sf9 cells were co-infected with recombinant baculovirus encoding the  $\alpha$ - and the  $\beta$ -subunit, grown, lysed, and a 10<sup>5</sup> g of supernatant was chromatographed on Q-Sepharose and Superdex 200 columns and the active fractions stored in multiple aliquots at -70 °C.

**Assay for Complex Formation between Rab1a and REP-1 on Gel Filtration Chromatography**—Reaction mixtures contained in a final volume of 50  $\mu$ l, 50 mM sodium Hepes (pH 7.2), 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 5  $\mu$ M unlabeled GGPP, 0.5  $\mu$ M RabGGTase, 2  $\mu$ M amounts of either Rab1a-CC, Rab1a-CS, Rab1a-SC, or Rab1a-SS, in the presence or absence of 2  $\mu$ M REP-1. After incubation for 15 min at 37 °C, reaction mixtures were loaded onto a Superdex 200 3.2/30 using a SMART system (Pharmacia Biotech Inc.). The column was equilibrated in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM dithiothreitol at a flow rate of 50  $\mu$ l/min, the samples were injected, and the material eluting between 0.75 ml and 1.95 ml was collected in 100- $\mu$ l fractions. An aliquot of fractions 2–11 was subjected to SDS-gel electrophoresis, transferred to nitrocellulose filters, and the proteins identified by immunoblot analysis using the ECL system (Amersham Corp.) as described (20).

**Assay for Complex Formation between Rab1a and REP-1 on Glycerol Gradient Ultracentrifugation**—Reaction mixtures contained 50 mM sodium Hepes (pH 7.2), 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10  $\mu$ M unlabeled GGPP or in some experiments 10.5  $\mu$ M [<sup>3</sup>H]GG pyrophosphate (1,650 dpm/pmol), in a final volume of 50  $\mu$ l, in the presence or absence of 4  $\mu$ M REP-1, 4  $\mu$ M RabGGTase, and 4  $\mu$ M amounts of either Rab1a-CC, Rab1a-CS, Rab1a-SC, or Rab1a-SS. After incubation for 15 min at 37 °C, reaction mixtures were diluted to 100  $\mu$ l with buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 7.5% glycerol, and 50  $\mu$ g each of the molecular weight standards catalase, aldolase, and ovalbumin and loaded onto 4 ml of 7.5–30% glycerol gradients in 20 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol. Glycerol gradients were spun for 16 h at 4 °C in a SW60 rotor (Beckman) at 50,000 rev/min. Fourteen fractions (0.3 ml) were collected from the top, and an aliquot was subjected to SDS-gel electrophoresis, transferred to nitrocellulose filters, and the proteins identified by immunoblot analysis using the ECL system (Amersham) as described (20). The internal standards were visualized by Ponceau S staining of the nitrocellulose filter and quantified with the Coomassie Plus Protein Assay Reagent (Pierce). The REP-1 protein used in these studies had undergone one extra step of purification on a Superdex 200 HR10/30 column (Pharmacia). One mg of REP-1 in 500  $\mu$ l was loaded on the gel filtration column equilibrated with buffer containing 50 mM sodium Hepes (pH 7.2) and 1 mM dithiothreitol at a flow rate of 0.25 ml/min. The major peak of eluted protein was collected and stored in aliquots at -70 °C.

#### RESULTS

Rab GGTase activity is stimulated by detergents such as Nonidet P-40 (15). Evidence indicates that detergents stimulate the reaction, because they act as acceptors of GG-Rab, the product of the reaction. In their absence, REP and GG-Rab form a stable complex, and REP is unable to undergo further rounds of catalysis. We wanted to determine if phospholipid vesicles would act similarly to stimulate the reaction. Fig. 1A shows an experiment where we measured the time-dependent transfer of [<sup>3</sup>H]GG to Rab1a. In the absence of detergents, the reaction reached completion when 2 pmol of [<sup>3</sup>H]GG were incorporated into Rab1a. In the presence of the detergent Nonidet P-40, the reaction progressed for up to 30 min, essentially as described before (12, 15). When phosphatidylcholine (PC) vesicles were used, a significant stimulation of the reaction was observed, comparable with that obtained with Nonidet P-40 (Fig. 1A). Under these conditions, 60 pmol of [<sup>3</sup>H]GG were incorporated into Rab1a or 30-fold stimulation over control reactions in the absence of detergents or phospholipids.

We used recombinant DNA techniques to generate Rab1a mutants that contain either one or both the carboxyl-terminal cysteines mutated to serines. The resulting proteins, Rab1a-CS, -SC, and -SS, can accept only one or no GG groups. When we analyzed mutant Rab1a-CS and Rab1a-SC in the same experiment, we observed that Nonidet P-40 or PC were very weak stimulators of GG transfer (Fig. 1, B and C). As shown in

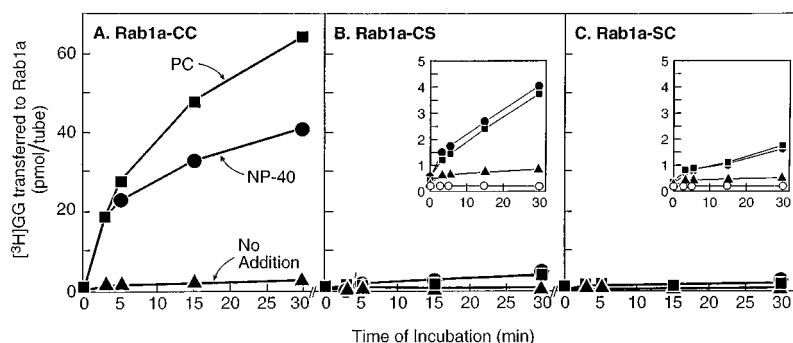


FIG. 1. **Geranylgeranylation of Rab1a wild-type and mutants: effect of detergent and phospholipid.** Each reaction mixture contained, in a final volume of 50  $\mu$ l, 5.5  $\mu$ M [ $^3$ H]GGPP (3,000 dpm/pmol), 1 pmol of RabGGTase, 1.4 pmol of REP-1, and either 2.5  $\mu$ M Rab1a-CC (A), Rab1a-CS (B), Rab1a-SC (C), or Rab1a-SS (insets). The reactions were incubated for the indicated times at 37  $^{\circ}$ C, in the absence (closed triangles) or presence of phosphatidylcholine vesicles (25  $\mu$ g/tube) (closed squares) or Nonidet P-40 (1 mM) (closed circles). After incubation, the amount of [ $^3$ H]GG transferred to Rab1a proteins was determined as described under "Experimental Procedures." Blank values determined at zero time (0.19 pmol/tube) were subtracted from each value. The insets in B and C show the same experiment plotted such that the y axis was rescaled to 5 pmol. A control reaction mixture containing 2.5  $\mu$ M Rab1a-SS (open circles) was included in the plot.

the inset to Fig. 1, B and C, the incorporation of [ $^3$ H]GG was only 4 pmol for Rab1a-CS and 1.8 pmol for Rab1a-SC after 30 min, which represented 3–4-fold stimulation over control reactions in the absence of lipids. This result suggested that mono GG-Rab1a forms a complex with REP and/or RabGGTase that is stable to detergent or phospholipid micelle destabilization, since the difference in -fold stimulation between wild-type and mutant proteins is much greater than can be accounted for by the reduced stoichiometry of geranylgeranylation of the Rab1a mutants (one-half of the wild-type).

If this hypothesis is correct and monoGG-Rabs are titrating essential components of the reaction, it should be possible to inhibit the prenylation of wild-type Rab1a with the Rab1a mutants. We tested this hypothesis in two experiments. In the first experiment, we used a fixed subsaturating amount of Rab1a-CC and added increasing amounts of mutant Rab1a proteins (Fig. 2). When increasing amounts of Rab1a-SS were added, we observed up to a 50% reduction in the amount of [ $^3$ H]GG incorporated into Rab1a-CC when the mutant protein was present at 4-fold higher concentration, consistent with previous results (13). When increasing amounts of Rab1a-CS were added, we observed a more striking inhibition. There was 65% inhibition of Rab1a-CC prenylation when both proteins were present at equimolar concentrations (Fig. 2). This finding is remarkable considering that the mutant protein is nevertheless a substrate for the reaction (Fig. 1B) and that the assay is measuring all of the [ $^3$ H]GG transferred. A similar, but somewhat less potent, effect was observed with Rab1a-SC mutant, while a related protein that is not a Rab GGTase substrate, Ha-Ras, did not inhibit the reaction.

The previous experiment demonstrated that monoGG-Rab is more potent than unprenylated Rab in inhibiting the prenylation of diGG-Rab, suggesting that it forms a more stable complex with the enzymatic components of the reaction. To test this hypothesis, we designed the following experiment (Fig. 3). We initiated the reaction with the mutant Rab proteins in the presence of enzyme (REP/Rab GGTase) so that stable association could occur. After a 10-min incubation, we added an excess of Rab1a-CC, and we measured the stimulation of the rate of the reaction upon this addition, as an indication of the ability of the wild type Rab1a to compete for the available enzyme in the presence of the Rab1a mutants. As shown in Fig. 3, Rab1a-CC efficiently competes with Rab1a-SS for [ $^3$ H]GG transfer (compare open and closed diamond curves), but is much more inefficient in overcoming the inhibition imposed by Rab1a-CS (compare open and closed triangle curves). Again, we obtained an intermediate effect with the Rab1a-SC mutant. These data

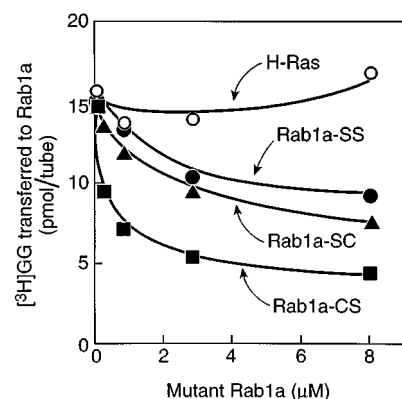


FIG. 2. **Inhibition of Rab1a-CC prenylation by mutant Rab1a proteins.** Each reaction mixture contained, in a final volume of 50  $\mu$ l, 1 mM Nonidet P-40, 5.5  $\mu$ M [ $^3$ H]GGPP (3,000 dpm/pmol), 1 pmol of RabGGTase, 1.4 pmol of REP-1, and 2.5  $\mu$ M Rab1a-CC. The reactions were incubated for 10 min at 37  $^{\circ}$ C, in the presence of the indicated concentrations of Rab1a-CS (closed squares), Rab1a-SC (closed triangles), Rab1a-SS (closed circles), or Ha-Ras (open circles). After incubation, the amount of [ $^3$ H]GG transferred to Rab1a proteins was determined as described under "Experimental Procedures." Blank values determined from parallel reactions in the absence of Rab1a proteins (0.29 pmol/tube) were subtracted from each value.

suggest that monoGG-Rab1a proteins are inhibitory for the wild-type diGG-Rab1a, because they form a relatively stable complex with the enzymatic components of the reaction.

To determine which component or components of the reaction are inactivated by mutant Rab1a proteins, we designed the following experiment (Fig. 4). We incubated Rab1a-CS under standard reactions conditions, with approximately 2 pmol each of RabGGTase and REP-1. After 5 min, Rab1a-CS incorporated 1 pmol of [ $^3$ H]GG. At this point, we made fresh additions to the reaction mixture, either RabGGTase, REP-1, or both. When REP-1 was added, either alone or in combination with RabGGTase, the incorporation of [ $^3$ H]GG into Rab1a-CS increased rapidly and was 2-fold higher than when RabGGTase or buffer control were added. These results suggest that REP-1 is the limiting component of the reaction when Rab1a-CS is added, because REP-1 and Rab1a-CS form a stable complex, as was demonstrated for REP-1 and Rab1a-CC in the absence of detergents (15).

If REP-1 is the limiting component in the reaction, then the amount of product formed should be proportional to the amount of REP-1 present in the reaction. In Fig. 5A, we show that the amount of REP-1 determines the amount of [ $^3$ H]GG incorpo-

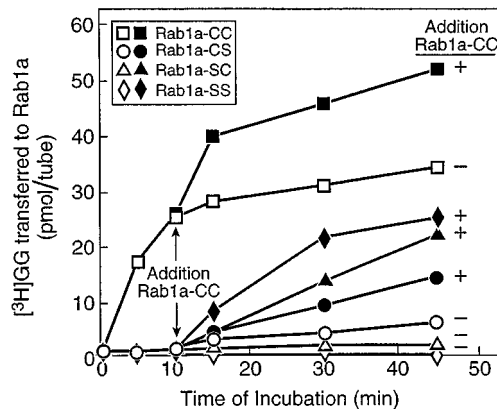


FIG. 3. Geranylgeranylation of Rab1a-CC in the presence of Rab1a mutants. Each reaction mixture, containing in a final volume of 50  $\mu$ l, 1 mM Nonidet P-40, 5.5  $\mu$ M [ $^3$ H]GGPP (3,000 dpm/pmol), 1 pmol of RabGGTase, and 1.4 pmol of REP-1, was incubated at 37  $^{\circ}$ C in the presence of 2.5  $\mu$ M either Rab1a-CC (squares), Rab1a-CS (circles), Rab1a-SS (triangles), or Rab1a-SS (diamonds). At 10-min incubation, 2.5  $\mu$ M Rab1a-CC was added (closed symbols, + curves) and at the indicated times, the amount of [ $^3$ H]GG transferred to Rab1a proteins was determined as described under "Experimental Procedures."

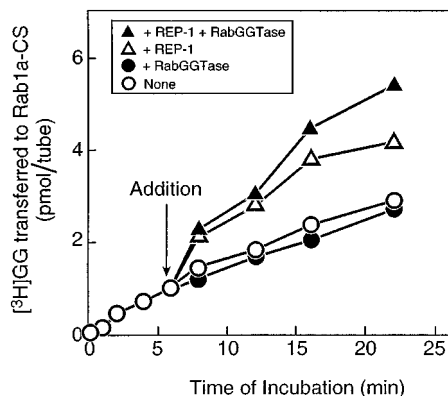


FIG. 4. Geranylgeranylation of Rab1a-CS: stimulation by REP-1. Each reaction mixture contained in a final volume of 50  $\mu$ l, 5.5  $\mu$ M [ $^3$ H]GGPP (3,000 dpm/pmol), 1.8 pmol of RabGGTase, 2.4 pmol of REP-1, and 2.5  $\mu$ M Rab1a-CS. After incubation for 5 min at 37  $^{\circ}$ C, one of the following additions was made: none (open circles), 4.5 pmol of RabGGTase (closed circles), 6 pmol of REP-1 (open triangles), or 4.5 pmol of RabGGTase with 6 pmol of REP-1 (closed triangles). At the indicated times, the amount of [ $^3$ H]GG transferred to Rab1a proteins was determined as described under "Experimental Procedures." Blank values determined at zero time (0.31 pmol/tube) were subtracted from each value.

rated into Rab1a-CS. Increasing amounts of REP-1, up to 10 pmol, resulted in increasing amounts of GG-Rab1a-CS formed, even when limiting amounts of RabGGTase (1 pmol) were present. There is a stoichiometry of approximately 0.5 pmol of [ $^3$ H]GG transferred for every pmol of REP-1, suggesting that one Rab binds a REP-1 dimer. These data also suggest that Rab GGase is not a stable component of the complex, since it can catalyze the reaction when present at much lower levels than REP. In the reverse experiment, increasing amounts of RabGGTase were unable to generate more [ $^3$ H]GG incorporation into Rab1a-CS, when limiting amounts of REP-1 were present (Fig. 5B).

Taken together, these results suggest that monoGG-Rab1a (Rab1a-CS) forms a tight complex with REP-1 that is resistant to dissolution by detergents or phospholipids. To demonstrate this binding directly, we incubated wild-type and mutant Rab1a with GGPP in a prenylation mixture containing RabGGTase, in the presence or absence of REP-1. Then, we loaded the reaction mixtures on Superdex 200 gel filtration

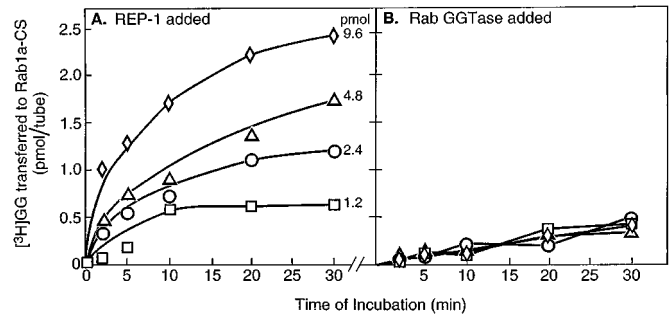
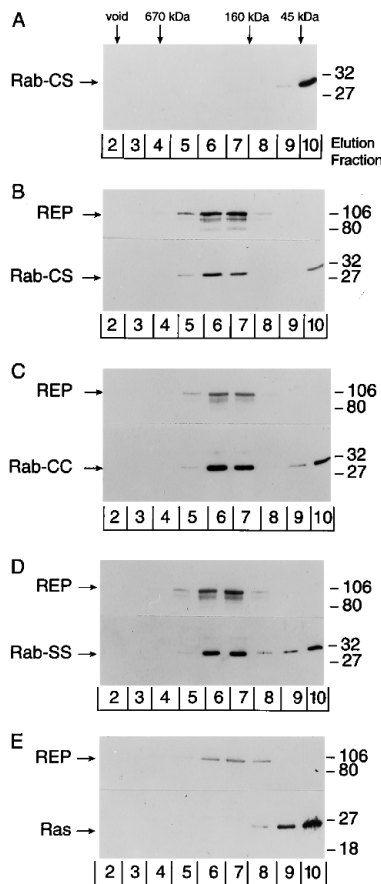


FIG. 5. Geranylgeranylation of Rab1a-CS in the presence of limiting amounts of REP-1 or RabGGTase. Each reaction mixture contained, in a final volume of 50  $\mu$ l, 1 mM Nonidet P-40, 5.5  $\mu$ M [ $^3$ H]GGPP (3,000 dpm/pmol), and 2.5  $\mu$ M Rab1a-CS. In A, the reaction mixture also contained 0.9 pmol of RabGGTase with 1.2 pmol (open squares), 2.4 pmol (open circles), 4.8 pmol (open triangles), or 9.6 pmol (open diamonds) of REP-1, and in B, the reaction mixture also contained 1.2 pmol of REP-1 with 0.9 pmol (open squares), 1.8 pmol (open circles), 4.8 pmol (open triangles), or 7.2 pmol (open diamonds) of RabGGTase. At the indicated times, the amount of [ $^3$ H]GG transferred to Rab1a proteins was determined as described under "Experimental Procedures." Blank values determined at zero time (0.31 pmol/tube) were subtracted from each value.

chromatography and determined the position of elution of REP-1 and Rabs by immunoblot following SDS-gel electrophoresis of the eluted fractions (Fig. 6). In the absence of REP-1, Rab1a-CS eluted from the gel filtration column at fraction 10 (Fig. 6A). This corresponds to the elution position of 30-kDa proteins and is consistent with elution as a monomer. In the presence of REP-1, a significant fraction of Rab1a-CS eluted earlier at fractions 6 and 7 (Fig. 6B). Fractions 6 and 7 also contained REP-1, and they correspond to elution position of 160-kDa proteins. This finding is consistent with the formation of a REP-1-Rab1a complex, since purified and recombinant REP-1 eluted from the same column with apparent molecular mass of 140 kDa (Ref. 13 and this study not shown). Rab1a-CC and Rab1a-SS also formed complexes with REP-1, as determined by co-elution upon gel filtration chromatography under the same conditions described above for Rab1a-CS (Fig. 6, C and D), but Ha-Ras did not (Fig. 6E).

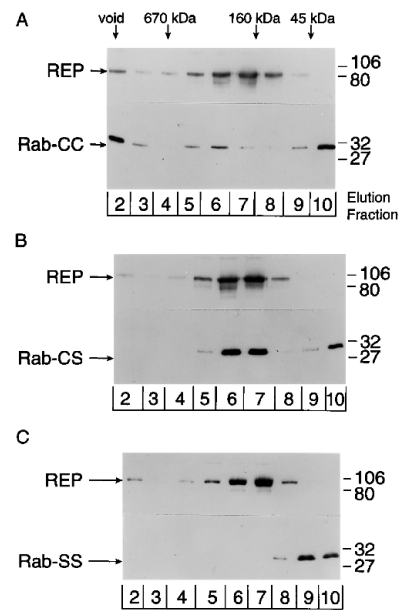
To demonstrate directly that REP-1 and monoGG-Rab1a formed a complex that was resistant to destabilization by phospholipids, we performed *in vitro* prenylation reactions as above in the presence of PC vesicles and subjected them to gel filtration chromatography (Fig. 7). When loaded on Superdex 200, PC vesicles eluted in the void of the column (fraction 2), clearly separated from the REP-Rab complex (fractions 6 and 7). In the presence of REP-1, wild-type Rab1a-CC now eluted at three different positions: in fraction 2 co-eluting with phosphatidylcholine vesicles, in fraction 6 co-migrating with REP-1, and in fraction 10 as a monomer (Fig. 7A, compare with Fig. 6C). Strikingly, under the same conditions Rab1a-CS was not found in fraction 2, but only in fractions 6–7 and 10, as was observed in the absence of phosphatidylcholine vesicles (Fig. 7B, compare with Fig. 6B). Rab1a-SS was present mostly in fraction 10, and very little was found co-migrating with REP-1 (Fig. 7C, compare with Fig. 6D). In the absence of REP-1, wild-type and mutant Rab1a proteins did not bind PC vesicles and eluted in fractions 9 and 10 (not shown). These data are consistent with the hypothesis that REP-1-monoGG-Rab1a complex is stable to disruption by phospholipids. The observation that the REP-Rab1a-SS complex is unstable in the presence of PC vesicles is surprising and may reflect a less stable association of REP-unprenylated Rab *versus* REP-prenylated Rab complexes. While the REP-Rab interaction remains to be defined in more detail, the available data suggest that there are at least two



**FIG. 6. Detection of REP-Rab1a complex by gel filtration chromatography.** Each reaction mixture contained, in a final volume of 50  $\mu$ l, 5  $\mu$ M GGPP, 0.5  $\mu$ M RabGGTase, and either 2  $\mu$ M Rab1a-CS (A and B), Rab1a-CC (C), Rab1a-SS (D), or Ha-Ras (E), in the absence (A) or presence of 2  $\mu$ M REP-1 (B–E). After incubation for 15 min at 37  $^{\circ}$ C, each sample was loaded onto a Superdex 200 3.2/30 column equilibrated and run as described under “Experimental Procedures.” An aliquot (30  $\mu$ l) of elution fractions 2–10 was subjected to SDS-gel electrophoresis on 12.5% minigels, the proteins transferred to nitrocellulose and detected with either J905 anti-REP-1 antibody (0.03  $\mu$ g/ml), D576 anti-Rab1a antibody (2.5  $\mu$ g/ml), or anti-Ha-Ras antibody (0.1  $\mu$ g/ml), as indicated, using the ECL system. The column was calibrated with thyroglobulin (670 kDa), aldolase (160 kDa), and ovalbumin (45 kDa), and vertical arrows on A denote the position of elution of the markers. Horizontal arrows denote the position of migration of REP, Rab1a, and Ha-Ras (left side) and the indicated molecular mass markers (right side) upon SDS-gel electrophoresis.

binding sites, one involving the prenyl groups and the COOH-terminal region and another involving one or more upstream Rab sequences (13, 18, 26, 27). This result suggests that the geranylgeranyl moiety is an important determinant of the REP-Rab interaction.

In order to study the stoichiometry of the REP-Rab complex, we performed glycerol gradient ultracentrifugation. We subjected reaction mixtures containing different combinations of REP, Rab GGTase, and wild-type and mutant Rab1a and determined the position of elution of these proteins by immunoblot following SDS-gel electrophoresis of the eluted fractions (Fig. 8). When subjected to density ultracentrifugation, REP migrated to fractions 5 and 6, consistent with a 60-kDa protein (Fig. 8A), Rab GGTase migrated to fraction 7, consistent with a 80-kDa protein (Fig. 8B) and Rab1a wild-type and mutants peaked at fraction 3, consistent with 30-kDa proteins (not shown). When Rab GGTase and Rab1a-CS were incubated together and applied on the glycerol gradient, no changes in migration were observed, suggesting that these proteins did not form a complex (Fig. 8C). However when Rab1a-CS was



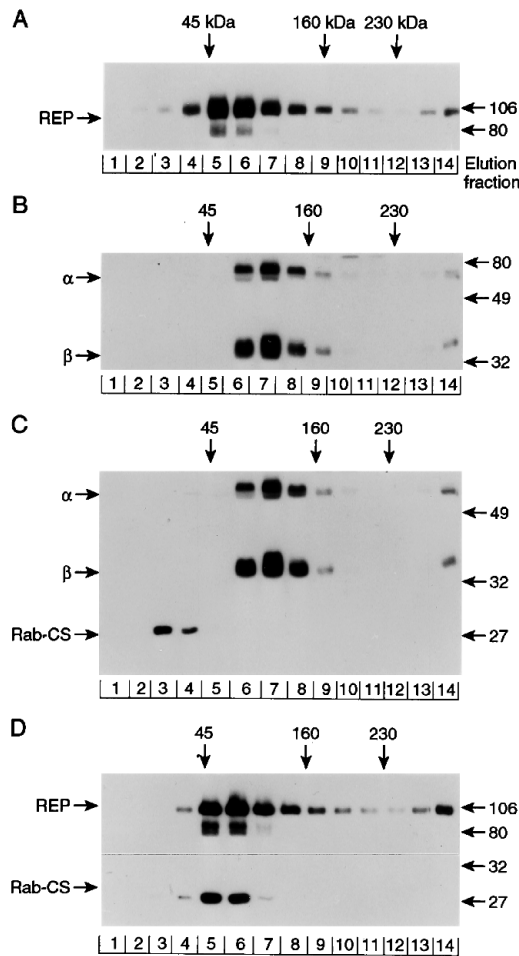
**FIG. 7. Gel filtration chromatography of Rab1a proteins after prenylation reaction in the presence of phospholipids.** Each reaction contained, in a final volume of 50  $\mu$ l, 25  $\mu$ g phosphatidylcholine vesicles, 5  $\mu$ M GGPP, 0.5  $\mu$ M RabGGTase, 2  $\mu$ M REP-1, and either 2  $\mu$ M Rab1a-CC (A), Rab1a-CS (B), or Rab1a-SS (C). After incubation for 15 min at 37  $^{\circ}$ C, each sample was loaded onto a Superdex 200 3.2/30 column equilibrated and run as described under “Experimental Procedures.” An aliquot (30  $\mu$ l) of elution fractions 2–10 was subjected to SDS-gel electrophoresis on 12.5% minigels, the proteins transferred to nitrocellulose and detected with either J905 anti-REP-1 antibody (0.03  $\mu$ g/ml) or D576 anti-Rab1a antibody (2.5  $\mu$ g/ml), as indicated, using the ECL system. The column was calibrated with thyroglobulin (670 kDa), aldolase (160 kDa), and ovalbumin (45 kDa), and vertical arrows on A denote the position of elution of the markers. Horizontal arrows denote the position of migration of REP, and Rab1a (left side), and the indicated molecular mass markers (right side) upon SDS-gel electrophoresis.

incubated with REP, its migration did shift significantly to fractions 5 and 6, co-migrating with REP (Fig. 8D). Similar results were obtained with Rab1a-CC and Rab1a-SS (not shown). We conclude that REP behaves as a monomeric protein upon density ultracentrifugation and that the migration of the REP-Rab complex is most consistent with a 1:1 stoichiometry.

To analyze the stoichiometry of the complex after prenylation, we included Rab GGTase in the reaction mixture and subjected the reaction mixtures to density ultracentrifugation. Under the same conditions described above, the migration of monoGG-Rab or diGG-Rab shifted and peaked in fraction 8 (Fig. 9, A and B). It is noticeable that REP migration is also significantly shifted toward later fractions. Under the same conditions, Rab1a-SS migrated to fractions 5 and 6 (Fig. 9C), the migration observed for unprenylated Rab1a in the presence of REP-1. We conclude that prenylation induces a change in the stoichiometry of the REP-Rab complex, likely to a dimeric complex.

#### DISCUSSION

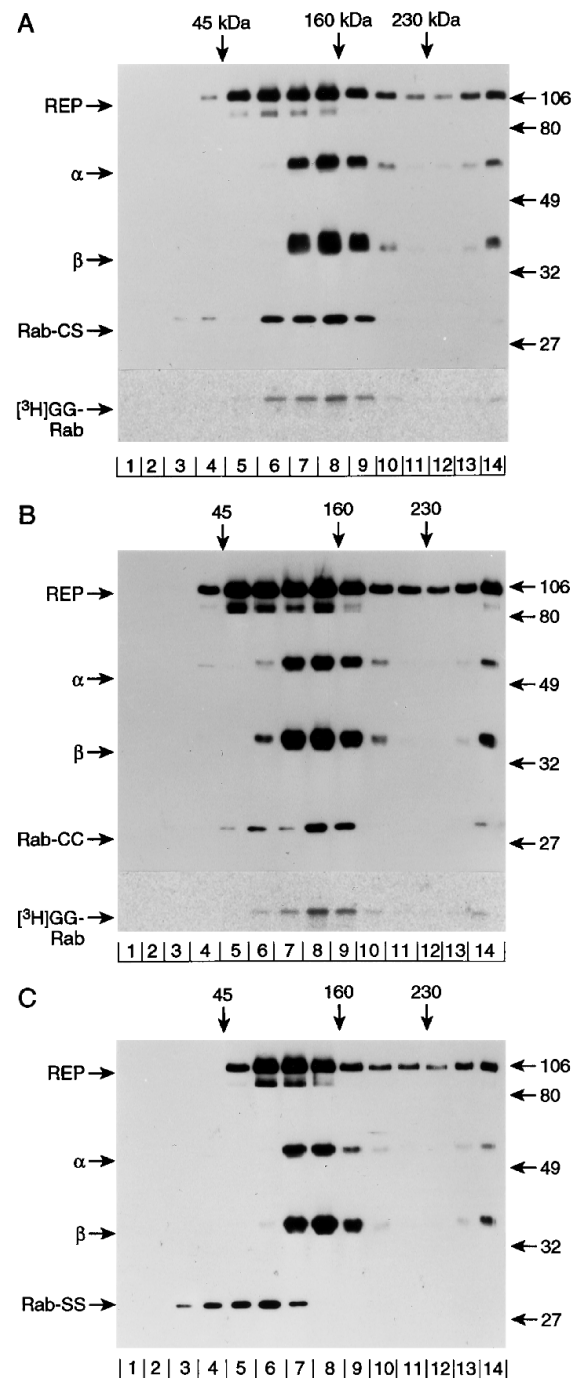
A possible mechanism by which Rab proteins are digeranylgeranylated is suggested by the current studies. We propose that each geranylgeranyl addition is an independent reaction that leads to the production of monoGG-Rab and diGG-Rab, respectively. However, the monoGG-Rab product does not accumulate, because it forms a complex with REP that is resistant to disruption by detergents and phospholipids, whereas the REP-Rab or the REP-diGG-Rab complex is not. The stability of the REP-monoGG-Rab complex prevents monoGG-Rab from dissociating from REP prior to the second geranylgeranylation



**FIG. 8. Glycerol Gradient Ultracentrifugation of REP, Rab GGTase and Rab1a.** Reaction mixtures contained 50 mM sodium Hepes (pH 7.2), 5 mM  $MgCl_2$ , 1 mM dithiothreitol, 10  $\mu M$  unlabeled GGPP in a final volume of 50  $\mu l$ , in the presence of 4  $\mu M$  REP-1 (A), 4  $\mu M$  RabGGTase (B), 4  $\mu M$  Rab1a-CS and 4  $\mu M$  RabGGTase (C), or 4  $\mu M$  Rab1a-CS and 4  $\mu M$  REP-1 (D). After incubation for 15 min at 37  $^{\circ}C$ , reaction mixtures were loaded onto 4 ml 7.5–30% glycerol gradients in 20 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol and spun as described under “Experimental Procedures.” An aliquot of each fraction (30  $\mu l$ ) was subjected to SDS-gel electrophoresis on 12.5% minigels and the proteins transferred to nitrocellulose and detected with either J905 anti-REP-1 antibody (0.03  $\mu g/ml$ ), H492 anti-Rab GGTase antiserum (1:5,000 dilution), or D576 anti-Rab1a antibody (8.6  $\mu g/ml$ ), as indicated, using the ECL system. Each gradient was calibrated with internal standards, catalase (230 kDa), aldolase (160 kDa), and ovalbumin (45 kDa), and vertical arrows on each panel denote the position of elution of the markers. Horizontal arrows denote the position of migration of REP, Rab GGTase  $\alpha$ - and  $\beta$ -subunits, and Rab1a (left side) and the indicated molecular mass markers (right side) upon SDS-gel electrophoresis.

reaction, ensuring efficient digeranylgeranylation of Rab substrates.

In the present work, we confirm and extend studies previously published. First, we provide a possible mechanism for the inefficient prenylation of mutant Rabs that can only accept one GG group, as reported *in vitro* (10, 22) or *in vivo* (28, 29). We show that the prenylation of Rab1a mutants is strictly dependent on, and stoichiometric with, the levels of REP present in the reaction. Furthermore, we demonstrate that REP and Rab associate in a stable complex, confirming previous observations (15). Rab GGTase appears not to be a stable component of the monoGG-REP complex. Rab GGTase is able to catalyze GG transfer even when present in much lower amounts than REP. Also, the position of co-elution of Rab1a and REP on gel filtration chromatography or glycerol gradient ultracentrifugation is



**FIG. 9. Stoichiometry of REP-Rab1a complex by glycerol gradient ultracentrifugation.** Reaction mixtures contained 50 mM sodium Hepes (pH 7.2), 5 mM  $MgCl_2$ , 1 mM dithiothreitol, 10.5  $\mu M$  [ $^3H$ ] GGPP, 4  $\mu M$  REP-1, and 4  $\mu M$  RabGGTase in a final volume of 50  $\mu l$ , in the presence of 4  $\mu M$  Rab1a-CS (A), 4  $\mu M$  Rab1a-CC (B), or 4  $\mu M$  Rab1a-SS (C). After incubation for 15 min at 37  $^{\circ}C$ , reaction mixtures were loaded onto 4 ml 7.5–30% glycerol gradients in 20 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol and spun as described under “Experimental Procedures.” An aliquot of each fraction (30  $\mu l$ ) was subjected to SDS-gel electrophoresis on 12.5% minigels and the proteins transferred to nitrocellulose and detected with either J905 anti-REP-1 antibody (0.03  $\mu g/ml$ ), H492 anti-Rab GGTase antiserum (1: 5,000 dilution), or D576 anti-Rab1a antibody (8.6  $\mu g/ml$ ), as indicated, using the ECL system. The same filters were exposed to a PhosphorImager plate for 18 h to visualize the [ $^3H$ ]GG-Rab1a protein. Each gradient was calibrated with internal standards, catalase (230 kDa), aldolase (160 kDa), and ovalbumin (45 kDa), and vertical arrows on each panel denote the position of elution of the markers. Horizontal arrows denote the position of migration of REP, Rab GGTase  $\alpha$ - and  $\beta$ -subunits, Rab1a and [ $^3H$ ]GG-Rab1a (left side), and the indicated molecular mass markers (right side) upon SDS-gel electrophoresis.



inconsistent with the presence of Rab GGTase as part of the complex. Therefore, we suggest that each prenylation reaction is an independent event that may involve dissociation-reassociation of Rab GGTase. Second, we provide a possible mechanism for the absence of accumulation of monoGG-Rab1a *in vitro*, as reported by Farnsworth *et al.* (21). As discussed above, we show the formation of a stable REP-monoGG-Rab complex that may prevent the dissociation of monoGG-Rab from REP until the second GG addition occurs.

Our results suggest that there is not an absolute order of addition of GG groups to the two adjacent cysteine residues in Rab1a, since either Rab1a mutant (CS or SC) can accept a prenyl group. However, the amino-terminal cysteine is somewhat preferred, since prenylation of that cysteine is more efficient than the carboxyl-terminal one. Also, Rab1a-CS is a more potent inhibitor of wild-type Rab1a prenylation (that is, REP recycling), than Rab1a-SC. Given the inherent flexibility of Rab GGTase, which is able to prenylate adjacent cysteines with or without a spacer amino acid in between the cysteine residues, it is possible that the digeranylgeranylation of Rab1a-CC *in vivo* is actually ordered. Further experiments will be needed to clarify this issue.

We obtained essentially the same results in identical biochemical experiments where we used wild-type and single cysteine mutants of Rab3a, a substrate that contains a XCXC motif rather than XXCC present in Rab1a.<sup>2</sup> This suggests that the mechanism of digeranylgeranylation is similar for all Rabs and involves a lipid-resistant transitional complex. However, several Rabs, including Rab8 and Rab13, contain only one cysteine residue within a carboxyl-terminal CAAX motif, where A is an aliphatic residue. Inasmuch as GG transfer to these Rabs could theoretically be catalyzed by either Rab GGTase or CAAX GGTase, it remains to be established which enzyme is actually responsible for the reaction under steady-state *in vivo* conditions.

The gel filtration experiments presented here with the recombinant protein (Figs. 6 and 7) and previously published with the purified protein (13) showed that REP elutes as a dimer. However, the behavior of a protein upon gel filtration is proportional to its Stokes radius rather than its molecular weight, and for nonglobular proteins, those two parameters are quite different. We attempted to dissociate the putative dimer by denaturation, but we were unable to shift REP migration on gel filtration chromatography from 140 to 70 kDa (not shown). It is noteworthy that REP migrates anomalously upon SDS-gel electrophoresis (to 95 kDa rather than its predicted molecular mass of 73 kDa) for unknown reasons. For these reasons, we used a more reliable method, namely density ultracentrifugation, to study the stoichiometry of the REP-Rab complex. Glycerol gradient ultracentrifugation suggested that REP is a monomer. The discrepancy in apparent molecular mass of REP-1 by gel filtration chromatography and density ultracentrifugation suggests that REP-1 is an elongated molecule with a large Stokes radius. The stoichiometry of the REP-Rab complex prior

to prenylation appears to be 1:1 (Figs. 8 and 9). However, upon prenylation we observed a significant shift in the migration of both REP-1 and Rab1a, consistent with the formation of a 2:2 or a 2:1 complex. We cannot distinguish between these two possibilities with the present experiments. We also cannot rule out that the shift is due to the binding of Rab GGTase to the REP-Rab complex, but this possibility is unlikely for the reasons detailed above. The significance of this shift in complex stoichiometry is unclear but may be important for the next step of the reaction, the REP-mediated delivery of diGG-Rab to intracellular membranes.

The issues raised by this work may be addressed with future studies detailing the kinetics of the prenylation reaction, biochemical studies on REP and the REP-Rab complex, and the role of REP in the delivery of prenylated Rabs to intracellular membranes.

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<sup>2</sup> F. Shen and M. C. Seabra, unpublished observations.