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Biochemistry

Rab geranylgeranyl transferase catalyzes the geranylgeranylation of adjacent cysteines in the small GTPases Rab1A, Rab3A, and Rab5A

(Ras/mass spectrometry/HPLC/membranes/isoprenoid)

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ABSTRACT **Rab proteins are Ras-related small GTPases that are geranylgeranylated on cysteine residues located at or near their C termini. They differ from other geranylgeranylated small GTPases in several important respects. (*i*) Most Rab proteins contain two adjacent cysteine residues within one of the following C-terminal sequence motifs: -XXCC, -XCXC, or -CCXX; (*ii*) a Rab protein that ends in a -XCXC motif has been shown to be geranylgeranylated on both adjacent cysteine residues; and (*iii*) Rab proteins are substrates of a unique Rab-specific geranylgeranyltransferase. Whether this enzyme catalyzes the geranylgeranylation of both cysteines is unknown. We addressed this question by direct structural analysis of *in vitro* prenylated proteins. We incubated recombinant Rab geranylgeranyltransferase, Rab escort protein, and [1-^{3}H]geranylgeranyl pyrophosphate with recombinant wild-type Rab1A (-XXCC), Rab3A (-XCXC), or Rab5A (-CCXX) and treated each labeled protein with trypsin. We then analyzed the resulting peptides by HPLC and electrospray mass spectrometry and found that for each protein both C-terminal adjacent cysteines were geranylgeranylated. These results indicate that Rab geranylgeranyltransferase/Rab escort protein catalyzes the geranylgeranylation of both cysteines in Rab proteins with three distinct C-terminal motifs and suggest that other Rab proteins with these motifs may be similarly modified.**

Rab proteins are Ras-related GTPases that mediate vesicle transport in the secretory and endocytotic pathways. Over 30 different Rab proteins have been identified, each one localized to a specific intracellular organelle. It has been proposed that Rab proteins cycle between soluble and membrane-bound forms as part of their regulatory role in protein trafficking (1, 2). Membrane localization is absolutely dependent on a posttranslational modification that occurs at the C terminus whereby a geranylgeranyl (GG) moiety is attached via a thioether bond to cysteine residues (3–5). Most Rab proteins have C termini that end in -XXCC (35%), -XCXC (37%), or -CCXX (15%). Two other motifs have also been identified, -CCXXX (8%) and -CXXX (5%). These latter motifs are similar to those present in proteins of the Ras and Rho subfamilies but contain atypical amino acid residues in the last and penultimate positions. Clearly, the presence of two adjacent cysteines is common to the C termini of most Rab proteins, and available evidence from labeling experiments suggests that at least one of these cysteines is geranylgeranylated (3, 5–7). However, complete structural studies have been done on only one native Rab protein. Mass spectrometry was used to demonstrate that the C terminus of brain Rab3A (-XCXC) is geranylgeranylated on each of the adjacent cysteine residues and that it is also carboxylmethylesterified (8). Whether other native Rab proteins are geranylgeranylated on adjacent cysteine residues is unknown.

Most Rab proteins appear to be substrates of the same Rab-specific geranylgeranyltransferase (9). The enzymatic reaction requires two components, a catalytic heterodimer designated Rab geranylgeranyltransferase (Rab GGTase) and a Rab escort protein (REP) (9–11). The catalytic component binds all-*trans*-GG pyrophosphate (GGPP) and subsequently transfers the GG moiety to a protein substrate (10). Two distinct REP proteins, REP-1 and REP-2, have been identified. REP-1 is the product of the choroideremia gene on the X chromosome, and its mutation results in retinal degeneration (10–12). Choroideremic lymphoblasts are deficient in Rab GGTase/REP-1 activity, which can be corrected *in vitro* by rat REP-1 (13). REP-2, an intronless gene on chromosome 1, can support the geranylgeranylation of most Rab proteins *in vitro* and therefore may substitute for REP-1 function in all tissues except the retina (14). REP proteins promote the geranylgeranylation reaction in at least two different ways: (*i*) by binding the apoprotein substrate and (*ii*) by forming a complex with Rab GGTase. It is not known whether the complex catalyzes the transfer of only one GG group per Rab protein or whether it can catalyze the transfer of a GG group to each of the adjacent cysteines of the C-terminal domain. In the present study, we addressed this question by incubating recombinant Rab GGTase/REP-1 with [1-^{3}H]GGPP and Rab proteins representative of three distinct C-terminal sequence motifs, -XXCC, -XCXC, and -CCXX. Upon examination of radiolabeled tryptic peptides by HPLC analysis and mass spectrometry, we found that Rab GGTase/REP-1 catalyzes the geranylgeranylation of both adjacent cysteines in Rab1A, Rab3A, and Rab5A.

MATERIALS AND METHODS

Materials. HPLC grade solvents (Baker), Nano Pure water (Barnstead), and sequencing grade HCl and CF_3COOH (Pierce) were used for all HPLC and mass spectral analyses. Unless otherwise specified, all other reagents were American Chemical Society reagent grade.

***In Vitro* Prenylation of Rab Proteins by Rab GGTase.** Recombinant histidine-tagged Rab proteins were produced and purified as described (14). Sequencing of the cDNA encoding human Rab5A revealed two discrepancies with the published sequence (15). Codon 81 reads CGA instead of GGA and codon 197 reads AGA instead of GGA. Both

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Abbreviations: GG, geranylgeranyl; Rab GGTase, Rab geranylgeranyltransferase; REP, Rab escort protein; GGPP, geranylgeranyl pyrophosphate.

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changes result in arginine for glycine substitutions. The canine version of the same gene also contains these arginines (16), suggesting that the original sequence contained two sequencing errors.

Recombinant histidine-tagged REP-1 and recombinant Rab GGTase produced in insect cells were purified to >90% purity as described (14) and were used in reaction mixtures containing the following concentrations of components in a final volume of 1000 μl: 50 mM NaHepes (pH 7.2); 1 mM dithiothreitol; 5 mM $MgCl_2$; 1 mM Nonidet P-40; 1 μM [1-^{3}H]GGPP (33,000 dpm/pmol; American Radiolabeled Chemicals, St. Louis); 3 μM Rab1A, Rab3A, or Rab5A; 1 μg of REP-1; and 1 μg of Rab GGTase. Protein concentrations were determined by the method of Bradford as modified (14). Some experiments were done in the absence of detergent and therefore permitted only a single round of catalysis. [Rab1A forms a tight complex with REP-1 that remains stable after geranylgeranylation unless detergent micelles are present that can act as prenylated protein acceptors (10).] After incubation at 37°C for 3 h, 100 μl of a buffer containing 50 mM $MgCl_2$, 10 mM Na_2EDTA, and 300 mM Tris·HCl (pH 8.0) were added to each sample, followed by the addition of 6 vol of −20°C acetone. The mixtures were quickly mixed in a Vortex, placed at −20°C for 1 h, and then centrifuged at 16,000 × g for 20 min at 4°C in an Eppendorf microcentrifuge. The resulting pellets were stored at −20°C.

Protein and Peptide Analysis. The protein pellets were proteolyzed as follows: (*i*) 50 μl of 4% CHAPS {3-[(3 cholamidopropyl)dimethylammonio]-1-propanesulfonate} (Pierce) in proteolysis buffer (100 mM ammonium bicarbonate, pH 8.2) was added to each pellet, and the samples were Vortex mixed; (*ii*) 50 μl of 8 M urea, Ultrapure grade (Baker), in proteolysis buffer was added and the samples were Vortex mixed again; (*iii*) 1.0 μl of 100 mM dithiothreitol and 1.0 μl of 500 mM $CaCl_2$ were added, followed by 15 μl of proteolysis buffer containing 4 mg of trypsin per ml that had been treated with TPCK (L-(1-tosylamido-2-phenylethyl chloromethyl ketone) (Worthington); (*iv*) samples were incubated for 24 h at 37°C. Each sample was then analyzed directly by C_{18} reverse-phase HPLC (8), and the radioactivity in 25-μl aliquots of each 0.5-ml fraction was analyzed by scintillation counting. Fractions containing peaks of radioactivity were pooled, dried under vacuum in a SpeedVac concentrator (Savant), resolubilized in 6 mM HCl/methanol, and analyzed with an electrospray mass spectrometer (Sciex, Toronto) as described (17, 18).

Synthetic peptides used to prepare geranylgeranylated peptide standards were synthesized by using fluoren-9-ylmethoxycarbonyl-protected amino acids on an Applied Biosystems synthesizer according to the manufacturer's procedures (Table 1). After the peptides were chemically geranylgeranylated with unlabeled GG chloride and purified by C_{18} reverse-phase HPLC (8), their structure was verified by electrospray mass spectrometry. Under the ionization conditions used here, the strongest peptide signal came from the $(m + 2H)^{2+}$ ions. Since the spectrometer separates and detects ions on the basis of mass/charge ratio, the value reported represents $(m + zH)/z$ where z is the number of charges ($z = 2$ in the present case).

Table 1. Selected C-terminal tryptic peptide sequences for Rab1A, Rab3A, and Rab5A

Protein	Sequence
Rab1A	
14-mer	-IQSTPVKQSGGGCC
18-mer	-SNVKIQSTPVKQSGGGCC
Rab3A	
18-mer	-QGPQLSDQQVPPHQDCAC
Rab5A	
18-mer	-GVDLTEPTQPTRNQCCSN
20-mer	-GRGVDLTEPTQPTRNQCCSN

RESULTS

HPLC Analysis of [1-^{3}H]GG-Labeled Rab Tryptic Peptides. To determine whether Rab GGTase/REP-1 can catalyze the transfer of a GG group to each of the adjacent C-terminal domain cysteines of Rab proteins, we incubated recombinant Rab GGTase/REP-1 and [1-^{3}H]GGPP with a wild-type recombinant form of Rab1A, Rab3A, or Rab5A; treated the labeled product with trypsin; and analyzed the resulting

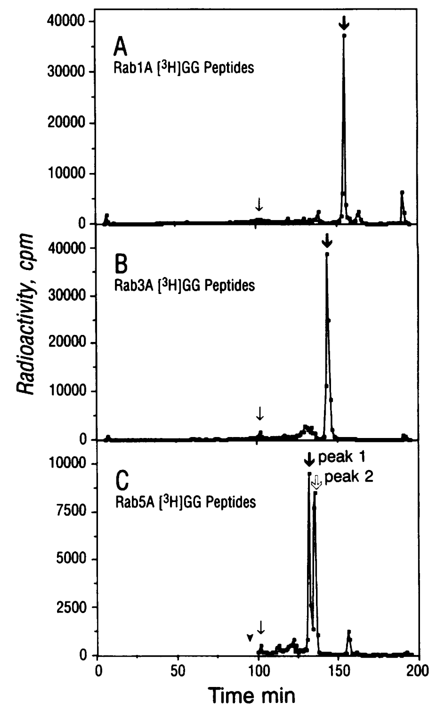

FIG. 1. C_{18} reverse-phase HPLC analysis of [1-^{3}H]GG-labeled Rab tryptic peptides. Rab1A, Rab3A, and Rab5A were incubated separately with [1-^{3}H]GGPP and Rab GGTase/REP-1 and digested with trypsin. Then the respective digestion mixtures were chromatographed by reverse-phase HPLC, and the radioactivity of each fraction was plotted versus elution time. For comparison, five different C-terminal peptides based on several potential tryptic cleavage sites were synthesized (see Table 1), chemically prenylated to form mono- and digeranylgeranylated peptide standards, and analyzed by HPLC. (*A*) Solid arrow indicates elution time of the synthetic, digeranylgeranylated Rab1A 14-mer. Thin arrow indicates elution time of the corresponding monoprenylated peptide. Elution time of the digeranylgeranylated Rab1A 18-mer was 141 min (data not shown). (*B*) Solid arrow indicates elution time of the synthetic, digeranylgeranylated Rab3A 18-mer. Thin arrow indicates elution time of the corresponding monoprenylated peptide. (*C*) Solid and open arrows, respectively, indicate elution times of the synthetic, digeranylgeranylated Rab5A 20-mer and the digeranylgeranylated Rab5A 18-mer. Thin arrow and arrowhead, respectively, indicate retention times of the corresponding monoprenylated 20-mer and 18-mer. Fractions 1–100 from this chromatogram were not analyzed for radioactivity. However, in a companion analysis using a different column, no counts above background were detected in this region except for the flow-through peak—i.e., fraction 7. Trypsin-treated Rab1A and Rab5A might have been expected to yield a labeled C-terminal 7-mer and 6-mer, respectively, in addition to the peptides indicated in Table 1. However, no major radiolabeled peaks were observed in the expected region of a diprenylated 7-mer or 6-mer (unpublished results). Recovery of total applied radioactivity from the column was 82%, 78%, and 75%, respectively, for the Rab1A, Rab3A, and Rab5A digestions shown. Elution of synthetic standards was monitored at 214 nm.

tryptic peptides by C_{18} reverse-phase HPLC and mass spectrometry (see *Materials and Methods*). Using this approach we found that the tryptic digests of [1-^{3}H]GG-labeled Rab1A and Rab3A each yielded a single major peak of radioactivity that was strongly retained by the C_{18} column and several very minor peaks of radioactivity that eluted earlier (Fig. 1 *A* and *B*). Furthermore, in each case the recovery of total label was high (82% and 78%, respectively). The tryptic digest of [1-^{3}H]GG-labeled Rab5A yielded two major peaks of radioactivity that were strongly retained by the C_{18} column (Fig. 1*C*, peaks 1 and 2) as well as minor peaks that eluted earlier. Subsequent experiments showed that the two major peaks, whose retention times differed by only 4 min, corresponded to peptides that had been formed by tryptic cleavage of the same geranylgeranylated protein product at two different sites (see below). Thus, for each of the three Rab substrates the Rab GGTase/REP-1 reaction resulted in the formation of a single major geranylgeranylated protein product.

To determine whether the labeled tryptic peptides from Rab1A, Rab3A, and Rab5A were mono- or digeranylgeranylated, we synthesized five different C-terminal peptides based on several potential tryptic cleavage sites (Table 1), chemically prenylated them to form mono- or digeranylgeranylated peptide standards, and analyzed them on the same reverse-phase column used for labeled tryptic digest analysis. In each case a digeranylgeranylated standard cochromatographed with the corresponding major radiolabeled peak or peaks (Fig. 1, solid and open arrows), whereas the monogeranylgeranylated standards eluted earlier (Fig. 1, small arrows and arrowhead). For each of the three proteins studied, >95% of the Rab GGTase/REP-1 product formed was digeranylgeranylated, whereas only a very small amount of monogeranylgeranylated product accumulated even though each assay was performed in the presence of detergent—i.e., under conditions that permit multiple rounds of catalysis (10).

In separate experiments, we labeled Rab1A or Rab3A in the absence of detergent—i.e., under single catalysis conditions—and analyzed the tryptic peptide products as described above. With this approach, labeled digests of Rab1A and Rab3A each yielded two peaks of radioactivity (data not shown). A large peak coeluted with the corresponding digeranylgeranylated synthetic peptide, and a smaller one coeluted with the corresponding monogeranylgeranylated synthetic peptide. Under these reaction conditions, ≈90% of the Rab GGTase/REP-1 product formed was digeranylgeranylated, whereas the monogeranylgeranylated species represented ≈10% of the total product formed. These results suggest that under some conditions it may be possible to trap monogeranylgeranylated protein intermediates.

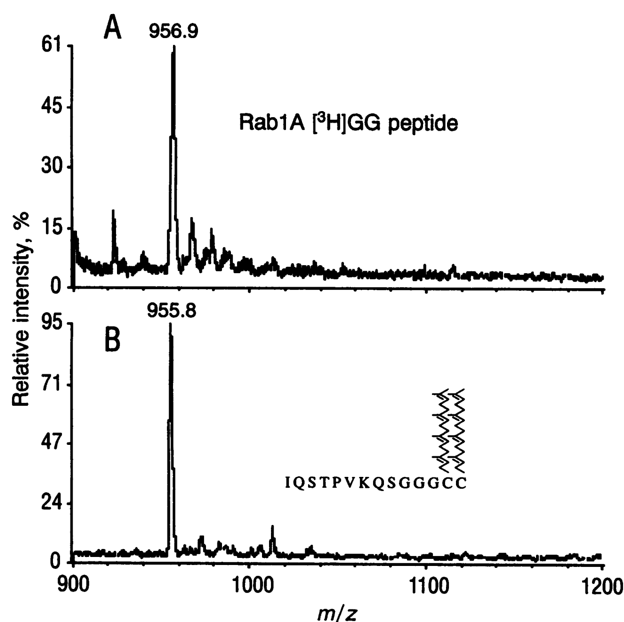

FIG. 2. Electrospray mass spectral analysis of the [1-^{3}H]GG-labeled Rab1A tryptic peptide and corresponding synthetic standard. (*A*) HPLC fractions corresponding to the peak of radioactivity in Fig. 1*A* were pooled and analyzed by electrospray mass spectrometry. (*B*) Corresponding synthetic standard was analyzed for comparison. Since the doubly protonated peptides gave the strongest detector signals, only the $(m + 2H)^{2+}$ ion spectra are shown.

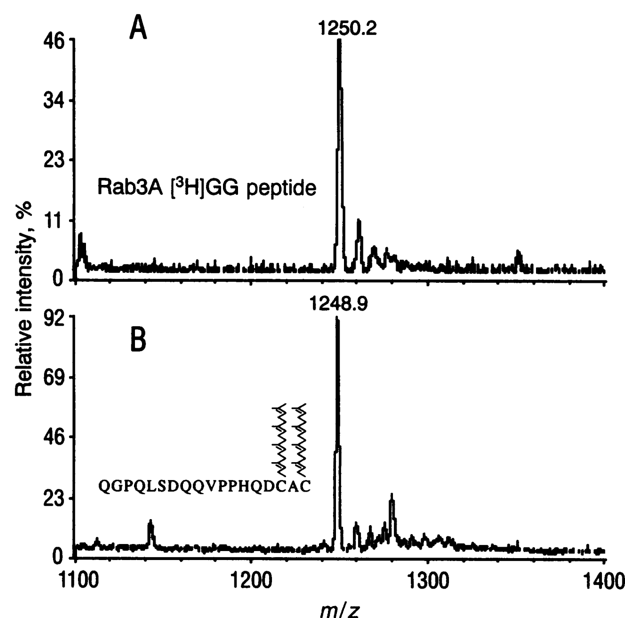

FIG. 3. Electrospray mass spectral analysis of the [1-^{3}H]GG-labeled Rab3A tryptic peptide and corresponding synthetic standard. (*A*) HPLC fractions corresponding to the peak of radioactivity in Fig. 1*B* were pooled and analyzed by electrospray mass spectrometry. (*B*) Corresponding synthetic standard was analyzed for comparison.

Mass Spectrometry of the C-Terminal Rab Tryptic Peptides. To obtain unequivocal evidence for digeranylgeranylation of the C termini of Rab1A, Rab3A, and Rab5A, we performed mass spectral analysis of the major radiolabeled peptide peaks and their corresponding standards obtained from the HPLC analyses. The mass spectra of the major labeled peptides were nearly identical with those of their standards (Figs. 2–4), and the molecular weights of the labeled peptides, determined from the *m*/*z* values of the $(m + 2H)^{2+}$ ion (see *Materials and Methods*), were all ≈2 mass units higher than those of their standards (Table 2). This 2-mass-unit difference between each labeled peptide and its corresponding unlabeled synthetic standard was due to the presence of ≈1 ^{3}H atom per peptide because the [1-^{3}H]GGPP used in these studies had a high specific activity (15 Ci/mmol, half of theoretical maximum; 1 Ci = 37 GBq). Therefore, the results of the peptide mass spectrometry provide definitive evidence that Rab GGTase/REP-1 transfers GG groups to both adjacent C-terminal domain cysteine residues of Rab1A, Rab3A, and Rab5A.

DISCUSSION

The fact that Rab GGTase/REP-1 can catalyze the digeranylgeranylation of these proteins probably accounts for the results of several previous studies. Thus, as mentioned earlier, Farnsworth *et al.* (8) showed that brain Rab3A is digeranylgeranylated. Andres *et al.* (10) incubated Rab GGTase/REP-1 with [1-^{3}H]GGPP and recombinant Rab1A and found that [1-^{3}H]GG was incorporated into the protein with

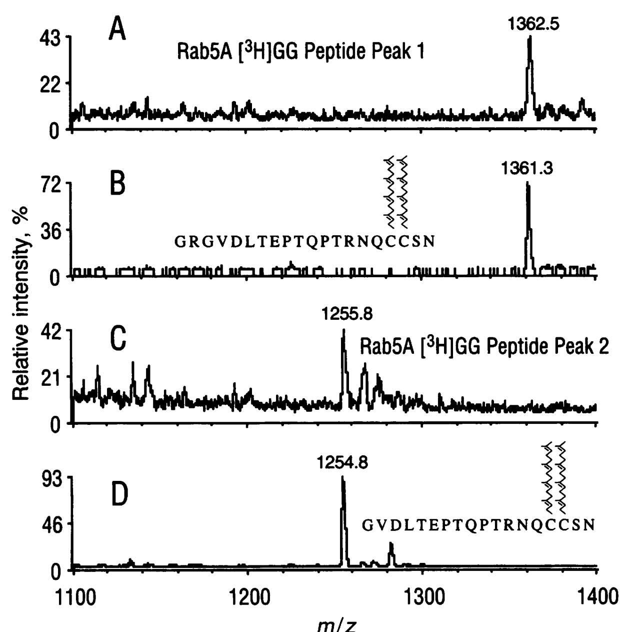

FIG. 4. Electrospray mass spectral analysis of the [1-^{3}H]GG-labeled Rab5A tryptic peptides and corresponding synthetic standards. (*A*) HPLC fractions corresponding to the radioactivity in Fig. 1*C*, peak 1, were pooled and analyzed by electrospray mass spectrometry. (*B*) Corresponding synthetic standard was analyzed for comparison. (*C*) Peak 2 of Fig. 1*C* was similarly analyzed and compared with the corresponding synthetic standard (*D*).

a molar ratio of at least 2:1. Giannakouros *et al.* (19) transfected COS cells with wild type or single cysteine mutants of the yeast counterpart of Rab5, Ypt5p, an -XCXC motif protein, incubated the cells with [^{3}H]mevalonic acid, and found that the wild-type Ypt5p incorporated at least twice as much label as the single cysteine mutants did. The simplest way to explain these results is to postulate that a single Rab-type GGTase catalyzed digeranylgeranylation of each of the native proteins.

However, apparently conflicting results obtained in labeling studies with yeast deserve comment. Molenaar *et al.* (20) and Newman *et al.* (7) overexpressed Ypt1p or Ypt3p (-XXCC motif proteins) in yeast, incubated the cells with [^{3}H]palmitic acid, and found that a C-terminal domain cysteine residue in each protein became labeled. However, when they did similar experiments with cells that expressed these proteins at normal levels, neither protein was palmitoylated. Thus, palmitoylation of cysteines in the C-terminal domains of Ypt1p and Ypt3p, which would be inconsistent with digeranylgeranylation, may have been an artifact caused by overexpression. One possible explanation of this effect is the following: The concentrations of nonprenylated Ypt1p and Ypt3p GTPases in the overexpression systems may have been increased manyfold by comparison with those found in normal cells. As a consequence, these species may have been better able to compete with monoprenylated Ypt1p and Ypt3p for geranylgeranylation by Rab GGTase/REP-1 (see below for a further discussion of the enzyme reaction mechanism). This in turn may have increased the concentrations of monoprenylated Ypt1p and Ypt3p and allowed these proteins to be palmitoyl thioesterified by a yeast palmitoyltransferase. In support of this possibility, farnesylation of Ras has been shown to be a prerequisite for palmitoylation (21), and other monoprenylated small GTPases may also be palmitoylated (22). However, the reaction conditions that normally control the lipidation of Rab proteins *in vivo* remain to be determined.

Table 2. M_r of [1-^{3}H]GG-labeled C-terminal tryptic peptides from Rab1A, Rab3A, and Rab5A and their corresponding synthetic standards

	Observed		Calculated
Sample	^{3}H labeled peptide	Synthetic peptide	Synthetic peptide
Rab1A	1911.8	1909.6	1909.5
Rab3A	2498.4	2495.8	2496.1
Rab5A			
Peak 1	2723.0	2720.6	2721.3
Peak 2	2509.6	2507.6	2508.1

Values of the $(m + 2H)^{2+}$ ions from Figs. 2, 3, and 4 are reported in terms of their molecular weights.

Further studies of the mechanism of the Rab GGTase/REP-1 reaction are needed. It is very uncommon for a single enzyme to catalyze the transfer of two lipid groups to different loci of a single acceptor substrate. The reaction is analogous to the phosphorylation of photoactivated rhodopsin by rhodopsin kinase, where a number of phosphates are incorporated at multiple sites at the C terminus of the acceptor protein (23). It is not analogous to DNA polymerase-catalyzed reactions, where the product of the addition of deoxyribonucleotide triphosphate to a primer is structurally very similar to the original substrate and only a single DNA binding site and a single deoxyribonucleotide triphosphate binding site are needed. In the case of the Rab GGTase/REP-1 reaction, the unmodified Rab protein, the monogeranylgeranylated Rab protein, and the digeranylgeranylated Rab protein are structurally very different, and it is likely that several binding sites are involved including one for an upstream region of unmodified Rab proteins (22), one or two binding sites for GGPP, and binding sites for the GG groups of the monogeranylgeranylated or digeranylgeranylated Rab protein. Furthermore, it is unclear how the enzyme is able to accept protein substrates that have different register positions of the two cysteines (-XXCC versus -CCXX) or cysteines that are in a different order (-XXCC versus -XCXC). Whether the Rab GGTase/REP-1 can accept additional Rab protein substrates, such as those that end in a -CCXXX or -CXXX motif, has not yet been examined.

Although the order of cysteine residues does not appear to affect the extent of Rab protein prenylation, it may play a role in the methylation of Rab proteins. Indeed, of those proteins examined, only -XCXC motif Rab and Ypt proteins were found to be methylated, whereas -XXCC motif Rab and Ypt proteins were not (7, 8, 19, 24). Furthermore, when Smeland *et al.* (25) incubated bovine brain membranes containing methyltransferase activity with *S*-[*methyl*-^{3}H]adenosyl-L-methionine and either digeranylgeranylated Rab3A or digeranylgeranylated Rab1A, only Rab3A was labeled. This result clearly depended on the order of cysteine residues in the two proteins because the opposite result was obtained when the digeranylgeranylated mutant proteins Rab3A-XXCC and Rab1A-XCXC were used. The basis of this selectivity is unknown.

For those Rab proteins that were studied here, it is not known whether the two adjacent cysteine residues are geranylgeranylated in a compulsory order or whether the first geranylgeranylation reaction may involve either one. Speculation based on relative kinetic parameters (for example, V_{max}) for the geranylgeranylation of Rab mutants in which one of the cysteines has been mutated to serine is of little value. There is ample precedent in the literature for enzymes that display large changes in V_{max} after their substrates have been structurally altered at sites removed from the site of chemical transformation. On the other hand, analysis of monogeranylgeranylated Rab proteins, obtained by incubating recombinant Rab proteins with Rab GGTase/REP-1

under single catalysis conditions as reported here, might be a useful experimental approach.

Whether monogeranylgeranylated Rab proteins must be geranylgeranylated on the adjacent cysteine residue before they can dissociate from Rab GGTase/REP-1 is yet another question. We detected little or no monogeranylgeranylated Rab protein in incubation experiments that were done under conditions that permitted multiple rounds of catalysis (Fig. 1). But two possible explanations for this must be considered. The rate of the second geranylgeranylation reaction might have been significantly faster than the rate of dissociation of the monogeranylgeranylated Rab protein substrate from the enzyme, or the rate of dissociation of the monogeranylgeranylated Rab protein might have been faster than that of the second geranylgeranylation reaction. In the latter case, the lack of significant buildup of monogeranylgeranylated product would imply that the k_{cat}/K_m for the second geranylgeranylation reaction is much larger than the k_{cat}/K_m for the first geranylgeranylation reaction. Further experiments are needed to resolve these possibilities.

In summary, we have shown that Rab GGTase/REP-1 can catalyze the *in vitro* digeranylgeranylation of Rab proteins that end in -XXCC, -XCXC, or -CCXXX motifs. Altogether, proteins with these motifs comprise 87% of all known Rab or Ypt proteins. Whether Rab GGTase/REP-1 can catalyze the geranylgeranylation of proteins that end in other motifs remains to be determined. Questions remain to be answered about the reaction conditions that lead to the lipidation of native Rab proteins *in vivo* and about the mechanism of the Rab GGTase/REP-1 reaction itself. Some of the experimental procedures developed in the present study may prove useful in this regard.

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