Determination of Structural Requirements for the Interaction of Rab6 with RabGDI and Rab Geranylgeranyltransferase*

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The importance of geranylgeranylation to the interaction of Rab proteins with RabGDI was investigated with a set of Rab6 mutants post-translationally modified by all known C-terminal lipid combinations. Rab6 proteins geranylgeranylated on CXC or CC motifs were found to be significantly better substrates for membrane extraction by RabGDI than either Rab6 proteins geranylgeranylated on CAAL motifs or Rab6 proteins that were farnesylated and palmitoylated. The methylation status of the CXC motif did not significantly affect interaction of wild type Rab6 with RabGDI. Rab6 protein sequences required for RabGDI interaction were then identified. Consistent with the significant homology between RabGDI and the Rab escort protein, a subunit of Rab geranylgeranyltransferase (RabGGTase), we show that there is an overlap between Rab6 motifs required for RabGDI binding and RabGGTase processing. The effector domain, loop3/β3 and the hypervariable region of Rab6 are all required for RabGDI binding, whereas loop3/β3 and the hypervariable region but not the effector domain are required for efficient processing of Rab6 by RabGGTase. Interestingly, however, loop3/β3 of Rab6 when introduced into H-Ras is sufficient to allow some in vivo processing of a C-terminal CSC motif.

Rab proteins are small (21–25 kDa), guanine nucleotide-binding proteins that seem to play a role in the trafficking of intracellular membranes (reviewed in Ref. 1). A growing number of this large family of proteins have been assigned to specific membranous organelles. Like other Ras-related proteins, they are synthesized as soluble precursors and must be posttranslationally modified by isoprenylation in order to associate with cellular membranes. The mechanism(s) by which the Rab proteins bind to their cognate compartment is poorly understood. The precise role of individual Rab proteins in cellular trafficking is also unclear. Although investigators have inferred from their intracellular localization, and in some cases demonstrated experimentally, that individual Rab proteins are involved in particular phenomena, such as lateral endosome fusion (Rab5) (2), endoplasmic reticulum to Golgi transport (Rab1B) (3), or late endosome to trans-Golgi network transport (Rab9) (4), their precise roles in processes such as the formation or cohesion of intracellular organelles or the control of transport, docking, or fusion of vesicles remain to be established.

Like other Ras-related proteins, each Rab protein can be regulated by three types of molecule: (i) a guanine nucleotide releasing factor that stimulates the release of GDP favoring the formation of a Rab-GTP complex, (ii) a guanine nucleotide dissociation inhibitor (GDI) that inhibits the release of GDP, and (iii) a GTPase-activating protein (GAP). A guanine nucleotide releasing factor has been identified for Rab3A and SEC4 (5–7) and a GAP for Rab3A and Rab6 characterized (7, 8); they seem to be relative specific for those individual Rab proteins. In contrast, only one RabGDI has been isolated (9) which interacts with a large number of Rab proteins (10). RabGDI forms a complex with prenylated Rab proteins in the GDP-bound state and decreases the rate of GDP dissociation. Interestingly, RabGDI can also solubilize the GDP form of Rab proteins from membranes (9–11). These data suggest that RabGDI either plays a role in recycling Rab proteins through the cytosol from an acceptor membrane, after GTP hydrolysis, back to a donor membrane. Or, that RabGDI keeps inactive Rab proteins in a soluble complex until nucleotide exchange is stimulated and the GDP-bound active form is released to bind to its specific membrane and initiate a particular trafficking event.

Rab proteins usually carry at their C terminus a CC or CXC sequence where C = cysteine and X = any amino acid. They are geranylgeranylated on these cysteines by a specific enzyme called RabGGTase (also known as GGTTase I) (12). The enzyme has α and β subunits which share homology with the α and β subunits of farnesyl transferase (FTase) and geranylgeranyltransferase I (GGTase I) but, requires a third component, the Rab escort protein (REP) for enzymatic activity (13, 14). REP, which is encoded by the gene that is defective in the human disease choroideremia, shares significant homology with RabGDI (15, 16). The binding of unprocessed Rab to REP is necessary for the α and β subunits to prenylate the C-terminal cysteines. Although the actual role played by REP in the catalysis is not known, recent data show that REP remains bound to geranylgeranylated Rab following prenylation and must deliver the processed Rab protein either to a membrane or to an acceptor molecule such as RabGDI (17). Taken together these results suggest that Rab proteins interact with RabGDI and REP in a similar fashion but that Rab/RabGDI binding may be more fastidious since it is nucleotide sensitive whereas Rab/REP binding is not (14). The prediction follows that some, but not all, mutations which perturb Rab/RabGDI binding should compromise Rab processing.

An interesting question is whether there are specific motifs in Rab proteins that mediate binding to REP and RabGDI or whether the overall conformation of the Rab protein is the sole

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1 The abbreviations used are: GDI, guanine nucleotide dissociation inhibitor; GAP, GTPase-activating protein; REP, Rab escort protein; PBS, phosphate-buffered saline; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; GTP·S, guanosine 5′-O-(thiotriphosphate); BSA, bovine serum albumin; GGPP, geranylgeranyl pyrophosphate; HVR, hypervariable region; GppNHp, 5′-guanylylimidodiphosphate; GDPPS, guanosine 5′-O-thiodiphosphate.
RabGDI and RabGGTase Interactions with Rab6

determinant of such interactions. These alternatives are not mutually exclusive although specific motifs that confer the ability to be processed by RabGGTase onto a non-Rab protein have not yet been identified. However, Rab sequences remote from the C terminus are clearly required for Rab processing (18), and certain effector domain point mutations reduce the in vitro prenylation of Rab1B (19). Using a combination of in vivo and in vitro assays, we have studied Rab/RabGDI binding and Rab/REP binding (i.e. Rab processing) in parallel to identify Rab motifs that are relevant to one or both interactions. In addition we have investigated to what extent Rab/RabGDI interactions are influenced by the nature and stoichiometry of C-terminal lipidation and methylation.

EXPERIMENTAL PROCEDURES

Rab-Ras Recombination—The C-terminal mutant Rab6 proteins were constructed as previously described (20). The Rab155Ras chimera was constructed by introducing a SpeI restriction site into the v-H-Ras coding sequence at the position encoding amino acid position 144 (without changing the amino acid at this new position) to correct a naturally occurring A to C substitution at codon 155. The C-terminal hypervariable domains of Rab6 and Ras were then exchanged, and the CVLS C-terminal motif of H-Ras was mutated to CSC by polymerase chain reaction. Other Rab/Ras chimeras were constructed by replacing sequences in Rab6 by the corresponding residues of v-H-Ras. Mutations were introduced either by oligonucleotide-directed mutagenesis in the coding sequence of the corresponding Rab6 or Rab6 sequences previously inserted in the pEX3V3 expression vector. Loop2 (amino acids D—PTIP-DYSKQ) and loop3/loop3 (E—TCCLIDIL) of v-H-Ras were introduced in place of loop2 (QATGIDPFLSR) and loop3/loop3 (GTVRLQW) of Rab6 and reciprocally, and the helix a5 of Rab6 (K—GLFRQVAAQPMS) was replaced by that of v-H-Ras (E—DAFYTVRLEIRQHK). All of these proteins, were N terminally tagged with a myc epitope (20) and expressed from the eukaryotic expression vector pEX3V; the entire coding region of Rab/Ras constructs were sequenced (Sequenase kit, U. S. Biochemical Corp.) to verify the mutations and confirm that no errors had been introduced during construction.

COS Cell Fractionation—COS cells were transfected by electroporation as described previously (21) and harvested 72 h later. After washing with phosphate-buffered saline (PBS), a 10-cm dish of cells was rinsed with 10 ml Tris-C1 pH 7.5, 10 mM NaCl, 5 mM MgCl2, 1 mM DTT and scraped into 0.5 ml of the same ice-cold buffer containing 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 100 µg/ml leupeptin. Cells were incubated on ice for 15 min then homogenized on ice with a Dounce homogenizer. Unbroken cells and nuclei were resuspended in 10 volumes of ice-cold 50 mM Tris-C1, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, 10 µg/ml aprotinin, and 100 µg/ml leupeptin. The crude extract was clarified by a 1,500 x g spin and membranes collected as described above for COS cells.

Yeast Membrane Preparation—Isogenic strains SM1188 (ste-14:TRP1) and SM1058 (wild type counterpart) were kindly provided by Susan Michaelis. Both of these strains were transformed with the yeast Rab6 expression vector pYNV-Rab6 (20). Extracts from 100-ml log phase cultures grown in synthetic medium were prepared by lysis with glass beads in 200 µl of 50 mM Tris-C1, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10 µg/ml aprotinin, and 100 µg/ml leupeptin. The extract was clarified by a 1,500 x g spin and membranes collected as described above for COS cells.

Yeast Methylation Assays—100-ml log phase cultures were harvested, washed in 5 ml of water, and resuspended to a final volume of 1 ml in growth medium containing 150 µCi of [3H]Sicysteine (Amersham Corp. SJ1523, 1300 Ci/mmol) and 125 µCi of [methyl-3H]adenosine-5'-monophosphate (New England Nuclear NET 155, 9 Ci/mmol) as described (25). The cells were incubated at 30 °C for 15 min with constant shaking and extracts prepared as described in the preceding section. Extracts were diluted into an equal volume of 2 x lysis buffer (200 mM NaCl, 1% Triton X-100, 2% Triton X-100, 200 mM Na2SO4, 40 mM Tris-C1, pH 7.5, 5 mM MgCl2, 2 mM DTT with 200 µg of leupeptin, 20 µg of aprotinin, and 20 µg of soy bean trypsin inhibitor/ml) and cleared with protein A-Sepharose beads for 2 h at 4 °C. The supernatants were removed after a 12,000 x g centrifugation, and Rab proteins were immunoprecipitated as described (20). Immunoprecipitates were washed six times with 1 x lysis buffer, once with PBS, and resolved by SDS-PAGE. Gels were fixed and fluorographed with Enlightening (NEN). Using the autoradiogram as a guide, gel slices containing Rab6 protein were cut out and a vapor phase equilibration assay performed as described previously (22). Briefly, the washed gel slices are incubated in 100 µl of 0.2 mM NaOH for 48 h at 37 °C in an open capped Eppendorf tube placed in a tightly capped vial of scintillation fluid. Any [3H]methionol released from the hydrolysis of a-carboxymethyl esters is collected by equilibration into the scintillation fluid surrounding the reaction tube. [3H]Sicysteine counts remaining in the gel slice after neutralization with 0.2 mM HCl indicate the total amount of protein captured in the immunoprecipitates.

RabGDI Purification—Human RabGDI was cloned from human lymphocyte cDNA by polymerase chain reaction using the bovine RabGDI sequence to design oligonucleotide primers. The polymerase chain reaction product was sequenced and cloned into pGEX-2T (Phar- macia). Protein expression, purification, and Bradford assay of the RabGDI fusion protein to yield pure RabGDI was carried out exactly as described recently for RhGDI (23). RabGDI was estimated as >85% pure by Coomasie staining, and after determining protein concentration by the Bradford refection RabGDI was stored at ~20 °C at 0.5 mg/ml in 50 mM Tris-C1, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 5 mM DTT, 50% glycerol.

RabGDI Extraction Assay—25 µl of membranes were pre-exchanged with GDP or GTP-S by adding 3.5 µl of 0.1 mM nucleotide, 0.5 µl of 0.2 mM DTT, and 2 µl of protease inhibitor mix (0.3 mM aprotinin, 0.3 mM soy bean trypsin inhibitor, 3 µg/ml leupeptin), 3.5 µl of 30 mM EDTA and incubating at 30 °C for 10 min. The reaction was stopped by adding 5 µl of 0.2 M MgCl2, 17 µl of exchanged membranes were then incubated with 5 µg of RabGDI (0.5 mg/ml) or 5 µg of BSA (0.5 mg/ml in RabGDI storage buffer) at 30 °C for 40 min. 13 µl of 10 mM Tris-C1, 150 mM NaCl, 5 mM MgCl2 was then added and the reactions centrifuged at 60,000 rpm at 4 °C for 50 min in a Beckman TL100 ultracentrifuge using a T100.3 rotor. The supernatant was removed to a tube containing 10 µl of 5% laemmli sample buffer and the pellet taken in 40 µl of 1 x Laemmli sample buffer. Samples were boiled for 4 min, resolved in 15% SDS-PAGE gels, and transferred to Immobilon membranes (Millipore) using a semi-dry blotting apparatus (Pharmacia) for Western blotting.

Western Blotting—Immobilin membranes were blocked overnight with 5% (w/v) dried milk in PBS + 0.2% Tween 20 (PBST). Since all Rab and Rabas chimeras expressed in COS cells were N terminally tagged with a myc epitope as described above, blots were probed with a 1:2000 dilution of 9E10 anti-myc hybridoma supernatant (10 mg/ml), and after washing, they were incubated in 15,000 anti-mouse-horseradish peroxidase conjugate (Amersham). Antibody incubations were performed for 1 h at room temperature in blocking buffer and membranes washed with PBST for 3 x 10 min. Western blots were developed using an ECL detection system (Amersham).

Expression of Rab/Ras Chimeric Proteins—A Glu-Glu epitope (MEYMPME) (24) was cloned onto the N terminus of wild type Rab6 and onto the N termini of the Rab/Ras chimeras. The cDNAs were assembled in pTc99C and transfected into DH5α Escherichia coli. For expression of the Glu-Glu tagged proteins, 50-µl cultures were incubated to saturation overnight in 2 x L-broth containing ampicillin 100 µg/ml, diluted 1:10 in 1 x L-broth (plus ampicillin) for Western blotting.
TABLE 1

| C-terminal mutant Rab proteins |
|--------------------------------|
| Actual | Type of motif | Modifications |
| C-terminal sequence | C<sub>1</sub> | C<sub>2</sub> | Methyl |
| CSC | C<sub>1</sub>C<sub>2</sub> | GG | GG | + |
| CC | C<sub>1</sub>C<sub>2</sub> | GG | (GG) | 0 |
| CVLS | C<sub>1</sub>AAX | 0 | F | + |
| CCL | C<sub>1</sub>AAL | 0 | GG | + |
| CTKVLS | C<sub>1</sub>AAX | Palm | F | + |
| CTKCL | C<sub>1</sub> | Palm | GG | |

Glu-Glu peptide and fractions analyzed by SDS-PAGE. Rab proteins were concentrated to approximately 2 mg/ml adjusted to 50% glycerol, 5 mM MgCl₂, and stored at -20°C. Purity was verified on SDS gels and protein concentration determined using the Bradford reaction.

Where required, GDP, GTPγS, or GppNHp was loaded onto the Rab proteins essentially as described above by incubating for 15 min at 30°C in the presence of 5 mM nucleotide, 5 mM MgCl₂, and 7 mM EDTA in a final volume of 100 μl. The reaction was stopped by adjusting the MgCl₂ to 15 mM. Unbound nucleotide was removed by diluting the sample 10-fold in 10 mM Tris-Chloride, 150 mM NaCl, 5 mM MgCl₂, and spin dialyzing to reduce the volume back to 100 μl. This procedure was then repeated resulting in a final free nucleotide concentration of ≤50 μM.

RabGGTase Purification—RabGGTase was partially purified as described (12). 50 T175 flask CV-1 (monkey kidney) cells were resuspended in 50 ml Tris-Chloride, pH 7.5, 20 μM ZnCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin and homogenized with 30 strokes of a Dounce homogenizer. The homogenate was centrifuged at 40,000 rpm in a Beckman Ti-45 rotor for 70 min. A 30-50% (NH₄)₂SO₄ precipitate was collected from the supernatant, homogenized with 30 strokes of a Dounce homogenizer. The homogenate was centrifuged at 40,000 rpm in a Beckman Ti-45 rotor for 70 min. A 30-50% (NH₄)₂SO₄ precipitate was collected from the supernatant, centrifuged at 15,000 rpm for 20 min, and the pellet was resuspended in 50 mM NaHepes, pH 7.2, 5 mM MgCl₂, 0.3 mM Nonidet P-40, 3 mM DTT, 0.5 μM [3H]geranylgeranylporphophate (ARC, ART348; 15Ci/mmol) for 30 min at 37°C. Reactions were stopped by adding 0.5 ml of 9:1 ethanol/HCl and incubating at room temperature for 30 min. The reaction plus 2 x 1 ml of ethanol washes of the reaction tube were filtered through glass fiber filters and the filters washed with 3 x 10 ml of 100% ethanol. Dried filters were counted in a scintillation counter. Background counts were determined by omitting enzyme.

RESULTS

Efficient Interaction of Rab and RabGDI Requires a CXC or CC Motif—We have previously described and characterized a series of Rab mutants in which the wild type CSC C-terminal amino acids have been replaced with five other C-terminal motifs (summarized in Table 1). One of these mutants, RabCC, is processed by RabGGTase, whereas the other mutants are processed by GGTase1, or FTase. Two of the mutants are additionally palmitoylated. Together this set of mutants represents all known possible combinations of C-terminal lipid modification present at the C termini of Ras-related proteins (20). To investigate whether RabGDI could interact with Rab proteins that have lipid modifications other than wild type, membranes were prepared from COS cells transiently expressing each mutant. The membrane-bound Rab proteins were pre-exchanged with GDP and incubated with recombinant RabGDI or an equivalent amount of BSA. Fig. 1A shows that RabGDI solubilized 50% Rab6 from the membrane while the control BSA protein had no effect. RabGDI did not solubilize Rab6 bound to GTPγS. In this assay RabGDI had an equivalent activity on the RabCC mutant, extracting 55% from the membrane, but had significantly reduced activity on all of the other membrane-bound Rab mutants, extracting only 10–15% in each case (Fig. 1, B and C). Thus, Rab6 geranyleranylated in the context of either a CXC or CC motif was a good substrate for RabGDI, whereas Rab6 geranyleranylated in the context of a CAAL motif was not. Palmitoylation of either geranyleranylated or farnesylated Rab6 did not enhance interaction with RabGDI.

**FIG. 1.** A, COS cell membranes expressing wild type myc-tagged Rab6 were prebound with GDP, GDPβS, or GTPγS and incubated with RabGDI or BSA. After repelleting, the membranes (p) and the supernatant (s) were resolved (9E10, an anti-myc monoclonal, RabGDI but not BSA extracts Rab6 from cellular membranes, but only when Rab6 is GDP-bound. B, COS cell membranes expressing myc-tagged Rab proteins with different C-terminal lipid modifications (see Table I) were prebound with GDP and incubated with RabGDI or BSA. After repelleting, the membranes (p) and the supernatant (s) were resolved by SDS-PAGE and Western blotted with 9E10. C, to better quantitate the experiment in B cells expressing the same mutant, Rab proteins were radiolabeled with [35S]methionine before preparation of membranes. After incubation with RabGDI or BSA and repelleting of the membranes, Rab6 proteins were immunoprecipitated from the s and p fractions with an anti-Rab6 serum. The amount of Rab protein immunoprecipitated was determined by β-scanning the SDS-PAGE gel. % extraction = counts/minute in solubilized + membrane-bound Rab6.
TABLE II

Methylation status of Rab6 in S. cerevisiae

| Yeast strain | [3H]MeOH | [35S]Cys |
|--------------|----------|----------|
| Wild type    | 172      | 705      |
| ste14 mutant | 0        | 306      |

Rab6 modified by farnesylation alone (RabCVLS) is not membrane associated and therefore could not be evaluated in the assay.

Methylation is a potentially reversible post-translational modification that may influence the avidity with which Ras-related proteins interact with membranes and with GDIs. To investigate whether this was the case for Rab6 and RabGDI, Rab6 was expressed in ste14 Saccharomyces cerevisiae, a yeast strain which lacks the methyltransferase (25) and also in an isogenic control strain with wild type methyltransferase activity. The methylation status of Rab6 expressed in these yeast strains was determined (Table II). Rab6 immunoprecipitated from membrane extracts of wild type yeast was methylesterified whereas Rab6 expressed in the ste14 mutant yeast is not.

Overlapping Domains in Rab6 Interact with RabGDI and RabGGTase—Next, using RabRas chimeras, we wished to determine which domains of Rab6, other than the C terminus, interact with RabGDI. A problem with this approach is that the Rab motifs required for processing have yet to be fully defined. And since RabGDI shares significant homology with the REP, a component of RabGGTase, the same Rab sequences could be required both for RabGDI binding and for processing. The analysis of RabGDI-binding domains therefore also becomes an analysis of Rab processing motifs.

In an attempt to maintain as normal a conformation of the mutant protein as possible, RabRas chimeras with a C-terminal CSC motif were constructed by replacing varying amounts of Rab6 sequence with the homologous sequence from H-ras. All of these chimeras bound GDP (as assessed with the purified bacterially produced proteins, data not shown), confirming that a relatively normal 3D structure was preserved. To determine if a chimera was processed in vivo, COS cells expressing the protein were lysed in Triton X-114 and the proportion of the N-terminal 71 amino acids of Rab6 was critical for processing. A comparison with other Ras-related proteins showed that residues 59-64, which form the C-terminal part of loop 3 (L3) and the N-terminal portion of β sheet 3 (β3), were conserved among Rab proteins and differed significantly from the corresponding sequence in Ras. When residues 59-64 in Rab155Ras were replaced with the corresponding Ras amino acids the protein (Rab155Ras+L3/β3Ras) was no longer processed in COS cells (Fig. 3). When, as a control, the effector domain of Rab155Ras was replaced with the effector domain (loop2-L2) of Ras, processing in COS cells was unaffected. The importance of L3/β3 for

FIG. 2. Membranes were prepared from wild type and ste14 mutant S. cerevisiae expressing myc-tagged Rab6 and used in the RabGDI extraction assay described in Fig. 1A. Proteins were visualized by Western blotting with 9E10. RabGDI extracts methylated and nonmethylated Rab6 with similar efficiency from yeast membranes.
Rab processing was further examined by replacing L3/β3 in full-length Rab6 with Ras L3/β3 and introducing RabL3/β3 into a Ras protein in which the C-terminal CAAX motif had been replaced with CSC. In COS cells the RabL3/β3 Ras chimera was processed to the same extent as wild type Rab6. Interestingly, however, the RasCSC+L3/β3Rab protein was also processed whereas the RasCSC control was not. Therefore, the L3/β3 region of Rab6 is sufficient to allow in vivo processing of a C-terminal CSC motif on a non-Rab protein and is required for processing the Rab155Ras chimera, but in the context of a full-length Rab protein, L3/β3 is not essential for processing in vivo.

We next examined which of the RabRas chimeras that were processed in COS cells, and which therefore bound to membranes, interacted with RabGDI in the membrane extraction assay. Fig. 4 shows that replacement of L2 or L3/β3 or the hypervariable domain of Ras with corresponding sequences from Rab blocked the ability of RabGDI to solubilize the chimeric protein from membranes. The chimera Rab65Ras, in which residues 153–168 of Ras (helix α5) replace the homologous amino acids in Rab6 was processed and extracted from membranes by RabGDI showing that not all Rab sequences affect Rab/RabGDI interactions. In summary these data indicate that the effector domain (L2), L3/β3, and the hypervariable domain of Rab6 are essential for recognition of the protein by RabGDI and that L3/β3 and the hypervariable domain, but not L2, are involved in the interaction of Rab6 with RabGGTase. Interestingly, L3/β3 is sufficient, when introduced into another Ras-related protein, v-H-Ras, to allow some in vivo processing of a C-terminal CSC motif. This is the first demonstration that a non-Rab Ras-related protein can be turned into a substrate for RabGGTase by the introduction of a short Rab sequence.

L3/β3 and the Hypervariable Domain Are Essential for in Vitro Processing—The experiments performed with COS cells only measure the steady state level of processed protein after 3 days of transient expression. Thus chimeras that were processed in COS cells could actually be poor substrates for RabGGTase. To further evaluate the efficiency with which the RabRas chimeras were processed, we measured the in vitro incorporation of [3H]geranylgeranyl pyrophosphate ([3H]GGPP) into bacterially expressed recombinant proteins. RabGGTase was partially purified from CV-1 (monkey kidney) cells; wild type Rab6 and selected RabRas chimeras were N terminally tagged with a Glu-Glu epitope and purified from E. coli by affinity chromatography. A filtration assay (15) was used to measure in vitro Rab geranylgeranylation. In pilot experiments the incorporation of [3H]GGPP into wild type Rab6 and each of the chimeras was found to be linear for 1–3 h in the conditions of the assay. All subsequent kinetic experiments were stopped after 30 min of reaction.

Fig. 5 shows that Rab155Ras and RabL3/β3Ras, which were processed to the same extent as wild type Rab6 in COS cells, were significantly compromised as substrates for RabGGTase in vitro. However, measures of the apparent $K_a$ and $V_{max}$ for these substrates (Table III) revealed different defects.

Rab155Ras had a relatively normal $K_a$, but a much reduced $V_{max}$, while RabL3/β3Ras had a significantly increased $K_a$ and a less perturbed $V_{max}$. RabL2Ras and Rab65Ras were good substrates for the enzyme although both were marginally compromised compared with wild type Rab6 (Table III). In prolonged incubations with RabGGTase, in vitro RasCSC+L3/β3Rab incorporated label from [3H]GGPP whereas the control protein RasCSC did not (Fig. 5C). The extent of labeling of RasCSC+L3/β3Rab was low compared with wild type Rab6 consistent with the reduced processing of this mutant in COS cells (Fig. 3).

Finally, the ability of RabGGTase to prenylate Rab6 in the GTP bound form was investigated. Fig. 6 shows that Rab6 is processed equally well in vitro whether complexed with GDP or

| Table III |
|---|
| Rab mutant | $K_a$ | Relative $V_{max}$ |
| Rab6 | 1.42 | 1.0 |
| Rab155 Ras | 1.0 | 0.1 |
| RabL2/β3 Ras | 40 | 0.6 |
| RabL2 Ras | 2.2 | 0.7 |
| Rab65 Ras | 2.2 | 0.6 |

Fig. 4. The RabRas chimeras that were processed and membrane associated in COS cells were assayed for interaction with RabGDI in the membrane extraction assay described in Figs. 1 and 2.

Fig. 5. In vitro processing by partially purified RabGGTase of recombinant wild type Rab6 (A) and two Rab Ras chimeric proteins (B). Incorporation of label from [3H]GGPP into the bacterially expressed protein was determined after a 30-min incubation at 37 °C in the presence of a fixed amount of RabGGTase (see "Experimental Procedures"). Each assay point was carried out in duplicate. The figure shows a typical result from a single experiment, C, in vitro geranylgeranylation of RasCSC+L3/β3Rab by RabGGTase. Incubations were carried out for 4 h at 37 °C with 1 μM [3H]GGPP and 5 μM substrate. RasCSC+L3/β3Rab undergoes geranylgeranylation whereas RasCSC does not.

In vitro processing of Rab6 mutant proteins Recombinant Rab proteins were used as substrates for partially purified RabGGTase in the assay described in Fig. 5 and under “Experimental Procedures.” $K_a$ and $V_{max}$ were calculated from double-reciprocal plots of 1/[V] versus 1/[Rab]. The $V_{max}$ data are expressed relative to the $V_{max}$ measured for wild type Rab6.
The nucleotide-loaded protein was then used as substrate in an *in vitro* geranylgeranylation assay. Incorporation of label from \(^{3}H\)GGPP was determined after a 30-min incubation at 37°C in the presence of a fixed amount of RabGGTase (see "Experimental Procedures"). Each assay point was carried out in duplicate. The figure shows a typical result from a single experiment. Identical results were obtained using GTP\(\gamma\)S in place of GppNHp.

GTP. In the experiment shown, Rab6 was loaded with the non-hydrolyzable GTP analogue GppNHp, but identical results were obtained when Rab6 was loaded with GTP\(\gamma\)S. It proved important to remove unbound nucleotide from the exchange reaction prior to incubating Rab6 with RabGGTase as free GTP\(\gamma\)S and GppNHp at concentrations >1 mM inhibited geranylgeranylation. Recently, it has been reported that Rab5 complexed to GTP is processed less well in a reticulocyte lysate than when complexed to GDP although these assays were performed in the presence of 0.4 mM GTP\(\gamma\)S (26). However, using purified reagents, in the absence of unbound GTP, Rab6 is an equally good substrate for RabGGTase in the GDP and GTP conformations. Moreover, Rab6 with an activating G23V mutation is membrane bound *in vivo*, indicating that geranylgeranylation of this constitutively GTP bound form also occurs normally in the intact cell.²

**DISCUSSION**

We have shown previously that the Golgi targeting and biological activity of Rab6 can be supported by geranylgeranylation in the context of CXC, CC, or CAAL motifs or by the combination of farnesylation and palmitoylation. These data imply that overall hydrophobicity rather than the specific lipid combination is important for Rab membrane binding (20). In contrast, RabGDI, interacts much more efficiently with Rab6 proteins terminating in CXC or CC motifs. CC motifs are doubly geranylgeranylated and methylated. CC motifs are probably also doubly geranylgeranylated although, because they are not methylated (27, 28), the presence of a C-terminal geranylgeranyl cysteine has been questioned (29). One interpretation of the preferential interaction of RabGDI with Rab-CXC and Rab-CC proteins is that RabGDI has a binding site for two geranylgeranyl moieties. Moreover, because substitution of one C\(\alpha\) lipid with palmitate compromises Rab6 binding to RabGDI the lipid-binding domain in RabGDI may be a specific C\(\alpha\) binding site rather than a nonspecific hydrophobic pocket.

Methylation is potentially a reversible post-translational modification on CAAAX proteins and influences the affinity with which Ras proteins bind to membranes (30, 31). Moreover, in resting neutrophils RhoGDI forms a cytosolic complex with non-methylated Rac2 until the cell is activated, whereupon Rac2 translocates to the membrane and is concomitantly methylated (32). An attractive synthesis of these data is that methylation may simultaneously decrease the affinity of a Ras-related protein for its GDI and increase its affinity for membranes and thus facilitate translocation between cytosol and membranes. *In vitro*, however, RhoGDI shows no preference for binding the non-methylated over the methylated form of Rac1 (23). Additionally, we show here that RabGDI can extract both methylated and nonmethylated Rab6 from membranes. Whatever the physiological role of C-terminal methylation may be, our data argue against this post-translational modification being an important determinant of Rab/RabGDI interactions.

Much work has been aimed at assigning functional interactions to the various structural domains of Ras-related proteins. For example the negative regulators p120GAP and neurofibromin as well as the Raf-1 serine/threonine kinase interact with loop2 (the effector domain) of Ras (32-35). And recent evidence suggests that residues between 100–110 are essential for the interaction of Ras with guanine nucleotide releasing factors (36). Three structural domains, namely loop2 (the effector domain), loop 7, and the HVR cooperate to specify Y314F function (37, 38). We have attempted to map domains involved in the interaction of Rab6 with RabGDI and the REP component of RabGGTase using a series of biochemically active RabRas chimeric proteins. Replacement of the a5 helix of Rab6 (i.e. the large helix preceding the C-terminal HVR) affected neither processing nor membrane binding *in vivo* and did not hinder RabGDI binding *in vitro*. Rab6 proteins with the HVR, loop3/β3, or the effector domain replaced by the homologous Ras amino acids were processed and membrane associated in COS cells to the same extent as wild type Rab6. However, none of these proteins could be solubilized from membranes by RabGDI. The combination of an HVR plus loop3/β3 replacement in the same protein blocked all *in vivo* processing whereas the combination of any other pair of replacements did not. Our interpretation of these data is that the HVR and the loop 3/β3 region, two regions of the protein that are spatially close to one another, are important motifs for Rab processing and that loop2 and a5 are not.

These predictions were confirmed when partially purified RabGGTase was used to geranylgeranylate recombinant Rab proteins *in vitro*. HVR and loop3/β3 replacement severely compromised Rab6 as a RabGGTase substrate whereas effector domain or a5 replacements had much less dramatic effects. Loop3/β3 replacement resulted in a 30-fold increase in the \(K_a\) whereas HVR replacement had a major effect on \(V_{max}\). These data suggest that loop3/β3 (and probably other N-terminal domains) but not the HVR of Rab are involved in binding to REP. The HVR of Rab proteins may need to be a certain overall length or have a certain flexibility to allow efficient coordination of the C-terminal CC/CXC motif with the catalytic site of the a/β subunit dimer of RabGGTase. Participation of the HVR in REP binding also seems unlikely because there is much diversity among Rab proteins in this region. A similar argument can be made concerning Rab/RabGDI binding. The Rab HVR may simply allow appropriate coordination of the C ter-

² B. Goud, personal communication.
minus in order to facilitate binding of the C-terminal lipids by RabGDI while the protein motifs that mediate the binding of Rab to RabGDI are N-terminal and are conserved among Rab proteins.

Recently, Wilson and Maltese (19) have shown that the effector domain mutations I41N and D44N significantly reduced the prenylation of Rab1B translated in reticulocyte lysates. In contrast we have shown here that the entire effector domain of Rab6 can be replaced by that of Ras without dramatically compromising Rab6 as a substrate for RabGGTase. It is possible that conformational changes outside of loop2/β2 induced by the I41N and D44N substitutions prevented these proteins from binding to REP. This was probably not an issue for the RabL2Ras chimera since replacement of the entire effector domain with I41N and D44N substitutions prevented these proteins from binding to REP. This hypothesis would also entertain the disparate observations that the processing of Rab5 may be nucleotide sensitive (26) whereas the processing of Rab3A (14) and Rab6 is not.

Although we have not carried out an exhaustive analysis of all Rab sequences, our data suggest that specific motifs do exist that are important determinants of Rab/RabGGTase interaction. One such motif is loop 3/β3 which is conserved among Rab proteins, but not other Ras-related proteins, and which is sufficient to permit some processing of a CSC motif tagged onto the C terminus of the H-Ras protein that is only 30% identical to Rab6. Given the sequence homologies between RabGDI and REP, some overlapping of the regions in Rab with which they interact was expected. The fact that processing is insensitive to the nucleotide bound to Rab, whereas RabGDI binding is restricted to the GDP-bound protein suggested that RabGGDI should also interact with a region of Rab whose conformation changes between GDP and GTP bound forms. Our data indeed show that mutations in Rab6 which compromise interactions with REP, i.e., processing, also block interaction with RabGDI, but that the interaction of Rab6 with RabGDI is more fastidious in that the effector loop, one of the regions exhibiting a major conformational change between the GDP- and GTP-bound forms of Ras-related proteins, is important for Rab/RabGDI binding but not for processing.

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