Newly Synthesized and Prenylated Rab5*  

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In vitro synthesis and post-translational prenylation of Rab5 is accomplished using reticulocyte lysate supplemented with prenyl precursors (Sanford, J. C., Pan, Y., and Wessling-Resnick, M. (1993) J. Biol. Chem. 268, 23773–23776). When Rab5 is translated in the presence of biotin-lysine-tRNA, it incorporates biotin-lysine into its peptide backbone and is efficiently prenylated; since this modification is dependent on guanine nucleotide binding, biotin-Rab5’s functional integrity must be maintained. Prenylated biotin-Rab5 associates with a 45-kDa reticulocyte GDP dissociation inhibitor (GDI), sedimenting as a ~70-kDa particle on 5–20% sucrose density gradients. The GDI-Rab5 complex can be captured using streptavidin-linked agarose beads. Only Rab5 peptides that are substrates for prenylation are found to cosediment with the lysate GDI on sucrose gradients. Post-translational association of Rab5 and GDI is a novel finding, since previous reports suggested Rab5 remains associated with Rab escort protein (REP) after prenylation (Alexandrov, K., Horiuchi, H., Steele-Mortimer, O., Seabra, M. C., and Zerial, M. (1994) EMBO J. 13, 5262–5273). Since post-translational prenylation is catalytically mediated by REP, our study suggests that a complex between Rab5 and this factor is transient in nature. Thus, newly synthesized and prenylated Rab5 is most likely escorted to its target membrane by a GDI acceptor molecule. Biotin-Rab5 provides a novel tool for future efforts to capture and characterize additional accessory factors required for Rab protein function in vesicle transport.

Intracellular vesicle transport involves a series of regulated budding and fusion events that are dependent on members of the Rab family of GTP-binding proteins (1–4). Rab5 is a particularly well-characterized member of this family. Rab5 is associated with early endosomes and the plasma membrane (5), and the internalization of transferrin (6) and uptake of horseradish peroxidase (7) have been shown to be enhanced by overexpression of this factor. In contrast, endocytosis is perturbed when the GTP-binding mutants Rab5G138V and Rab5G134V are overexpressed (6–8). Cell-free systems also have demonstrated a role for Rab5 in endosome-endosome fusion (2, 7, 9). Although the precise function of Rab proteins in membrane transport remains undefined, it has been proposed that these GTPases act as regulators of the assembly and disassembly of protein complexes involved in the docking and fusion of vesicles (10).

Important structural elements required for Rab function are prenyl groups that are post-translationally attached to the protein’s C-terminal cysteines (11, 12). Rab5 mutants lacking these key cysteine residues fail to stimulate endocytosis in vivo (7) and do not support vesicle fusion activity in cell-free assays (2). Non-prenylated Rab5 that contains the C-terminal cysteines also fails to stimulate endosome-endosome fusion in vitro (9). Rab proteins, including Rab5, are post-translationally prenylated by geranylgeranyl transferase I (13, 14). This enzyme is composed of two components: an αβ dimer with homology to farnesyl transferase (15) and geranylgeranyl transferase I (16), and Rab escort protein (REP) component A (13). REP was originally identified to have significant homology with the gene product responsible for the retinal degenerative disorder, chorioderemia (17). REP forms a complex with Rab proteins to enable functional prenylation by the catalytic αβ subunits of geranylgeranyl transferase I (18). More recently, REP has been assigned a role in the delivery of newly synthesized Rab5 to membranes. Alexandrov et al. (19) have demonstrated that Rab5 can be modified in a reconstitution assay using purified prenylation reaction components and that Rab5 remains associated with REP after modification to be delivered to membranes in the absence of other cytosolic factors. These observations help to explain why the in vitro prenylation reaction is limited in the absence of detergents, which help to dissociate the Rab-REP complex; delivery and release of Rab proteins to membranes subsequent to prenyl transfer would help to recycle REP and thus to recover catalytic activity of geranylgeranyl transferase I. Otherwise, this enzyme appears to act in a stoichiometric fashion in vitro (18).

The apparent ability of REP to target Rab proteins to membranes resembles a functional characteristic of another Rab binding factor, GDP dissociation inhibitor (GDI). GDI was first identified and purified by its ability to inhibit GDP release (20). The binding of Rab proteins to GDI is dependent on their post-translational prenylation (21, 22). GDI also has been shown to selectively recruit Rab proteins to their target membranes (23, 24); release of the Rab from GDI at the membrane surface is accompanied by GDP/GTP exchange (24, 25). Finally, GDI has a well-characterized role in the retrieval of Rabs from membranes and is responsible for maintaining a soluble pool of the GDP-binding proteins (20, 26, 27).

We have devised a novel method to capture protein complexes in association with biosynthetically biotinylated Rab5. Using this approach, we find that newly synthesized and pre-
nylated Rab5 associates with a cytosolic GDI. In contrast to the findings of Alexandrov et al. (19), stable complexes between REP and newly synthesized Rab5 are not observed in the cell-free reticulocyte lysate system. Since the assembly of the Rab5-GDI complex is dependent on protein prenylation and occurs in the absence of membranes, we conclude that immediately after geranylgeranylation, Rab5 must rapidly dissociate from REP to bind GDI in the reticulocyte lysate. These novel findings indicate that biosynthetic biotinylation can be a useful tool to explore protein–protein interactions of members of the Rab family and their accessory factors, including yet-to-be-identified guanine nucleotide exchange factors and GTPase-activating proteins (GAPs).

EXPERIMENTAL PROCEDURES

Materials—[35S]Methionine (1200 Ci/nmol) was purchased from Du Pont NEN. Restriction endonucleases and other enzymes were purchased from Boehringer Mannheim and New England Biolabs; RNasin, RQI DNAse, tRNAnscend™ (biotin–lysine–tRNA), streptavidin-linked alkaline phosphatase and reticulocyte lysate were from Promega; streptavidin-linked agarose was from Pierce. Bovine Rab3A GDI and GD12-peptide antibodies were generously provided by Drs. Suzanne Pfeffer (27) and Asa Shisheva (28), respectively. Anti-REP1 antibody was a generous gift from Dr. Miguel Seabra (18).

In Vitro Biosynthesis, Biotinylation, and Prenylation of Rab5—The construction and subcloning of Rab5WT and Rab5Q79L for in vitro synthesis and prenylation have been described previously (29). Rab5S34N was constructed via the method of Kunkel (30) using the antisense oligonucleotide 5′-AAGCACTAGGCTGTTTTTGCCAACAGC. This construct was subcloned into pAGA in a manner identical to that detailed for Rab5WT (29). Plasmids containing Rab5WT, Rab5S34N, and Rab5Q79L were linearized with HindIII and transcripts were synthesized with T7 RNA polymerase (29). Reticulocyte lysate was programmed with these transcripts to translate peptides in the presence of [35S]methionine (150,000 cpn/pmol) for Rab5WT (29). The amount of peptide synthesized was determined by trichloroacetic acid precipitation. Biosynthesis of Rab5WT with lysine derivatized with biotin was accomplished by supplementing the translation reaction with biotin–lysine–tRNA; 1 μl of the stock provided by the supplier (Promega) was added per 20 μl of the translation reaction mixture. Biotinylated and nonbiotinylated Rab5 peptides were posttranslationally modified with prenyl groups by supplementation of the lysate with 100 μM mevalonate or geranylgeranylation pyrophosphate and subsequent incubation at 37 °C for times of limited length in the figure legends.

Sucrose Density Gradient Analysis—Reticulocyte lysate containing [35S]labeled proteins was fractionated on 4.8 ml of 5–20% continuous sucrose gradients in 50 mM HEPES, pH 7.5, 1 mM MgCl2, 1 mM DTT, and 5 μM GDP, with ultracentrifugation at 160,000 × g for 17 h at 4 °C. One hundred fifty μl aliquots were collected from the bottom of the gradient and 30–μl aliquots were analyzed on urea-acrylamide gradient SDS gels (29). Standard proteins used to determine the molecular mass of complexes in the 5–20% sucrose gradient were: cytochrome c (12.5 kDa), chymotrypsinogen (25 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), and aldolase (158 kDa).

Identification of Biotin-Rab5 Complexes with Lysate Proteins—Sucrose gradient fractions containing prenylated biotin-Rab5 were combined and concentrated using a Centricon-30 device (Amicon). The sample was then incubated with 20 μl of streptavidin-linked agarose for 16 h at 4 °C. The beads were pelleted by microcentrifugation and washed three times before resuspension in Laemmli buffer. Samples were then heated at 100 °C for 10 min, chilled on ice, and then microcentrifuged again; supernatants were analyzed on urea-acrylamide gradient SDS gels.

Immunoblotting—After SDS-PAGE of cytosol from K562 cells, bovine brain, or rabbit reticulocytes, proteins were electrophoretically transferred to nitrocellulose. The Western blots were incubated for 1 h in a blocking solution containing 20 mM Tris–Cl, pH 8.0, 150 mM NaCl, 5% bovine serum albumin, and 0.1% or 0.02% Tween 20, depending on whether the blots were to be probed with anti-GDI or anti-REP1 antibodies, respectively. Biotin-Rab5 was detected directly using avidin–biotinylated alkaline phosphatase, while anti-GDI and anti-REP1 immunocomplexes were identified using goat anti-rabbit IgG–linked alkaline phosphatase as the secondary antibody. Brieﬂy, immunoblots were incubated with alkaline phosphatase-linked conjugates in 100 mM Tris–Cl, pH 8.0, 150 mM NaCl, and then incubated with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The alkaline phosphatase reaction was quenched by washing the blot with distilled H2O.

Miscellaneous Methods—Protein concentration was determined by the method of Bradford (31) using bovine serum albumin as a standard. Urea-acrylamide SDS-gel electrophoresis was carried out exactly as described previously (29). Brieﬂy, a continuous gradient of 4–8% urea and 10–15% acrylamide was formed in the absence of SDS with a 6% stacking gel prepared as described by Laemmli (32). The following molecular size markers were used to calibrate the gels: lysozyme (34.4 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), and bovine serum albumin (66 kDa). Cytosolic fractions from K562 cells and bovine brain were prepared as described previously (33).

RESULTS

Biosynthetic Biotinylation of Rab5—Attempts to isolate and characterize factors that interact with Rab proteins have met with limited success (34, 35). In order to develop an alternate approach to this problem, we have taken advantage of the tight association between biotin and streptavidin to capture biosynthetically biotinylated Rab5 in complex with proteins present in rabbit reticulocyte lysate. Biosynthetic biotinylation can be achieved by in vitro translation in lysate containing biotinylated lysine–tRNA (Promega). The addition of excess tRNA does not alter the efficiency of peptide synthesis; translation in the presence of biotin–lysine–tRNA typically yields ~100 fmol of biotin–Rab5 μl of the in vitro reaction. As shown in Fig. 1, the biotinylated translation product can be detected on Western blots using streptavidin–linked alkaline phosphatase. A single 25-kDa band is labeled by the alkaline phosphatase reaction (panel A), corresponding to the synthetic 35S-labeled product of translation (panel B). Thus, the only biotinylated species detected in the lysate under these conditions is newly synthesized Rab5. Since this molecule contains 13 lysine residues and the lysate contains endogenous lysine–tRNA, it is difficult to determine exactly how many biotinylated amino acids are incorporated into the synthetic product. Under our labeling conditions, we estimate that 1–2 biotin–lysines are biosynthetically incorporated into every nascent peptide chain.

FIG. 1. Biotin-Rab5 is prenylated. Translation of Rab5 was carried out in the presence of [35S]methionine (50,000 cpn/pmol) and biotin–lysine charged tRNA (Promega) for 30 min at 30 °C. For post-translation processing, reaction mixtures were adjusted to 40% reticulocyte lysate with 12 mM Tris–Cl, pH 8.0, 0.6 mM DTT, 3.0 mM MgCl2, and 100 μM mevalonate was added. Incubation was continued at 37 °C for 16 h. After translation, synthesis and processing, Rab5 peptides (250 fmol) were electrophoresed on urea-acrylamide gradient gels and transferred to nitrocellulose. The immunoblot was then incubated with streptavidin–linked alkaline phosphatase (Avidin-AP, left panel). The biotin-Rab5/streptavidin–linked alkaline phosphatase complex was detected using NBT and BCIP as described under “Experimental Procedures.” The immunoblot was also exposed to film (1 day of exposure), and the autoradiograph is shown (right panel). The mass of electrophoresed molecular size markers is shown on the left in kDa.
Biosynthetically Biotinylated Rab5 Complexes with GDI

Our previous work has demonstrated that newly synthesized Rab5 becomes post-translationally modified with two geranylgeranyl groups and that the prenylated isoform can be detected by its characteristic mobility shift on urea-acrylamide gradient gels (36). Accordingly, further incubation of 35S-labeled biotin-Rab5 at 37 °C with the isoprenoid precursor mevalonate added to the lysate results in conversion of the peptide to a greater mobility isoform (Fig. 1). Since geranylgeranylation of Rab5 is dependent on its guanine nucleotide binding capacity (29), the fact that biotin-Rab5 is fully processed into this isoform indicates the functional integrity of the GTP-binding protein is not perturbed by the incorporation of biotinylated lysine residues.

Biotin-Rab5 Complexes with Lysate Proteins—To identify complexes between Rab5 and reticulocyte lysate proteins, the translation reaction mixture was fractionated on continuous 5–20% sucrose density gradients as detailed under “Experimental Procedures.” In vitro translated and prenylated biotin-Rab5 was found to sediment with an apparent Mₐ of ~70,000 (Fig. 2). Unmodified biotin-Rab5, which can be distinguished from the prenylated species by its lower mobility on the urea-acrylamide gradient gels, sediments with its native Mₐ of ~25,000. This sedimentation pattern is not unique to the biotinylated product, since non-biotinylated Rab5 displays the same behavior (see below). Alexandrov et al. (19) have shown that subsequent to post-translational modifications with purified geranylgeranyl transferase II, Rab5 remains associated in a 120-kDa complex with REP. In contrast, based on the apparent Mₐ of both the prenylated and nonprenylated forms of Rab5, it is unlikely that stable complexes with the 95-kDa REP are formed by the nascent product in the lysate. Since a species immunologically related to human REP1 is present in the rabbit reticulocyte cytosol (Fig. 3), and the biotinylated product becomes fully modified during post-translational processing in a reaction that requires REP activity (13, 14), the failure to detect a 120-kDa Rab5-REP complex is clearly not due to the absence of this factor. It is possible that a minor amount of Rab5-REP complex forms, but cannot be distinguished from the predominant 70-kDa complex identified on the sucrose gradient.

Figs. 2. Biotin-Rab5 sediments in a ~70-kDa complex. Rab5 was synthesized, biotinylated, and prenylated as described in Fig. 1. The reticulocyte lysate containing biotin-Rab5 was then layered on a continuous 5–20% sucrose gradient and centrifuged at 165,000 × g for 17 h at 4 °C. One hundred and fifty-ul fractions were collected from the bottom of the gradient and were incubated with streptavidin-linked agarose for 16 h at 4 °C to collect biotin-Rab5. Protein captured on streptavidin-agarose was boiled in Laemmli buffer and electrophoresed on a urea-acrylamide gradient SDS gel. The arrow at right indicates unprocessed Rab5, and the arrowhead at right designates the prenylated isoform. The positions of molecular size markers in the gradient are indicated by arrows at the top of the figure.

Fig. 3. Detection of REP in reticulocyte lysate. Reticulocyte lysate and K562 cell cytosol (600 µg/lane) were electrophoresed 8% SDS-PAGE gels. Proteins were transferred to nitrocellulose and incubated with anti-REP1 (1:400 dilution) in 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5% bovine serum albumin, and 0.02% Tween 20 for 1 h. Immunocomplexes were detected with goat anti-rabbit IgG-linked alkaline phosphatase as described under “Experimental Procedures.”

To verify that Rab5WT and the 45-kDa GDI isoform exist in the predicted 1:1 complex in the reticulocyte lysate, gradient fractions containing biosynthetically biotinylated and prenylated Rab5WT were incubated with streptavidin-linked agarose beads. Proteins captured by the beads were electrophoresed and transferred to nitrocellulose, and the immunoblot was then probed with anti-GDI2 antibodies. Autoradiography confirms the isolation of 35S-labeled biotin-Rab5 on the streptavidin-linked beads as demonstrated by the results of Fig. 6 (left panel). Furthermore, biotin-Rab5 bound to the streptavidin-agarose was indeed associated with the 45-kDa GDI2-like species (right panel). These results indicate that complex formation does not interfere with the binding of the biotinylated product to the beads. Conversely, biosynthetic biotinylation apparently does not disrupt Rab5-GDI1 interactions. Incubation of immunoblots of the same sample with anti-REP1 antibodies failed to detect this protein in complex with biotin-Rab5 (data not shown). These observations are consistent with the apparent Mₐ of prenylated Rab5 on sucrose gradients and confirm that the sedimentation pattern observed is due to a 1:1 complex of the newly synthesized product with the GDI2-like isoform.

Rab5-REP Complexes May Be Transient in Nature—In an attempt to identify Rab5-REP complexes that may be formed during early stages of co- and post-translational processing, time course experiments were performed. Aliquots of in vitro translation and prenylation reactions were removed at the times indicated in Fig. 7 and fractionated on sucrose gradients. As shown by sedimentation profiles of nascent and post-translationally modified Rab5, even at very early times with less than 5% of the 35S-labeled protein processed, the GTP-binding protein sediments with an apparent Mₐ of ~25,000 corresponding to the unprocessed isoform. Upon geranylgeranylation of the nascent peptide, association with GDI is quite rapid as noted by the appearance of the 70-kDa complex in the gradient with 35S-labeled Rab5. Larger molecular mass complexes due to Rab5-REP association are not observed during early or late
lysate was precleared at 350,000 g followed by its immediate retrieval by GDI, the reticulocyte lysate provides for the release of Rab5 from REP. However, Alexandrov et al. (19) were also able to demonstrate that REP can mediate the transfer of Rab5 to membranes. Therefore, to rule out the possibility that contaminating membranes provide for the release of Rab5 from REP followed by its immediate retrieval by GDI, the reticulocyte lysate was precleared at 350,000 g for 15 min prior to prenylation reactions. Results obtained with precleared lysate were found to be identical to those shown in Figs. 2 and 5 (data not shown).

To further investigate potential Rab5-REP complex formation during synthesis and processing, a series of Rab5 mutants was studied. We have previously demonstrated that a GTPase-defective mutant, Rab5S34N, is poorly processed in vitro (29). For the purposes of this study, we further investigated the post-translational processing of Rab5S34N, a point mutant that constitutively binds GDP (28). Fig. 8 compares the time course of prenylation determined for Rab5WT, Rab5S34N, and Rab5Q79L.

Our results clearly contradict the idea that, subsequent to prenylation, newly synthesized Rab5 remains associated with membranes. Therefore, to rule out the possibility that contaminating membranes provide for the release of Rab5 from REP followed by its immediate retrieval by GDI, the reticulocyte lysate was precleared at 350,000 g for 15 min prior to prenylation reactions. Results obtained with precleared lysate were found to be identical to those shown in Figs. 2 and 5 (data not shown).

For these experiments, translated peptides were adjusted to 10 nM in the in vitro prenylation reaction and 100 μM mevalonate was added. Aliquots of each reaction were removed, quenched in Laemmli buffer, and electrophoresed on urea-acrylamide gradient gels (panel A). The amount of modified product was determined by densitometric scanning of the unprocessed (upper band) and prenylated (lower band) isoforms. As shown in panel B, Rab5S34N is modified with the same rate and efficiency as wild type, unlike Rab5Q79L. This observation confirms that the GDP-bound state of Rab5 is indeed the preferred conformation for geranylgeranyl transferase function. Thus, Rab5S34N is predicted to interact preferentially with REP. Nonetheless, sucrose density gradient analysis (Fig. 9) demonstrates that Rab5S34N sediments in the same 70-kDa complex observed for wild type. The hydrolysis-defective mutant Rab5Q79L exhibits the same sedimentation behavior. Finally, a C-terminal truncation mutant, Rab5Δ211, which lacks the cysteine acceptor sites for prenylation, was also translated and fractionated on a sucrose density gradient. Although this peptide is an inhibitor of Rab5WT prenylation in vitro (29), most
likely due to competitive interactions with REP, only a M₀ ~25,000 isofrom is observed in the gradient sedimentation analysis (Fig. 9). Rab5S34N does not associate with the GD12-like isofrom, since this interaction would require prenylation of C-terminal cysteines (21). We conclude that stable Rab5-REP complexes cannot be identified by sucrose density ultracentrifugation and that the association between Rab5 and REP may be transient in nature. All of our results are consistent with the rapid transfer of newly synthesized and prenylated Rab5 from the REP/geranylgeranyl transferase II complex to the 45-kDa GDI. An alternate explanation of our data is that the GD12 isofrom may actually provide the Rab escort function during post-translational modifications and that the association between Rab5 and REP may appear to be unimpaired (29). Thus, Rab5-REP can serve as a novel reagent to capture and identify factors that regulate GTP-binding protein's activity. Our results demonstrate that the only GDI isoform present in reticulocyte lysate, a 45-kDa GDI2-like species, forms a complex with nascent prenylated biotin-Rab5. This complex sediments as a 70-kDa particle and can be captured on streptavidin-linked agarose beads through biotin adduct(s) on Rab5.

Cytosolically disposed Rab proteins are known to complex with GDIs (23, 28). Rab3A GDI was first purified from bovine brain based on its ability to decrease the rate of GDP release (20). A closely related 55-kDa homolog, GD11, and a second isoform of 45-kDa, GD12, were subsequently identified by molecular cloning of mouse GDIs (28). Nishimura et al. (37) have also cloned two GDI isofroms from rat brain, called GD1α and GD1β. The latter shares amino acid sequence with a 47-kDa protein purified from CHO cells (38); thus, two classes of GDI isofroms are known to exist: mouse GD12, rat GD1β, and the 47-kDa CHO GDI represent lower molecular mass isofroms, while bovine Rab3A GDI, mouse GD11, and rat GD1α display higher mobility on SDS gels and closer sequence similarity (38). This is clearly a multigene family, and at least five distinct mouse GDI genes are predicted based on Southern analysis (39). Our results show that Rab5 associates with a GD12-like isofrom that can be detected, this result is not surprising. Shishheva et al. (28) have demonstrated that both GD11 and GD12 extract membrane-bound Rab5 with the same efficiency. Furthermore, the results of Yang et al. (38) indicate that Rab proteins partition to form complexes with the most abundant GDI isoform available. The latter observation suggests there is little functional difference between the various GDIs, an idea that is strongly supported by biochemical studies (28, 37).

One unexpected finding made in our study is the observation that newly synthesized Rab5 associates with the reticulocyte GDI. The inability to detect a predominant Rab5-REP complex is particularly perplexing, since Zerial and co-workers have previously documented that Rab5 remains associated with REP upon prenylation (19). In the latter study, the complex between REP and Rab5 was identified by gel filtration chromatography over Superose 12; accordingly, we also have fractionated the products of our in vitro synthesis and prenylation reaction over Superose 12 but do not observe the hallmark ~120-kDa complex to distinguish REP-Rab5 interactions. A singular difference between our study and the report by Alexandrov et al. (19) is that while the reticulocyte lysate contains the necessary complement of factors required for post-translational modification, the latter group employed purified components, and therefore GDI was not available during the course of Rab5 prenylation. Subsequent to its prenylation, Alexandrov et al. (19) demonstrated the apparent release of Rab5 from REP to membranes. However, this process potentially could have been mediated by a membrane-associated form of GDI, consistent with its established role in Rab protein targeting (23). Although REP is thought to associate with both prenylated and closely related bovine Rab3A GDI cannot substitute for REP in in vitro assays of geranylgeranyl transferase II function (19).

DISCUSSION

Biosynthetic biotinylation of Rab5 can be accomplished by its in vitro synthesis in the presence of biotin-lysin tRNA. Since incorporation of biotin-lysin does not interfere with the post-translational modification of Rab5 with geranylgeranyl groups, the guanine nucleotide binding properties of biotin-Rab5 appear to be unimpaired (29). Thus, Rab5-REP can serve as a novel reagent to capture and identify factors that regulate GTP-binding protein's activity. Our results demonstrate that the only GDI isoform present in reticulocyte lysate, a 45-kDa GDI2-like species, forms a complex with nascent prenylated biotin-Rab5. This complex sediments as a 70-kDa particle and can be captured on streptavidin-linked agarose beads through biotin adduct(s) on Rab5.

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non-prenylated Rab proteins (18), our findings agree with evidence characterizing the ability of GDI to recognize only prenylated Rabs (22), particularly since non-modified Rab5 and Rab51–211 do not assemble into the multimeric 70-kDa complex.

If REP were to mediate the association of a newly synthesized Rab protein with membranes, one would predict that a round of GTP hydrolysis would be required prior to its retrieval and binding by GDI. This idea stems from observations that reveal the concomitant exchange of GDP for GTP upon membrane binding of Rab proteins and the selective recruitment of GDP-bound Rabs by GDI from membranes (24–27). Therefore, two additional lines of evidence argue that GDI serves as a GDP-bound Rabssby GDI from membranes (24–27). Therefore, our results suggest that GTP hydrolysis is not required for association of nascent Rab proteins with GDI. Moreover, since the kinetics of Rab5$^{34N}$ prenylation are identical to those observed for the wild-type protein, GTPase function may not be required for post-translational prenylation. This issue is raised because in contrast to Rab5WT and Rab5$^{34N}$, the GTPase-defective mutant Rab5Q79L is insufficiently processed.

Based on the results of the present study, this effect is most likely due to the predominant GTP-binding state of this mutant. Although we cannot completely rule out the possibility of Rab5-GAP activity capable of stimulating GTP hydrolysis by Rab5Q79L, this conclusion is consistent with our previous data, which indicated that the GDP-bound form of Rab5 is the preferred substrate conformation for the Rab geranylgeranyl transferase and possibly REP interactions (29). These observations suggest that Rab5’s GTPase activity does not play a major role in its post-translational modification and insertion into membranes.

Finally, our study clearly demonstrates the utility of biosynthetically biotinylated Rab5 complexes with GDI as an approach to capturing Rab protein accessory factors. Biotin-Rab5 provides a useful tool to isolate key factors involved in vesicle transport. Candidate elements include guanine nucleotide exchange factors and GAPs, which are thought to interact specifically with individual Rab family members (40, 41). Future experiments will focus on endosomal membrane proteins that may be identified associated with biotin-Rab5 and captured on avidin-linked beads, much like the soluble complex with reticulocyte lysate GDI isolated and characterized in this study.

REFERENCES

1. Plutner, H., Cox, A. D., Pind, S., Khosravi-Far, R., Bourne, J. R., Schwaninger, R., Der, C. J., and Balch, W. E. (1991) J Cell Biol 115, 31–43
2. Gorvel, J.-P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) Cell 64, 913–925
3. Huber, L. A., Pimplikar, S., Parton, R. G., Viruta, H., Zerial, M., and Simons, K. (1993) J Cell Biol 123, 35–45
4. Lombardi, D., Soldati, T., Riederer, M. A., Goda, Y., Zerial, M., and Pfeffer, S. R. (1993) EMBO J 12, 3635–3642
5. Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K., and Zerial, M. (1990) Cell 62, 317–329
6. Bucci, C., Parton, R. G., Mather, J., Sturzenberg, H., Simons, K., Hofack, B., and Zerial, M. (1992) Cell 70, 715–728
7. Li, G., and Stahl, P. D. (1993) J Biol Chem 268, 24475–24480
8. Stenmark, H., Parton, R. G., Steede-Mortimer, O., Lütöke, A., Gruenberg, J., and Zerial, M. (1994) EMBO J 13, 1287–1296
9. Hoffenberg S., Sanford, J. C., Liu, S., Daniel, D. S., Tuvin, M., Knol, B. J., Westling-Resnick, M., and Dickey, B. F. (1995) J Biol Chem 270, 5048–5056
10. Brennwald, P., Kearns, B., Champion, K., Keränen, S., Bankaitis, V., and Novick, P. (1994) Cell 79, 245–258
11. Farum, M. C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M. H., and Glomset, J. A. (1991) Proc Natl Acad Sci U S A 88, 6196–6200
12. Kisselev, B. T., and Maltese, W. A. (1991) J Biol Chem 266, 8540–8544
13. Seabra, M. C., Goldstein, L. J., Südhof, T. C., and Brown, M. S. (1992) J Biol Chem 267, 81–88
14. Seabra, M. C., Reiss, Y., Casey, P. J., Brown, M. S., and Goldstein, L. J. (1991) Cell 65, 429–434
15. Seabra, M. C., Brown, M. S., Slaughter, C. A., Südhof, T. C., and Goldstein, J. L. (1992) Cell 70, 1049–1057
16. Andres, D. A., Seabra, M. C., Brown, M. S., Armstrong, S. A., Smelant, T. E., Creners, F. P. M., and Goldstein, J. L. (1993) Cell 73, 1091–1099
17. Alexander, K., Horiuichi, H., Steele-Mortimer, O., Seabra, M. C., and Zerial, M. (1994) EMBO J 13, 5262–5273
18. Araki, S., Kikuchi, A., Hata, Y., Isomura, M., and Takai, Y. (1990) J Biol Chem 265, 13007–13015
19. Araki, S., Kikuchi, K., Sasaki, T., Hata, Y., and Takai, Y. (1991) Mol Biol Cell 2, 1348–1347
20. Reiss, J., Kikuchi, A., Takai, Y., and Wollheim, C. B. (1992) J Biol Chem 267, 17512–17519
21. Dirac-Svejstrup, A. B., Soldati, T., Shapiro, A. D., and Pfeffer, S. R. (1994) J Biol Chem 269, 15427–15430
22. Ulrich, O., Horiuichi, H., Bucci, C., and Zerial, M. (1994) Nature 368, 157–160
23. Soldati, T., Shapiro, A. D., Dirac-Svejstrup, A. B., and Pfeffer, S. R. (1994) Nature 369, 76–78
24. Ulrich, O., Stenmark, H., Alexander, K., Huber, L. A., Kikuchi, K., Sasaki, T., Takai, Y., and Zerial, M. (1993) J Biol Chem 268, 18143–18150
25. Soldati, T., Riederer, M. A., and Pfeffer, S. R. (1993) Mol Biol Cell 4, 425–434
26. Shiroma, A., Südhof, T. C., and Czech, M. P. (1994) Mol Cell Biol 14, 3459–3468
27. Sanford, J. C., Pan, Y., and Westling-Resnick, M. (1993) J Biol Chem 268, 8173–8177
28. Kunkel, T. A., Robert, J. D., and Zakour, R. A. (1987) Methods Enzymol 154, 367–382
29. Bradford, M. M. (1976) Anal Biochem 72, 248–254
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. Westling-Resnick, M., and Braell, W. A. (1990) J Biol Chem 265, 16751–16759
32. Kurzchalia, T. V., Gorvel, J.-P., Dupree, P., Parton, R., Kelner, R., Houtheeve, T., Gruenberg, J., and Zerial, M. (1992) J Biol Chem 267, 18419–18423
33. Shirakata, H., Kikuchi, K., Yamaguchi, T., Wada, K., Horiuichi, H., and Takai, Y. (1992) J Biol Chem 267, 10946–10949
34. Sanford, J. C., Foster, L., Kapadia, Z., and Westling-Resnick, M. (1995) Anal Biochem 234, 547–556
35. Nishimura, N., Nakamura, H., Takai, Y., and Sano, K. (1994) J Biol Chem 269, 14191–14198
36. Yang, C., Slepnev, V. I., and Goud, B. (1994) J Biol Chem 269, 31891–31899
37. Janoueix-Lerosey, I., Jollivet, F., Camonis, J., Marche, P. N., and Goud, B. (1995) J Biol Chem 270, 14801–14808
38. Burstein, E. S., Linko-Stentz, K., Lu, Z., and Macara, I. G. (1991) J Biol Chem 266, 2689–2692
39. Horichi, H., Giner, A., Hofack, B., and Zerial, M. (1995) J Biol Chem 270, 11257–11262