Modification of Rab5 with a Photoactivatable Analog of Geranylgeranyl Diphosphate*

Received for publication, May 15, 2001, and in revised form, July 9, 2001
Published, JBC Papers in Press, August 24, 2001, DOI 10.1074/jbc.M104398200

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A photoprobe analog of geranylgeranyl diphosphate (2-diazo-3,3,3-trifluoropropionyloxy-farnesyl diphosphate or DATFP-FPP) inhibits mevalonate-dependent prenylation of in vitro translated Rab5 in rabbit reticulocyte lysate, suggesting that it competes for lipid binding to the Rab geranylgeranyl transferase. Modification of Rab5 with DATFP-FPP, demonstrated by gel mobility shift and Triton X-114 phase separation experiments, confirms that the enzyme uses the analog as a substrate. The sedimentation of DATFP-modified Rab5 as a larger mass complex on sucrose density gradients indicates that it binds to other factors in rabbit reticulocyte lysate. Most importantly, DATFP-Rab5 cross-links to these soluble factors upon exposure to UV light. Immunoprecipitation with antibodies raised against proteins known to interact with Rab5 reveals that the cross-linked complexes contain Rab escort protein and GDI-1. DATFP-Rab5 also associates with membranes in a guanosine-5’-O-(3-thiotriphosphate)-stimulated manner. However, although prenylated Rab5 can be cross-linked to two unknown membrane-associated factors by the chemical cross-linker disuccinimidyl suberate, these proteins fail to be UV cross-linked to membrane-bound DATFP-Rab5. These results strongly suggest that membrane-associated factors bind Rab5 through protein-protein interactions rather than protein-prenyl interactions. The modification of Rab5 with DATFP-FPP establishes a novel photoaffinity technique for the characterization of prenyl-binding sites.

Many mammalian proteins are post-translationally modified with prenyl groups, including the Ras-like superfamily of small molecular weight GTPases (1). Farnesyl (15-carbon) or geranylgeranyl (20-carbon) groups are covalently attached to such proteins via a thioether linkage to cysteine residues near the C terminus. Three different enzymes are known to catalyze these irreversible modification reactions. Protein farnesyl transferase (PFT) uses farnesyl diphosphate to prenylate Ras, the nuclear lamins, and other proteins with the C-terminal consensus sequence CaaX (where a is any aliphatic residue and X is Met, Ser, or Gln). Protein geranylgeranyl transferase type I (PGGTI) modifies members of the Rho family and most of the γ-subunits of the heterotrimeric G proteins using geranylgeranyl diphosphate. These proteins have a different C-terminal CaaX sequence where X is typically Leu. Finally, newly synthesized members of the Rab family of small GTPases bind to the Rab escort protein (REP) to form a complex recognized by Rab geranylgeranyl transferase (RabGGT, also known as PGGTI). This enzyme then modifies the Rab proteins at both cysteines in the C-terminal sequences XXCC, CXC, or CCXX, where X can be any amino acid residue.

Prenylated proteins require post-translational modification for their cellular function and membrane binding (2–4). The magnitude of the hydrophobicity of the modification has been shown to be an important determinant of prenylated protein function, suggesting that hydrophobicity mediates membrane attachment (5). However, a binding activity for prenylated Ras in the plasma membrane has been characterized that can be inhibited by isoprenoid analogs (6). Furthermore, prenylated peptides bind microsomal membranes with high affinity (Kd = ~30 nM) (7). Both of these results support the idea that specific membrane factors recognize the lipid moiety itself. The importance of prenylation for cytosolic protein-protein interactions is also well established. For example, prelamin A must be prenylated for recognition by the endoprotease activity that catalyzes its processing to a mature form (8). Furthermore, Rho and Rab proteins interact with their soluble guanine nucleotide dissociation inhibitors (GDIs) only when post-translationally modified (9, 10). The nature of such protein-lipid interactions is only beginning to become understood. Recently, photoprobe analogs of the isoprenoid diphosphates have been used to study protein-prenyl interactions. The ω-isoprenyl units of these photoprobe analogs are replaced with a diazotrifluoropropionyloxy group (Fig. 1). Previous work has shown that these analogs competitively inhibit purified or recombinant PFT and PGGTI and cross-link to their β subunits upon activation with UV light (11–13). Yeast PFT has been further shown to use the farnesyl analog as a substrate for the in vitro modification of recombinant Ras protein (14). One aim of this investigation was to determine whether RabGGT recognizes the geranylgeranyl DATFP analog as a substrate to modify Rab proteins, specifically Rab5, in a similar fashion. Rab5 facilitates both receptor-mediated and fluid phase endocytosis in vivo (15, 16) and stimulates homotypic endosome fusion in vitro (17, 18). The precise role of Rab5 in these events is not yet fully understood; however, the function of Rab pro-inhibitor; GTPγS, guanosine-5’-O-(3-thiotriphosphate); PGGTI, protein geranylgeranyl transferase type I; RabGGT, Rab geranylgeranyl transferase; REP, Rab escort protein; TBS, Tris-buffered saline.
teins is closely tied to their GTPase cycle (19, 20). Cytosolic
Rabs, in their GDP-bound form, exist as a complex with GDI
(21). Delivery of Rabs to membranes by GDI requires a GDI
displacement factor, which dissociates the Rab-GDI complex
and places the Rab in the membrane. A guanine nucleotide
exchange factor then catalyzes the release of GDP and the
binding of GTP. In the active GTP-bound form, Rab proteins
bind and recruit the protein components of the machinery
that mediates budding, movement, and fusion of membrane vesi-
cles. After membrane fusion, a GTPase-activating protein ac-
celerates the rate of GTP hydrolysis by the Rab to regenerate
its GDP-bound form. In a process hypothesized to involve a
membrane-bound Rab-GDI recycling factor (22), GDI then binds
the GDP-bound Rab from the membrane for return to its donor
compartment. Although it is established that Rab protein pre-
nylation is required for association with GDI and membranes
(2), it is not known whether protein-prenyl interactions are
involved in other steps of the GTPase cycle. The modification of
Rab5 with the photoactivatable isoprenoid analog DATFP-FPP
reported here is the first step toward this analysis employing
UV cross-linking techniques.

**EXPERIMENTAL PROCEDURES**

**Materials—**DATFP-FPP was chemically synthesized as described
previously (12) and stored at −20 °C in the dark in 2 mM NH4OH at 485
μM. [35S]Methionine (1175 Ci/mmol) was purchased from PerkinElmer
Life Sciences. RNAsin, RQ1 DNase, and rabbit reticulocyte lysate were
from Promega (Madison, WI). Disuccinimidyl suberate (DSS) was pur-
chased from Pierce and prepared as a 10 mg/ml stock solution in Me2SO
just before use. Triton X-114 was purchased from Roche Molecular
Biochemicals and prepared as a 10% stock in Tris/NaCl/EDTA as de-
scribed (23). A rabbit polyclonal antisera was raised (Pocono Rabbit
Farm and Laboratory, Canadensis, PA) against recombinant human
Rab5. Bovine Rab5A GDI (24), GDI2-peptide (25), and REP1 (26) anti-
bodies were generously provided by Drs. Suzanne Pfeffer (Stanford
University, CA), Assia Shisheva (Wayne State University, MI), and
Miguel Seabra (Imperial College, UK), respectively. Anti-GDI-1 peptide
antibody was purchased from Zymed Laboratories Inc. (San Francisco,
CA).

**In Vitro Biosynthesis and Prenylation of Rab5—**The plasmids pAGA-
Rab5 and pAGA-Rab5 were purified by CsCl density ultracent-
trifugation and linearized with HindIII (27). Run-off transcripts were
synthesized using T7 RNA polymerase. Rabbit reticulocyte lysate con-
taining 20 μM amino acids and 20 mM KCl was programmed with these
transcripts (90 μg/ml to translate peptides in the presence of [35S]methi-
ionine (200,000 cpm/μl for characterization and 800,000 cpm/μl for
cross-linking experiments). Translation reactions to prepare proteins
for cross-linking experiments also contained protease inhibitor cocktail
(1 μg/ml each of phenylmethylsulfonyl fluoride, aprotinin, leupeptin,
and pepstatin A). After incubation for 30 min at 30 °C, translation was
terminated with addition of 50 μg/ml RNase A. The amount of peptide
synthesized was determined by trichloroacetic acid precipitation. Post-
translational modification with mevalonate or DATFP-FPP was per-
formed at 37 °C. These prenylation reaction mixtures included 40% (v/v)
rabbit reticulocyte lysate, 12 mM Tris-Cl, pH 7.5, 3 mM MgCl2, 0.6
mM dithiothreitol, and 50 μg/ml RNase A.

**Triton X-114 Extractions—**A 2-μl aliquot of protein was diluted into
200 μl of 1% Triton X-114 in NaCl/EDTA. The samples were vortexed
and incubated on ice for 1 h. Phase separation was induced by
incubation at 37 °C for 3 min and centrifugation at 10,000 × g for 1 min
at room temperature (23). The top aqueous phases were transferred
in new tubes and re-extracted with 20 μl of 1% Triton X-114 in TBS. The
lower detergent phases were re-extracted with 100 μl of TBS. The final
phases were made up to the same volume and detergent concentration
by adding 200 μl of TBS to the detergent phases and 20 μl of 10% Triton
X-114 in TBS to the aqueous phases. Fifty-μl samples of each fraction
were mixed with Laemmli buffer before characterization on a urea-
acrylamide gradient gel.

**Sucrose Density Gradient Analysis—**Reticulocyte lysate containing
radioactive Rab5 was fractionated on 4.8 ml of 5–20% continuous
sucrose gradients in 50 mM HEPES, pH 7.5, 1 mM MgCl2, 1 mM dithi-
obreitol, and 5 μM GDP (28). After ultracentrifugation at 165,000 × g
for 17 h at 4 °C using a Beckman SWTI 55 rotor, 150-μl fractions were
collected from the bottom of the gradient. Thirty-μl aliquots of odd-
numbered fractions were analyzed on a urea-acrylamide gradient gels.
To determine the molecular mass of complexes in the sucrose gradients,
the following standard proteins were used: carbonic anhydrase (29
kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), and aldol-
ase (158 kDa).

**Membrane Binding Assay—**Prenylated protein was desalted into
assay buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 85 mM sucrose, 20 μM
EGTA) through Micro Bio-Spin 6 chromatography columns (Bio-Rad) at
4 °C. The concentration of radioabeled protein was determined by
trichloroacetic acid precipitation. The membranes were prepared from
K562 cell post-nuclear supernatant by centrifugation at 16,000 × g
for 15 min at 4 °C (29). The protein concentration was determined by
the Folin/Lowry assay. 35S-Labeled Rab5 (3 nM) was mixed with mem-
branes (2 mg/ml) and incubated at 37 °C for 30 min in the presence of
protease inhibitor cocktail and 1 mM GDP or GTPγS. Membrane pellet
and supernatant fractions were isolated by centrifugation (16,000 × g,
4 °C, 15 min). The pellets were washed and resuspended with an equal
volume of assay buffer. To determine the fraction of membrane bound
35S-labeled Rab5, the pellet and supernatant fractions were separated
on urea-acrylamide gradient gels, and the amount of modified protein in
the dried gel was quantified by phosphorimaging analysis (Bio-Rad).

**Cross-linking—**Proteins were chemically cross-linked using 0.5
mg/ml DSS for 1 h on ice. The reaction was stopped by adding 50 mM
Triton X-114, pH 7.4, and incubating on ice for 30 min. For UV cross-linking,
the proteins were diluted 10-fold in assay buffer into quartz cuvettes
and exposed to a short wave (254 nm) 4-W UV lamp at a 1-cm distance
for 1 h at 4 °C.

**Immunoprecipitation—**The samples were adjusted to 1.0% Triton
X-100, diluted to 500 μl with IP buffer (assay buffer with 1.0% Triton
X-100 containing protease inhibitor cocktail, vortexed, and incubated
on ice for 60 min. Five-μl aliquots of the appropriate antisera (Rab5,
GDI-1, GDI-2, or REP) or 50 μg of purified antibody (GDI) was added,
and incubation on ice continued for 90 min. Nonspecific complexes were
removed by centrifugation (10,000 × g, 4 °C, 10 min), and 100 μl of 10%
(v/v) protein A-agarose (Calbiochem, La Jolla, CA) in IP buffer was
added. The samples were then rocked at 4 °C for 1 h. The beads were
collected (10,000 × g, 4 °C, 1 min) and washed three times with 1 ml of

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**Fig. 1. Structure of the DATFP-isoprenoid photoprobe.** The structures of two isoprenoid diphenolates (OPP) and their photoactivatable
analogues are depicted. OPP, geranyl diphenol; OPP, farnesyl diphenol; OPP, geranylglycerol diphenol.

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brane binding reactions were analyzed by urea-acrylamide gradient gel electrophoresis (30). Briefly, a continuous gradient of 4–8% urea and 10–15% acrylamide was formed in the absence of SDS. The samples were prepared in Laemmli buffer but were not boiled. These gels were fixed and dried before exposure to phosphorimaging screens. Cross-linked products were characterized on gels with a continuous gradient of 5–15% acrylamide containing 0.1% SDS. The gels were processed for fluorography by saturating them in Me₂SO followed by a 5-min incubation in 20% (w/v) 2,5-diphenyloxazole in the same solvent. The gels were then washed extensively with water before they were dried and exposed to film. The following molecular size markers were used to calibrate gels: cytochrome c (12.5 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97 kDa), and β-galactosidase (116 kDa).

**RESULTS**

**DATFP-FPP Inhibits Rab5 Prenylation and Modifies Rab5 in Vitro**—Allen and co-workers (12) have previously demonstrated that the photoactivatable analog of geranylgeranyl diphosphate, DATFP-FPP, inhibits PGGTI activity in vitro. To determine the effect of DATFP-FPP on RabGGT activity, the analog was added to prenylation reaction mixtures containing in vitro translated [35S]-labeled Rab5. Reaction products were analyzed using a urea-acrylamide gradient gel system that separates prenylated product from the less mobile unmodified Rab5 (Fig. 2A). Rab5 is prenylated in a temperature-dependent manner (lane 1 versus lane 4). A fraction of the Rab5 (∼20%) is modified by endogenous lipid donors, but the addition of mevalonate to the assay effects complete prenylation of the [35S]-labeled protein (lane 2 versus lane 4). However, the presence of increasing concentrations of DATFP-FPP reduces the amount of prenylated product (lanes 4–8). An increase in the amount of unmodified substrate is observed as well as the appearance of a new species of intermediate mobility. Thus, the photoprobe inhibits native prenylation of Rab5 by rabbit reticulocyte RabGGT. Because DATFP-FPP competitively inhibits PGGTI activity (12), it is likely that the analog also competes as a lipid substrate for RabGGT. The mobility-shifted reaction product that appears in the presence of DATFP-FPP supports this idea. This product is more apparent in the reactions that are not supplemented with mevalonate (lane 3). With or without mevalonate, this radiolabeled species represents roughly 50–60% of the total material. The unique band does not represent a normal intermediate in the post-translational modification of Rab5 because it is not observed in assay reactions containing other inhibitors of RabGGT activity, such as N-acetyl-S-geranylgeranyl-cysteine or N-acetyl-S-farnesyl-cysteine (Fig. 2B). Thus, the appearance of this species of intermediate mobility in assays containing DATFP-FPP strongly suggests that RabGGT uses the photoprobe as a substrate to modify Rab5.

To verify that Rab5 is modified with the hydrophobic photoprobe, the protein product was extracted with Triton X-114 for phase separation experiments. As shown in Fig. 3, unmodified Rab5 (that is, untreated after translation) fails to partition into the detergent phase and is exclusively found in the aqueous phase. In contrast, the majority of prenylated protein can be extracted into the detergent phase. The [35S]-labeled product of intermediate mobility produced in the presence of DATFP-FPP partitions into the detergent phase to the same extent as prenylated Rab5. Thus, this Rab5 band of intermediate mobility must represent protein that has been modified by the hydrophobic isoprenoid, DATFP-FPP. It should be noted that the stoichiometry of DATFP modification could not be determined from this analysis. The gel mobility of monogeranylgeranylated Rab51–212 is the same as digeranylgeranylated Rab5WT (30), suggesting that the band of intermediate mobility is not necessarily a monoprenylated form of Rab5. Thus, the altered gel mobility likely reflects the more polar nature of DATFP, whether Rab5 is modified with one or two groups.

**DATFP-Rab5**

Fig. 2. In vitro modification of Rab5 with DATFP-FPP. Rabbit reticulocyte lysate was programmed with Rab5WT mRNA in the presence of [35S]methionine (0.2 mM). The concentration of Rab5, as determined by trichloroacetic acid precipitable counts, was adjusted to 6 mM in the absence (−) or presence (+) of 10 µM mevalonate with the indicated concentrations of DATFP-FPP (0, 1, 5, 10, and 50 µM) (A) or 0.5 mM N-acetyl-S-geranylgeranyl-cysteine or N-acetyl-S-farnesyl-cysteine (B). Incubations were performed at 4 or 37 °C under reduced room light for 1 h. A 3-µl aliquot from each sample was diluted into 77 µl of Laemmli buffer, and half of these mixtures were electrophoresed on a urea-acrylamide gradient gel. The gel was fixed, dried, and exposed to a phosphorimaging screen for 3 h. AGGC, N-acetyl-S-geranylgeranyl-cysteine; AFC, N-acetyl-S-farnesyl-cysteine.

Fig. 3. Triton X-114 extraction of DATFP-Rab5. Rab5 was translated and [35S]methionine-labeled as described in the legend to Fig. 2, diluted to 16 µM, and left unmodified on ice or prenylated with 24 µM mevalonate or DATFP-FPP at 37 °C for 1 h. The samples were extracted as described under “Experimental Procedures.” Fifty-µl aliquots of the final aqueous (A) and detergent (D) phases of each sample were mixed with Laemmli buffer and electrophoresed on a urea-acrylamide gradient gel. The dried gel was exposed to a phosphorimaging screen overnight.
both prenylated and DATFP-modified Rab5 sediment in a larger mass complex. These results suggest that like prenylated Rab5 (28), DATFP-Rab5 can form a complex with other reticulocyte lysate proteins. It is possible that the larger mass complex with DATFP-Rab5 reflects an oligomer of Rab5 molecules interacting via the hydrophobic modification. However, prenylated Rab5 that is not complexed to other proteins is insoluble (31). To directly determine whether reticulocyte lysate factors bind to DATFP-Rab5 and are in direct contact with the prenyl analog, protein-prenyl cross-linking was performed by UV irradiation. As shown in Fig. 5, several UV cross-linked products containing 35S-labeled Rab5 are detected. Importantly, these complexes are specific because they are not observed when DATFP-Rab5 was left in the dark. DATFP-Rab5 therefore binds and UV cross-links to cytosolic proteins in the rabbit reticulocyte lysate in a prenylation-dependent manner.

Membrane Association of DATFP-Rab5 Is Nucleotide-dependent—One hallmark of Rab activity is the coupled process of membrane binding and nucleotide exchange (GDP for GTP) (32, 33). GTP*S, the slowly hydrolyzable analog of GTP, is known to enhance the membrane binding of Rab proteins, stabilizing the active membrane-bound form and reducing the amount of GDP-bound Rab available for GDI-mediated release (33). Therefore, to test the ability of DATFP-Rab5 to bind membranes, prenylated or DATFP-modified Rab5 in rabbit reticulocyte lysate was mixed with a crude membrane preparation in the presence of GDP or GTP*S. After a 30-min incubation at 37°C, membrane and supernantant fractions were separated by centrifugation. The amount of modified Rab5 in each fraction was determined by phosphorimaging proteins separated by gel electrophoresis. The Rab51−211 truncation mutant is not recruited to the membrane fraction in control assays (data not shown); therefore binding activity requires the prenylation of Rab5. Membrane binding of both native prenylated and DATFP-Rab5 is enhanced by GTP*S (Fig. 6); however, the overall extent of DATFP-Rab5 binding is reduced relative to prenylated Rab5 in the presence of either GDP or GTP*S. The DATFP-Rab5 bound to membranes does not represent contaminating mono- or di-prenylated Rab5 because the isoprenoid photoprobe completely inhibits native prenylation under the conditions used to produce 35S-labeled protein for this assay (Fig. 2A, lane 3). Therefore, DATFP-Rab5 binds membranes in a nucleotide-dependent fashion, albeit to a lesser extent than the native prenylated Rab5.

DATFP-Rab5 Binds but Does Not UV Cross-link to Membrane Proteins—The ability of membrane-bound DATFP-Rab5 to UV cross-link proteins was compared with the chemical cross-linking of native prenylated Rab5 with disuccinimidyl suberate (DSS). DSS is a bifunctional amine-reactive lipid-soluble reagent with an 11.4-angstrom linker. In contrast, DATFP will UV cross-link at a 2–3-angstrom distance. Briefly, membrane fractions with Rab5 bound as described above were recovered by centrifugation after UV or DSS cross-linking. Detergent-solubilized Rab5 complexes were then immunoprecipitated and separated by gel electrophoresis. As shown in Fig. 7, DSS promotes cross-linking of both native prenylated Rab5 and DATFP-Rab5 into two complexes of ~45 and 70 kDa in mass. These results confirm that DATFP-Rab5 binds to the same membrane-associated factors as native prenylated Rab5 and is thus appropriately oriented in the membrane. However, UV irradiation does not promote cross-linking of DATFP-Rab5 into these or any other complexes. If membrane-associated proteins directly interact with the prenyl moiety, additional bands would have been expected upon UV irradiation of DATFP-Rab5. The absence of such bands strongly suggests that protein-prenyl interactions do not occur in the membrane. These results fail to identify any specific “prenyl receptors” and therefore imply that Rab5 interacts with the membrane through hydrophobic interactions between the geranylgeranyl groups and the membrane lipids as well as through protein-protein interactions between Rab5 and membrane-associated factors such as those observed upon DSS cross-linking.

DATFP-Rab5 UV Cross-links to REP and GDI-1—Because DATFP-Rab5 does not UV cross-link to membrane-associated factors, the soluble cross-linked complexes observed in rabbit reticulocyte lysate were further characterized. To identify the soluble proteins that cross-link to Rab5, the supernatant fractions of the membrane binding assays described above were immunoprecipitated using antibodies raised against candidate proteins, GDI-1, GDI-2, and REP (Fig. 8). Antibodies recognizing REP immunoprecipitates a 170-kDa complex containing DATFP-Rab5 (right panel). Interestingly, this complex cannot be detected by chemical cross-linking of native prenylated Rab5 with DSS (left panel). Two different polyclonal antibodies against GDI-1 precipitate 90- and 105-kDa complexes from

**Fig. 4.** DATFP-Rab5 incorporates into larger molecular weight complexes. Rab5WT and Rab51−211 were synthesized in vitro, and the 35S-labeled protein (5 nM) was modified with 10 μM mevalonate or DATFP-FPP for 3 h. Ten-μl aliquots were layered onto continuous 5–20% sucrose gradients and ultracentrifuged at 165,000 × g for 17 h at 4 °C. The fractions (150 μl) were collected from the bottom of the gradient, and 30-μl aliquots of odd fractions were electrophoresed on urea-acrylamide gradient gels that were then fixed, dried, and exposed to phosphorimaging screens for 40 h. The sedimentation positions of standard proteins are indicated at the top of the figure: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), and aldolase (158 kDa).

**Fig. 5.** DATFP-Rab5 cross-links to proteins in rabbit reticulocyte lysate upon exposure to UV light. Ten-μl aliquots of lysate containing 30 nM 35S-labeled Rab5 modified with 48 μM DATFP-FPP were diluted 10-fold. The samples were exposed to UV light with a 254 nm, 4-W bulb in a quartz cuvette at 4 °C for 30 min (+) or left wrapped in aluminum foil on ice (−). The samples were then mixed with Laemmli buffer and electrophoresed on an acrylamide gradient SDS gel. The gel was fixed, dried, and exposed to a phosphorimaging screen for 48 h. The sizes (in kDa) and migration of standard proteins are shown on the left. The calculated molecular masses of the Rab5 complexes are indicated on the right.
FIG. 6. DATFP-Rab5 binds membranes in a nucleotide-dependent manner. 35S-Labeled Rab5 (35 nM) was modified with 24 μM mevalonate or DATFP-FPP for 4 h, and the extent of prenylation was quantified by the mobility shift observed on urea-acrylamide gradient gels (100 and 50%, respectively). The Rab5 mixtures were then desalted into assay buffer and adjusted to the same content of modified Rab5 (3 nM) with 40% (v/v) rabbit reticulocyte lysate. This preparation was incubated at 37 °C for 30 min with K562 cell membranes (2 mg/ml) in the presence of 1 mM GDP or GTP·S. Membrane-bound Rab5 was then separated by centrifugation (16,000 × g at 4 °C for 15 min), and the amount of modified Rab5 in the pellet and supernatant fractions was determined by phosphorimaging analysis of the radiolabeled protein electrophoresed on urea-acrylamide gradient gels. The mean fraction of membrane-bound Rab5 (± S.D., n = 6) is shown.

**DISCUSSION**

Novel photoactivatable prenyl analogs have been synthesized wherein the ω-isoprene unit is replaced with a DATFP group. These compounds have been previously shown to interact with both PFT and PGGTI (11–13), and this study further demonstrates the utility of DATFP-FPP as a substrate analog of Rab geranylgeranyl transferase. The photoprobe isoprenoids have been reported to inhibit purified PFT and PGGTI at concentrations similar to the Kₘ for the native prenyl substrates (0.02 μM) (12), but DATFP-FPP appears to inhibit Rab-GGT in rabbit reticulocyte lysate at higher concentrations (IC₅₀ = ~5 μM). However, the half-maximal concentration of geranylgeranyl diphosphate for Rab5 prenylation in this system is also ~5 μM (data not shown). Importantly, DATFP-FPP can serve as a lipid substrate for Rab5 modification in vitro. The DATFP-modified Rab5 remains functional, binding to cytosolic proteins and associating with membranes in a guanine nucleotide-dependent manner. These features are critical because they permit further analysis of factors that associate with Rab5 using UV cross-linking to capture protein-prenyl interactions.

One goal of these studies was to utilize DATFP-Rab5 to identify protein-prenyl interactions between the GTPase and membrane-associated factors. Thus, the lack of specific UV cross-linked complexes in membrane fractions is disappointing. Although the extent of binding is less than that observed for native prenylated Rab5, DATFP-Rab5 associates with membranes in a nucleotide-dependent manner. Furthermore, it chemically cross-links to the same membrane-associated factors as native prenylated Rab5, an observation indicating that DATFP-Rab5 is properly oriented in the membrane. Thus, it is unlikely that prenyl receptors exist that mediate Rab5 membrane binding through protein-prenyl interactions. Rather, the results of these experiments support the hypothesis that Rab protein prenyl groups associate with the lipid bilayer directly through hydrophobic interactions. These cross-linking experiments do demonstrate protein-protein interactions with Rab5 and other membrane-associated factors. Chemical cross-linking with DSS identifies 45- and 70-kDa complexes containing...
Rab5. Although the results suggest that the formation of these complexes is nucleotide-dependent, a greater amount of GTPyS-bound Rab5 is membrane-associated. Thus, it is not clear whether the cross-linked Rab5 interactors are recruited or bind to the GAPase in a regulated manner. Because there are no obvious candidates for these novel Rab5 membrane interactors, further studies are warranted to define these elements and their interactions with the GAPase.

Despite the lack of membrane-bound complexes in these binding assays, several UV cross-linked complexes were found in the supernatant fractions containing reticulocyte lysate. One of the factors that was clearly identified to be UV cross-linked to DATFP-Rab5 is GDI-1. The observed association supports a large body of evidence demonstrating the ability of GDI to bind to prenylated Rab proteins as a soluble complex (33–36). Although previous attempts to detect GDI-1 in rabbit reticulocyte lysate were unsuccessful, these new data confirm its presence and its association with prenylated Rab5 in this in vitro system. Earlier studies did show that biosynthetically biotinylated Rab5 associates with GDI-2 in rabbit reticulocyte lysate (28). However, immunoprecipitation experiments in the present study fail to detect this interaction despite the presence of a cross-linked species of the expected mass (Figs. 5 and 8). It is possible that the recognition epitope of the anti-GDI-2 antibody is blocked by interactions with native prenylated Rab5 (as detected by chemical cross-linking with DSS) and the DATFP-modified protein (as detected by UV cross-linking). Rab5 can nonetheless be UV cross-linked to GDI-1 through protein-prenyl interactions.

Because the expected mass of a 1:1 complex between Rab5 (24 kDa) and GDI-1 (55 kDa) is 79 kDa, the immunoprecipitation of the larger 90- and 105-kDa cross-linked species may indicate that the Rab5-GDI complex interactors with additional factors. It is unlikely that these much larger cross-linked species result from post-translational modification(s) (such as phosphorylation) of GDI-1 and/or Rab5 (37, 38). In particular, phosphorylation of serine 121 on bovine GDI-1 does not cause an alteration of the apparent electrophoretic mobility of the protein (39). Based on known Rab5 interactors, possible small mass candidate(s) for ternary complex formation with Rab5-GDI include the 17-kDa guanine nucleotide exchange factor M84 (40) and the 24-kDa prenylated Rab acceptor 1 (41). However, preliminary attempts to immunoprecipitate Rab5 cross-linked complexes using M84 or prenylated Rab acceptor 1 antiserum have been unsuccessful (data not shown).

If the speculation that DATFP-Rab5 forms a ternary complex between GDI and additional unknown factor(s) is correct, then the results further suggest that prenyl interactions occur with each of these proteins and Rab5. Because of the fact that the photoactivatable analog cross-links within a 2–3-angstrom distance to a single target, a UV cross-linked ternary complex can only be formed if DATFP-Rab5 is modified at both cysteines and each of the prenyl groups binds to two different proteins. A caveat is that the actual stoichiometry of Rab5 modification with DATFP-FPP cannot be determined from these experiments. Nonetheless, these observations raise the exciting possibility that the 90- and 105-kDa species represent intermediate complexes formed during the GAPase cycle of Rab5. During this process, the protein-prenyl interactions of GDI with the geranylgeranyl groups must become disrupted such that a role for additional prenyl-binding sites could readily be envisioned.

The second factor identified to be UV cross-linked to DATFP-Rab5 is REP, verifying its role in the post-translational prenylation of the GAPase (26). Interestingly, chemical cross-linking with DSS does not capture this complex. These combined observations suggest that the DATFP group must be in direct contact with REP. However, the observed mass of the Rab5-REP complex (~170 kDa) is also much larger than the expected size (~120 kDa), once again suggesting that a ternary complex is formed because of the binding of two DATFP prenyl groups with two separate factors. Previous studies using DATFP-geranyl diphosphate and DATFP-FPP have identified UV cross-linking to the β subunits of PFT and PGGTI, respectively (11–13). Based on the observed mass of the Rab5-REP complex, a candidate for a third component in the UV cross-linked complex is the β subunit of RabGDI (38 kDa). This idea is supported by the high degree of homology between the β subunits of the protein-prenyl transferases (20–30% identity and 50–60% similarity) and by the fact that the RabGDI functionally modifies Rab5 with DATFP-FPP. Zhang et al. (42) have demonstrated that RabGDI only binds one molecule of geranylgeranyl diphosphate, suggesting that the two geranylgeranyl groups are transferred to Rab proteins in independent and consecutive reactions. The results of this study support the notion that REP must bind the lipid moiety of monogeranyl-geranylated Rab while the enzyme catalyzes the second prenylation reaction. UV cross-linking of DATFP-Rab5 appears to have trapped an intermediate wherein the second lipid moiety is attached to Rab5 but remains in the RabGDI active site yielding a ternary complex of ~170 kDa in mass.

The UV cross-linked complexes of DATFP-Rab5 provide a basis to further characterize molecular interactions within the
prenyl-binding pockets for GDI, REP, and the RabGGT β subunit. In the structure of GDI obtained by Balch and co-workers (43), one domain (defined by α-helices I, A, N, and C and by β-sheets a and c) has structural similarity with flavin adenine dinucleotide-binding domains. A groove in these domains represents the flavin adenine dinucleotide-binding site, and it has been proposed that the corresponding GDI groove may be a potential prenyl-binding pocket. In support of this hypothesis, regions of homology between REP and GDI (~30% identity overall) include this domain. Future peptide mapping experiments may help to characterize this putative prenyl-binding pocket through the UV-cross-linking approaches used here. Structural information for the protein prenyl transferases has more precisely defined their prenyl-binding sites. In a deep cavity of PFT, a cluster of aromatic residues (including Trp102 and Tyr154) surround the isoprene unit of a farnesyl diphosphate analog in a ternary complex with a CaaX peptide (44,45). A cavity also exists in the structure of RabGGT lined with residues that, based on sequence alignment, correspond to the cavity-lining residues of PFT. However, Zhang et al. (42) have indicated that a molecule of geranyleranyl diphosphate can be docked into the RabGGT cavity in two different ways. Analysis of the DATFP-FPP or DATFP-Rab5 UV cross-linking to the β subunit of RabGGT may help distinguish between these two possibilities. Thus, UV cross-linking approaches utilizing DATFP-FPP as a photoactivatable prenyl analog provide a novel and attractive means to resolve a more precise definition of prenyl-binding sites.

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