MODIFICATION OF RAB5 WITH A PHOTOACTIVATABLE ANALOG OF GERANYLGERANYL DIPHOSPHATE

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ABSTRACT

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 crosslinks to these soluble factors upon exposure to UV light. Immunoprecipitation with antibodies raised against proteins known to interact with Rab5 reveals that the crosslinked complexes contain Rab Escort Protein and GDI-1. DATFP-Rab5 also associates with membranes in a GTPγS-stimulated manner. However, while prenylated Rab5 can be crosslinked to two unknown membrane-associated factors by the chemical crosslinker disuccinimidyl suberate (DSS), these proteins fail to be UV-crosslinked 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.
INTRODUCTION

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 carboxyl 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 (GGPP). 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 PGGTII). 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 (K_d ~ 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, prelimin 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-isoprene units of these photoprobes are replaced with a diazotrifluoropropionyloxy group (Figure 1). Previous work has shown that these analogs competitively inhibit purified or recombinant PFT and PGGTI and crosslink 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 \textit{in vitro} modification of recombinant Ras protein (14). One aim of this investigation was to determine if 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 \textit{in vivo} (15, 16) and stimulates homotypic endosome fusion \textit{in vitro} (17, 18). The precise role of Rab5 in these events is not yet fully understood, however, the function of Rab proteins 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 (GDF), which dissociates the Rab-GDI complex and places the Rab in the membrane. A Guanine nucleotide Exchange Factor (GEF) 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 vesicles. After membrane fusion, a GTPase Activating Protein (GAP) accelerates the rate of GTP hydrolysis by the Rab to regenerate its GDP-bound form. In a process hypothesized to involve a membrane-bound Rab
Recycling Factor (RRF) (22), GDI then extracts the GDP-bound Rab from the membrane for return to its donor compartment. Although it is established that Rab protein prenylation 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 towards this analysis employing UV-crosslinking techniques.
MATERIALS AND METHODS:

Materials: DATFP-FPP was chemically synthesized as previously described (12) and stored at –20°C in the dark in 2 mM NH₄OH at 485 µM. [³⁵S]-Methionine (1175 Ci/mmol) was purchased from DuPont NEN (Boston, MA). RNAsin, RQ1 DNase, and rabbit reticulocyte lysate were from Promega (Madison, WI). Disuccinimidyl suberate (DSS) was purchased from Pierce (Rockford, IL) and prepared as a 10 mg/ml stock solution in DMSO just before use. Triton X-114 was purchased from Boeringer Mannheim (now Roche Molecular Biochemicals; Indianapolis, IN) and prepared as a 10% stock in Tris / NaCl / EDTA (TNE) as described (23).

A rabbit polyclonal antiserum was raised (Pocono Rabbit Farm and Laboratory, Canadensis, PA) against recombinant human Rab5. Bovine Rab3A GDI (24), GDI2-peptide (25), and REP1 (26) antibodies 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 (South San Francisco, CA).

In Vitro Biosynthesis and Prenylation of Rab5: The plasmids pAGA-Rab5WT and pAGA-Rab5¹²¹ were purified by CsCl density ultracentrifugation and linearized with HindIII (27). Run-off transcripts were synthesized using T7 RNA polymerase. Rabbit reticulocyte lysate containing 20 µM amino acids and 20 mM KCl was programmed with these transcripts (90 µg/ml) to translate peptides in the presence of [³⁵S]-methionine (200,000 cpm/µl for characterization and 800,000 cpm/µl for crosslinking experiments). Translation reactions to prepare proteins for crosslinking experiments also contained PIC (Protease Inhibitory Cocktail, 1 µg/µl each of PMSF, 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 performed at 37 °C. These prenylation reaction mixtures included 40 % (v/v) rabbit reticulocyte lysate, 12 mM Tris-Cl, pH 7.5, 3 mM MgCl$_2$, 0.6 mM DTT, and 50 µg/ml RNAse A.

**Triton X-114 Extractions:** A 2-µl aliquot of protein was diluted into 200 µl 1% Triton X-114 in TNE. 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 x g for 1 min at room temperature (23). The top aqueous phases were transferred to new tubes and re-extracted with 20 µl 1% Triton X-114 in TBS. The lower detergent phases were re-extracted with 100 µl TBS. The final phases were made up to the same volume and detergent concentration by adding 200 µl TBS to the detergent phases and 20 µl 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 radiolabeled peptides was fractionated on 4.8 ml of 5-20% continuous sucrose gradients in 50 mM HEPES, pH 7.5, 1 mM MgCl$_2$, 1 mM DTT, and 5 µM GDP (28). After ultracentrifugation at 165,000 x 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 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 aldolase (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 (BioRad, Hercules, CA) at 4 °C. The concentration of radiolabeled
protein was determined by trichloroacetic acid precipitation. Membranes were prepared from
K562 cell post-nuclear supernatant by centrifugation at 16,000 x g for 15 min at 4 °C (29).
Protein concentration was determined by the Folin/Lowry assay. [35S]-Rab5 (3 nM) was mixed
with membranes (2 mg/ml) and incubated at 37 °C for 30 min in the presence of PIC and 1 mM
GDP or GTPγS. Membrane pellet and supernatant fractions were isolated by centrifugation
(16,000 x 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 (BioRad, Personal
FX).

Crosslinking: Proteins were chemically crosslinked using 0.5 mg/ml DSS for 1 h on ice. The
reaction was stopped by adding 50 mM Tris-Cl, pH 7.4 and incubating on ice for 30 min. For
UV-crosslinking, proteins were diluted 10-fold in assay buffer into quartz cuvettes and exposed
to a shortwave (254 nm) 4-W UV lamp at a 1-cm distance for 1 h at 4 °C.
**Immunoprecipitation:** 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 PIC, vortexed, and incubated on ice for 60 min. Five-µl aliquots of the appropriate antisera (Rab5, GDI-1, GDI-2, and REP) or 50 µg purified antibody (GDI) was added, and incubation on ice continued for 90 min. Nonspecific complexes were removed by centrifugation (10,000 x g, 4 °C, 10 min), and 100 µl 10 % (v/v) Protein A agarose (Calbiochem, La Jolla, CA) in IP buffer was added. Samples were then rocked at 4 °C for 1 h. Beads were collected (10,000 x g, 4 °C, 1 min) and washed three times with 1 ml IP buffer. Proteins were released from the beads and denatured by boiling for 10 min in 50 µl Laemmli buffer, and then resolved on acrylamide gradient gels for phosphorimaging analysis.

**Gel Electrophoresis:** The products of *in vitro* prenylation and membrane binding reactions were analyzed by urea-acrylamide gradient gel electrophoresis (30). Briefly, a continuous gradient of 4 - 8 M urea and 10 –15 % acrylamide was formed in the absence of SDS. Samples were prepared in Laemmli buffer but were not boiled. These gels were fixed and dried before exposure to phosphorimaging screens. Crosslinked products were characterized on gels with a continuous gradient of 5 – 15 % acrylamide containing 0.1 % SDS. Gels were processed for fluorography by saturating them in DMSO followed by a 5-min incubation in 20% (w/v) 2,5-diphenyloxazole (PPO) in the same solvent. 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 coworkers (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 (Figure 2A). Rab5 is prenylated in a temperature-dependent manner (lane 1 versus 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 (lanes 2 versus 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 (AGGC or AFC, Figure 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 Figure 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 $^{35}$S-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 Rab5$^{1-212}$ is the same as digeranylgeranylated Rab5$^{WT}$ (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 binds and UV-crosslinks to cytosolic proteins.**

To examine whether modification of Rab5 with DATFP-FPP affects its function, the association of DATFP-Rab5 with other cytosolic factors was evaluated by sucrose density gradient ultracentrifugation. Previous studies have demonstrated the prenylation-dependent association of Rab5 with GDI-2 by this method (28). As shown in Figure 4, the carboxyl terminal truncation mutant Rab5$^{1-211}$, which lacks the prenylation motif, sediments as the expected monomeric species of ~ 24 kDa, while 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 crosslinking was performed by UV irradiation. As shown in Figure 5, several UV crosslinked products containing $^{35}$S-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-crosslinks 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). GTPγS, 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 GTPγS. After a 30-min incubation at 37 °C, membrane and supernatant fractions were separated by centrifugation. The amount of modified Rab5 in each fraction was determined by phosphorimaging proteins separated by gel electrophoresis. The Rab5$^{1-211}$ 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 GTPγS (Figure 6); however, the overall extent of DATFP-Rab5 binding is reduced relative to prenylated Rab5 in the presence of either GDP or GTPγS. The
DATFP-Rab5 bound to membranes does not represent contaminating mono- or di-prenylated Rab5 since the isoprenoid photoprobe completely inhibits native prenylation under the conditions used to produce \[^{35}\text{S}^\]labeled protein for this assay (Figure 2, 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 crosslink to membrane proteins.**

The ability of membrane-bound DATFP-Rab5 to UV crosslink proteins was compared to the chemical crosslinking 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-crosslink at a 2 - 3 angstrom distance. Briefly, membrane fractions with Rab5 bound as described above were recovered by centrifugation after UV or DSS crosslinking. Detergent-solubilized Rab5 complexes were then immunoprecipitated and separated by gel electrophoresis. As shown in Figure 7, DSS promotes crosslinking 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 crosslinking 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 crosslinking.

**DATFP-Rab5 UV crosslinks to REP and GDI-1.**

Because DATFP-Rab5 does not UV-crosslink to membrane-associated factors, the soluble crosslinked complexes observed in rabbit reticulocyte lysate were further characterized. To identify the soluble proteins which crosslink 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 (Figure 8). Antiserum recognizing REP immunoprecipitates a 170-kDa complex containing DATFP-Rab5 (right panel). Interestingly, this complex cannot be detected by chemical crosslinking of native prenylated Rab5 with DSS (left panel). Two different polyclonal antibodies against GDI-1 precipitate a 90- and 105-kDa complex from assays containing either DSS-crosslinked prenylated Rab5 or UV-crosslinked DATFP-Rab5 (both panels). The expected mass of complex between Rab5 (24 kDa) and GDI-1 (55 kDa) is 79 kDa, so why larger-than-expected 90- and 105-kDa complexes are crosslinked to Rab5 is not clear. Although an antibody specific for GDI-2 (45 kDa) did not precipitate any Rab5 complexes, the 67-kDa crosslinked complex observed in rabbit reticulocyte lysate (Figure 5) is consistent with a Rab5-GDI-2 complex. It is possible that this antibody may not be able to immunoprecipitate the complex because crosslinking blocks the major antigenic epitope. Nonetheless, the immunoprecipitation experiments rigorously demonstrate that soluble DATFP-Rab5 binds and UV crosslinks with at least two known factors, REP and GDI-1.
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 DAFTP-FPP as a substrate analog of Rab Geranylgeranyl Tranferase. The photoprobe isoprenoids have been reported to inhibit purified PFT and PGGTI at concentrations similar to the K_m for the native prenyl substrates (0.02 µM) (12), but DATFP-FPP appears to inhibit RabGGT in rabbit reticulocyte lysate at higher concentrations (IC_50 ~ 5 µM). However, the half-maximal concentration of geranylgeranyl diphosphate for Rab5 prenylation in this system is also approximately 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-crosslinking 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-crosslinked 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 crosslinks to the same membrane-associated factors as native prenylated Rab5, an observation that indicates 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 crosslinking experiments do demonstrate protein-protein interactions with Rab5 and other membrane-associated factors. Chemical crosslinking with DSS identifies 45- and 70-kDa complexes containing Rab5. Although the results suggest the formation of these complexes is nucleotide-dependent, a greater amount of GTPγS-bound Rab5 is membrane-associated. Thus, it is not clear whether the crosslinked Rab5 interactors are recruited or bind to the GTPase 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 GTPase.

Despite the lack of membrane-bound complexes in these binding assays, several UV-crosslinked complexes were found in the supernatant fractions containing reticulocyte lysate. One of the factors that was clearly identified to be UV-crosslinked 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 crosslinked species of the expected mass (Figures 5 and 8). It is possible that the anti-GDI-2 antibody’s recognition epitope is blocked by interactions with native prenylated Rab5 (as detected by chemical crosslinking with DSS) and the DATFP-modified protein (as detected by UV-crosslinking). Rab5 can nonetheless be UV-crosslinked 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 crosslinked species may indicate that the Rab5-GDI complex interacts with additional factors. It is unlikely that these much larger crosslinked 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 αGDI does not cause an alteration of the protein’s apparent electrophoretic mobility (39). Based on known Rab5 interactors, possible small mass candidates for ternary complex formation with Rab5-GDI include the 17-kDa GEF MSS4 (40) and the 24-kDa Prenylated Rab Acceptor 1 (PRA1) (41). However, preliminary attempts to immunoprecipitate Rab5-crosslinked complexes using MSS4 or PRA1 antisera 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. Due to the fact that the photoactivatable analog crosslinks within a 2 –3 angstrom distance to a single target, a UV-crosslinked 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 GTPase cycle of Rab5. During this process, GDI’s protein-prenyl interactions 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-crosslinked to DATFP-Rab5 is REP, verifying its role in the post-translational prenylation of the GTPase (26). Interestingly, chemical crosslinking
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 due to the binding of two DATFP prenyl groups with two separate factors. Previous studies using DATFP-GPP and DATFP-FPP have identified UV-crosslinking to the $\beta$ 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-crosslinked complex is the $\beta$ subunit of RabGGT (38 kDa). This idea is supported by the high degree of homology between the $\beta$ subunits of the protein-prenyl transferases (20-30 % identity and 50-60 % similarity) and by the fact that the RabGGT functionally modifies Rab5 with DATFP-FPP. Zhang et. al. (42) have demonstrated that RabGGT only binds one molecule of GGPP, 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 monogeranylgeranylated Rab while the enzyme catalyzes the second prenylation reaction. UV-crosslinking of DATFP-Rab5 appears to have trapped an intermediate wherein the second lipid moiety is attached to Rab5 but remains in the RabGGT active site yielding a ternary complex of ~ 170 kDa in mass.

The UV-crosslinked complexes of DATFP-Rab5 provide a basis to further characterize molecular interactions within the prenyl-binding pockets for GDI, REP, and the RabGGT $\beta$ subunit. In the structure of GDI obtained by Balch and coworkers (43), one domain (defined by $\alpha$-helices I, A, N, and C and by $\beta$-sheets a and c) has structural similarity with FAD-binding domains. A groove in these domains represents the FAD-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-crosslinking 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 W102 and Y154) surround the ω-isoprene unit of an FPP 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 FPT. However, Zhang et al. (42) have indicated that a molecule of GGPP can be docked into the RabGGT cavity in two different ways. Analysis of the DATFP-FPP or DATFP-Rab5 UV-crosslinking to the β subunit of RabGGT may help distinguish between these two possibilities. Thus, UV-crosslinking 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.
REFERENCES


ABBREVIATIONS

AFC, N-acetyl-S-farnesyl-cysteine; AGGC, N-acetyl-S-geranylgeranyl-cysteine; DATFP-FPP, 2-diazo-3,3,3-trifluoropropionyloxy-farnesyl diphosphate; DMSO, dimethylsulfoxide; DSS, disuccinimidyl suberate; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid; FAD, flavin adenine dinucleotide; FPP, farnesyl diphosphate; GAP, GTPase Activating Protein; GDF, GDI-Displacement Factor GDI, Guanine nucleotide Dissociation Inhibitor; GDP, guanosine diphosphate; GEF, Guanine nucleotide Exchange Factor; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; GTP, guanosine triphosphate; GTPγS, guanosine-5′-O-(3-thiotriphosphate); PIC, Protease Inhibitor Cocktail; PFT, Protein Farnesyl Transferase; PMSF, pheylmethylsulfonyl fluoride; PGGTI, Protein Geranylgeranyl Transferase Type I; PPO, 2,5-diphenyloxazole; RabGGT, Rab Geranylgeranyl Transferase; REP, Rab Escort Protein; RRF, Rab Recycling Factor; TNE, Tris / NaCl / EDTA; Tris, trishydroxymethylaminomethane; UV, ultraviolet.
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**Fig. 1. Structure of the DATFP-Isoprenoid Photoprobes.** The structures of two isoprenoid diphosphates (OPP) and their photoactivatable analogs are depicted. GPP = geranyl diphosphate, FPP = farnesyl diphosphate, GGPP = geranylgeranyl diphosphate, DATFP = 2-diazo-3,3,3-trifluoropropionyloxy-.

**Fig. 2. In Vitro Modification of Rab5 with DATFP-FPP.** Rabbit reticulocyte lysate was programmed with Rab5\(^{WT}\) mRNA in the presence of \[^{35}\text{S}\]-methionine (0.2 mCi/ml). The concentration of Rab5, as determined by trichloroacetic acid precipitatable counts, was adjusted to 6 nM in the absence (-) or presence (+) of 10 µM mevalonate with the indicated concentrations of DATFP-FPP (0, 1, 5, 10, 50 µM) (A) or 0.5 mM AGGC or AFC (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 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.

**Fig. 3. Triton X-114 Extraction of DATFP-Rab5.** Rab5 was translated and \[^{35}\text{S}\]-methionine labeled as described in Figure 2, diluted to 16 nM and left unmodified on ice or prenylated with 24 µM mevalonate or DATFP-FPP at 37 °C for 1h. Samples were extracted as described in Materials and Methods. 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.
Fig. 4. DATFP-Rab5 Incorporates into Larger Molecular Weight Complexes. Rab5\(^{WT}\) and Rab5\(^{1-211}\) were synthesized \textit{in vitro}, and the \[^{35}\text{S}\]-labeled protein (5 nM) was modified with 10 \(\mu\text{M}\) mevalonate or DATFP-FPP for 3 h. Ten-\(\mu\text{L}\) aliquots were layered onto continuous 5-20\% sucrose gradients and ultracentrifuged at 165,000 \(x\ g\) for 17 h at 4 \(^{\circ}\)C. Fractions (150 \(\mu\text{L}\)) were collected from the bottom of the gradient, and 30-\(\mu\text{L}\) aliquots of odd fractions were electrophoresed on urea-acrylamide gradient gels which 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 Crosslinks to Proteins in Rabbit Reticulocyte Lysate upon Exposure to UV Light. Ten-\(\mu\text{L}\) aliquots of lysate containing 30 nM \[^{35}\text{S}\]-labeled Rab5 modified with 48 \(\mu\text{M}\) 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 \(^{\circ}\)C for 30 min (+) or left wrapped in Al foil on ice (-). 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 size (in kDa) and migration of standard proteins are shown on the left. The calculated molecular mass of the Rab5 complexes is 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 GTPγS. Membrane-bound Rab5 was then separated by centrifugation (16,000 x 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 (+/- SD, n=6) is shown.

Fig 7. DATFP-Rab5 Binds but does not UV Crosslink to Membrane Proteins. Assay mixtures containing prenylated Rab5 or DATFP-Rab5 with (+) or without (-) GTPγS were left untreated (none) or crosslinked for 60 min at 4 °C with DSS (0.5 mg/ml) or UV light (254 nm, 4 W bulb) as indicated. Membrane fractions were then isolated by centrifugation, and Rab5 complexes were immunoprecipitated using polyclonal rabbit anti-human Rab5. After boiling in Laemmli buffer, the crosslinked complexes were separated by acrylamide gradient gel electrophoresis. The gel was fixed, soaked in scintillant, dried, and exposed to film for 2 wk. The size (in kDa) and migration of standard proteins are shown on the left. The calculated molecular mass of the complexes is indicated on the right. Asterisks mark crosslinker independent bands.
**Fig. 8. Rab5 Crosslinks to REP and GDI-1.** Assay mixtures containing prenylated Rab5 or DATFP-Rab5 were crosslinked for 60 min at 4 °C with DSS (0.5 mg/ml) or UV light (254 nm, 4 W bulb) as described for Figure 7. Supernatant fractions isolated by centrifugation were immunoprecipitated with antibodies raised against the indicated protein (Rab5, GDI, GDI-1, GDI-2, or REP). After boiling in Laemmli buffer, the crosslinked complexes were separated by acrylamide gradient gel electrophoresis. The gel was stained, soaked in scintillant, dried, and exposed to film for 2 wk. The size (in kDa) and migration of standard proteins are shown on the left. The calculated molecular mass of the complexes is indicated on the right.
Figure 1.
Figure 2A.

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<tr>
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Figure 2B.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Modification of Rab5 with a photoactivatable analog of geranylglyceranyl diphosphate
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