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Synthesis of Fluorescently Labeled Mono- and Diprenylated Rab7 GTPase

Thomas Durek,[†] Kirill Alexandrov,[†] Roger S. Goody,[†] Alexandra Hildebrand,[§] Ines Heinemann,[‡,||] and Herbert Waldmann*[,‡,||]

Contribution from the Max-Planck-Institut für Molekulare Physiologie, Abteilung Physikalische Biochemie, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany, Institute of Biochemistry, Romanian Academy, Spl. Independentei, 296, sect. 6, CP: 17-37, 777 000 Bucharest 17, Romania, Max-Planck-Institut für Molekulare Physiologie, Abteilung Chemische Biologie, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany, and Universität Dortmund, Otto-Hahn-Strasse 6, 44227 Dortmund, Germany

Received June 29, 2004; E-mail: herbert.waldmann@mpi-dortmund.mpg.de

Abstract: Modification of proteins with isoprenoid lipids is a widespread phenomenon in eukaryotic organisms that has received much attention due to its involvement in the progression of several diseases including cancer. Progress in studies of prenylated proteins has been hampered by difficulties associated with isolation of these proteins from native or recombinant sources. Small GTPases of the Rab family represent a particularly difficult example since they are doubly C-terminally geranylgeranylated and in some cases methylated. Here, we report an efficient and versatile strategy for the synthesis of mono- and digeranylgeranylated fluorescent RabGTPases using a combination of chemical synthesis and expressed protein ligation. Using this approach we generated fluorescent mono- and diprenylated Rab7 proteins that display near-native properties and form stoichiometric complexes with their natural chaperone REP-1. We demonstrate that the complex formed from semisynthetic monoprenylated Rab7 and REP-1 represents a genuine intermediate of the Rab prenylation reaction and thus provides a unique tool for studies of the Rab prenylation mechanism. Semisynthetic Rab7 proteins were used to develop a novel fluorescence-based in vitro prenylation assay. Using this assay we dissected the mechanism of the Rab7 double-geranylgeranylation reaction mediated by Rab geranylgeranyl transferase. We conclude that the reaction follows a random sequential mechanism. These results highlight the usefulness of the semisynthetic reaction intermediates in the study of protein posttranslational modification.

Introduction

Since its discovery over a decade ago, it has become apparent that covalent modification of proteins with isoprenoid lipids is a widespread phenomenon affecting up to 2% of all eukaryotic proteins.[1,2] The importance of protein prenylation is underscored by the nature of the proteins it affects, many of which participate in signal transduction pathways controlling cell growth and differentiation, cytoskeletal rearrangement, and vesicular transport. In protein prenylation, either a farnesyl or a geranylgeranyl moiety is donated by soluble phosphoisoprenoids and attached to one or two C-terminal cysteine residues of the target protein via a thioether linkage. This type of reaction can be catalyzed by three different protein prenyl transferases: protein farnesyltransferase (FTase), protein geranylgeranyl transferase-I (GGTase-I), and Rab geranylgeranyl transferase (RabGGTase or GGTase-II) (for a review see ref 3). The closely related FTase and GGTase-I transfer prenyl groups from prenyl pyrophosphates to proteins that contain a C-terminal CAAX motif, also known as a CAAX box (C is cysteine, A is usually an aliphatic amino acid, and X can be a variety of amino acids). The X residue of this motif largely determines the choice of the isoprenoid. RabGGTase is quite different from the above-mentioned enzymes both functionally and structurally. Like other prenyltransferases, mammalian RabGGTase is a heterodimer composed of α and β subunits, the latter bearing the active site. The enzyme transfers the geranylgeranyl moiety onto two C-terminal cysteines of Rab GTPases in a broad context of amino acids[3] and requires an additional factor for its activity known as Rab escort protein (REP). Unlike other prenyltransferases, RabGGTase recognizes not a specific cysteine-containing C-terminal sequence but a complex of RabGTPase with REP. The ensuing diprenylation reaction is a multistep process that was proposed to follow a random sequential mechanism where one cysteine is somewhat preferred for the first prenylation.[4] However, in all cases monocysteine mutants of Rab proteins were used that provide only an approximation of the

[†] Max-Planck-Institut für Molekulare Physiologie, Abteilung Physikalische Biochemie.
[‡] Universität Dortmund.
[§] Romanian Academy.
[||] Max-Planck-Institut für Molekulare Physiologie, Abteilung Chemische Biologie.

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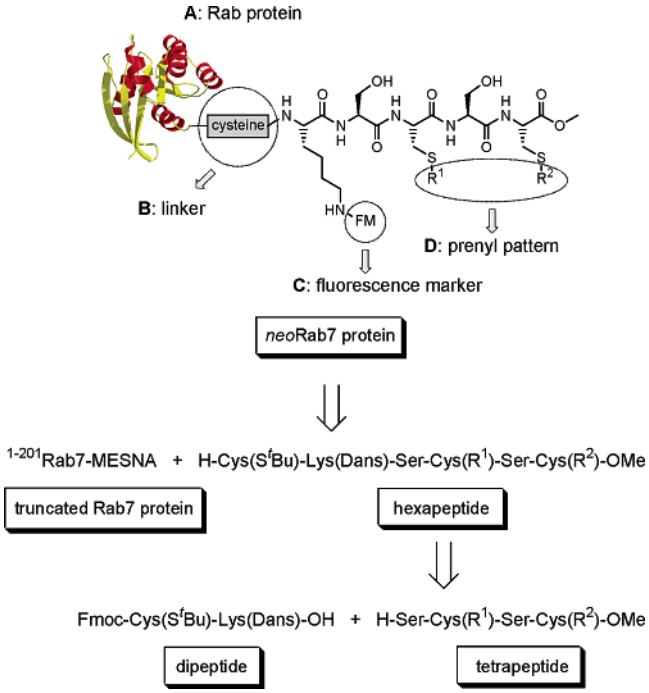
native situation. Moreover, the available reports dispute the exact sequence of isoprenoid addition.[4,5] It is also not entirely clear to which extent the monoprenylated Rab:REP complex dissociates from RabGGTase between two rounds of prenylation.[5] Upon diprenylation the conjugated isoprenoids are removed from the active site to bind to a lipid-binding site on REP, inducing a conformational change leading to dissociation of the complex.[6,7] Despite broad biological and clinical significance[8] as well as extensive efforts aimed toward a better understanding of GTPase function, the role of prenyl modifications in their biogenesis remains only partially understood. It is particularly unclear how the choice and number of isoprenoids associated with a specific GTPase are determined. This reflects a familiar general situation in biology where, on the one hand, the last 20 years have brought major progress in the synthesis of small biologically active molecules that are rivaled only by advances in protein engineering. On the other hand, only limited progress has been achieved in the construction of posttranslationally modified proteins. At least in part this is due to the limited applicability of the otherwise powerful methods of organic chemistry to large and sensitive molecules such as proteins.

The recently developed expressed protein ligation method provides the means for solving many of these problems.[9] Central to this method is the ability of certain protein domains (inteins) to excise themselves from a precursor protein in which they are flanked by exteins.[10] In an engineered intein expression system, the role of a cysteine in the intein is replaced by a thiol reagent, which induces cleavage and leaves a reactive thioester group on the C-terminus of the protein of interest.[11] The isolated C-terminally thioester-tagged target protein can then be used to couple essentially any polypeptide harboring an N-terminal cysteine to the protein of interest by forming a native peptide bond.[12] Using this approach synthetic peptides bearing unnatural groups can be incorporated into virtually any position of the protein.[9] In contrast to traditional modification methods, this technology provides a means for versatile and precise modification of protein molecules and allows departure from the restrictions of standard peptide chemistry. Recently we described the application of the expressed protein ligation method to form a modified Rab protein derivative by coupling a synthetic lipopeptide fragment to a recombinant Rab protein, which was later used for biochemical and structural studies.[13−15] Building on our preliminary data, in this report we present the synthesis of fluorescently labeled and biologically active mono- and diprenylated Rab7 proteins. We demonstrate that monoprenylated GTPases represent genuine intermediates of the Rab diprenylation reaction.

Scheme 1. Retrosynthetic Analysis for the Synthesis of the Prenylated Rab7 Proteins



Results

For the construction of fluorescent prenylated Rab7 proteins, hexapeptides corresponding to the C-terminal region of Rab7 were synthesized incorporating all desired modifications. These were subsequently coupled to recombinant Rab7 C-terminally truncated by six amino acids. To make the semisynthetic proteins amenable to spectroscopic characterization, we attached a fluorescent group in the proximity of the geranylgeranylated cysteine. As a reporter group, one of the smallest known fluorescent markers, the 5-(dimethylamino)-1-naphthalene sulfonyl (dansyl) moiety, was chosen. The amino acid following the N-terminal cysteine essential for in vitro protein ligation was changed from glutamic acid to lysine. This substitution is known to have no impact on the interaction of Rab7 with the subunits of RabGGTase.[13]

Due to flexibility considerations for the synthesis of the desired hexapeptides, a modular strategy was adopted whereby a N-terminal dipeptide was coupled to differently prenylated tetrapeptides. The reductively cleavable *S-tert*-butyl moiety was chosen as the protecting group of both the N-terminal and unprenylated cysteines. The deprotection can be carried out in situ during protein ligation with an excess of thiol agent (Scheme 1).[16]

N-Terminal Dipeptide. The synthesis began with the generation of the ϵ-N-fluorescently labeled lysine **5**. The protected lysine **2** containing a hydrogenolytically cleavable protecting group on both the α-amino group and the carboxy group was synthesized first (Scheme 2). Selective labeling of the amino group and hydrogenolysis furnished the desired lysine **5** in an excellent overall yield of 52%. Condensation with the cysteine **6** finally provided the fluorescently labeled dipeptide **7**.

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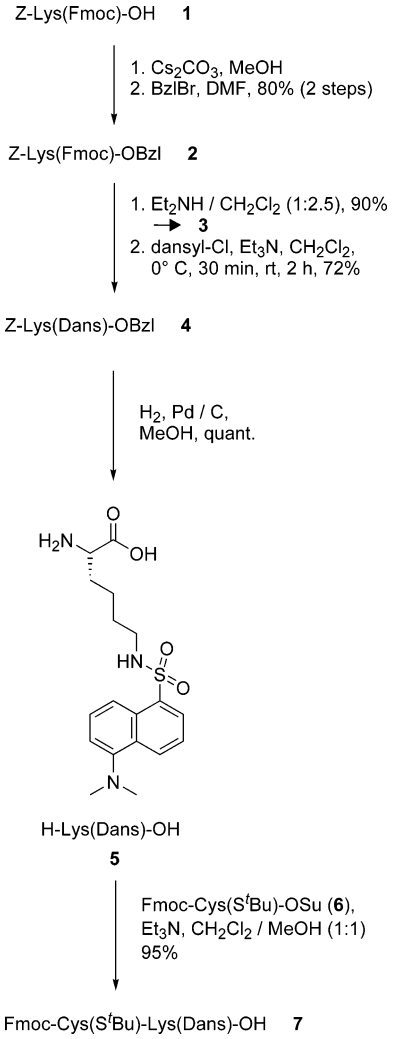
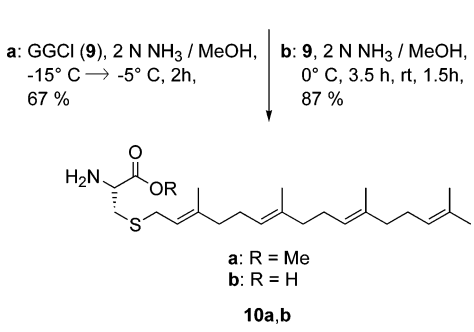
Scheme 2. Formation of the N-Terminal Dipeptide **7**



Scheme 3. Synthesis of the Geranylgeranylated Cysteines **10a**,**b**



Prenylated Tetrapeptides. For the synthesis of the differently prenylated tetrapeptides, geranylgeranylated cysteine methyl ester **10a** and geranylgeranylated cysteine **10b** were generated based on a known literature procedure (Scheme 3).[17] To this end, geranylgeraniol was activated by Corey−Kim chlorination[18] followed by reaction of the prenyl chloride **9** (GGCl) with the cysteines **8a**,**b** employing 2 N ammonia in methanol as solvent. This procedure provided the desired compounds in high yield.

In general, the synthesis of the three prenylated tetrapeptides **19a**−**c** followed standard peptide coupling reactions. Synthesis of the C-terminally geranylgeranylated tetrapeptide **19a** started with the protected cysteine **11** (Scheme 4). Six coupling and deprotection steps, including a block condensation of the tripeptide **17** with geranylgeranylated cysteine methyl ester **10a**, furnished the desired tetrapeptide in an overall yield of 21%.

For the synthesis of the internally geranylgeranylated tetrapeptide **19b**, a convergent strategy was adopted (Scheme 4). Condensation of the dipeptides **20** and **24** and subsequent removal of the Fmoc protecting group provided the tetrapeptide **19b** in high yield. Due to the pronounced acid lability of the geranylgeranyl moiety and the lability of an N-terminally deprotected unprotonated dipeptide methyl ester, a convergent strategy for the synthesis of the diprenylated tetrapeptide was unsuccessful. Therefore, starting with the prenylated dipeptide **20**, a subsequent chain elongation toward the C-terminus and final deprotection of the N-terminus was applied, yielding the digeranylgeranylated tetrapeptide **19c** in 33% yield over four steps (Scheme 5).

Prenylated Hexapeptides. With efficient access to the N-terminal fluorescently labeled dipeptide **7** as well as a sufficient supply of the three prenylated tetrapeptides **19a**, **b**, and **c** in hand, the coupling reactions to the target hexapeptides **30** were investigated (Scheme 6). Each condensation was performed essentially under the same conditions (utilizing EDC and HOBt as coupling reagents), and all reactions provided the hexapeptides in moderate to high yields. Fmoc deprotection finally furnished the desired compounds in excellent yields. Confirmation of structure for each compound was obtained by ^{1}H and COSY NMR as well as by MALDI and ESI mass spectrometry. A possible epimerization of individual amino acid residues in the coupling steps could not be detected by high-field NMR techniques.

Adapting the in Vitro Ligation Procedure to Prenylated Proteins. To test whether the generated prenyl peptides could be covalently attached to the recombinant Rab7 protein we performed in vitro ligation assays. In one of these assays thioester-tagged Rab7ΔC6-MESNA[13] was incubated for 8 h at room temperature with test peptide CK(Dans)C(GG)-OMe generated using the strategy outlined above. The resulting mixture was then resolved on SDS−PAGE, and the progression of the ligation reaction was monitored by the appearance of fluorescent bands in the 20−30 kDa molecular weight range. Although the in vitro ligation reaction is typically robust and independent of the type of peptide used, no fluorescent product was formed. This was not due to degradation of the thioester group on the Rab7 since the protein readily ligated to a nonprenylated peptide (not shown). We assumed that the hydrophobic isoprenoid residues reduce the peptide's solubility, leading to micelle formation or aggregation that interferes with the ligation reaction. However, addition of detergents to the ligation mixture such as Triton X-100, Nonident P40, CHAPS, or *n*-octylglucoside either alone or in combination with chaotropic agents did not lead to product formation. We tested a range of other additives and serendipitously found that SDS−PAGE running buffer was able to support the ligation reaction. Analysis of the buffer components revealed that SDS alone was sufficient for efficient ligation of the lipidated peptide to the thioester-tagged Rab7 protein. Since exposure to SDS leads to

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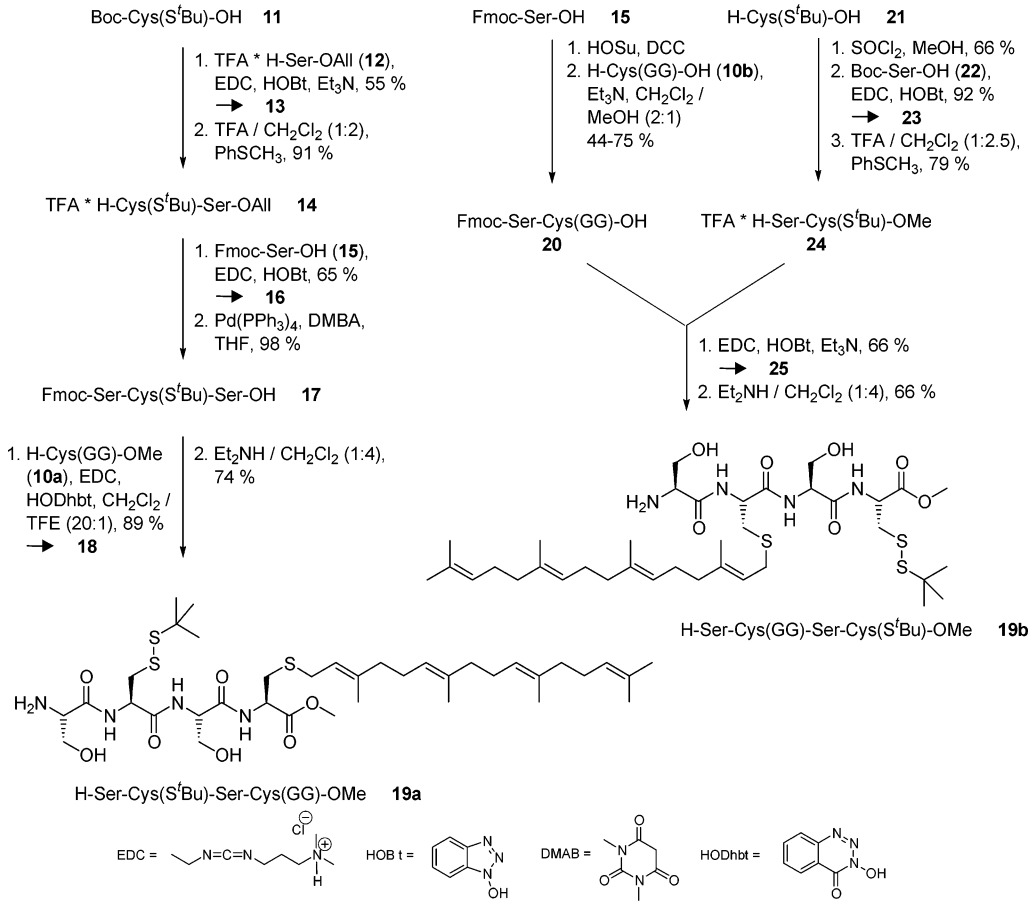
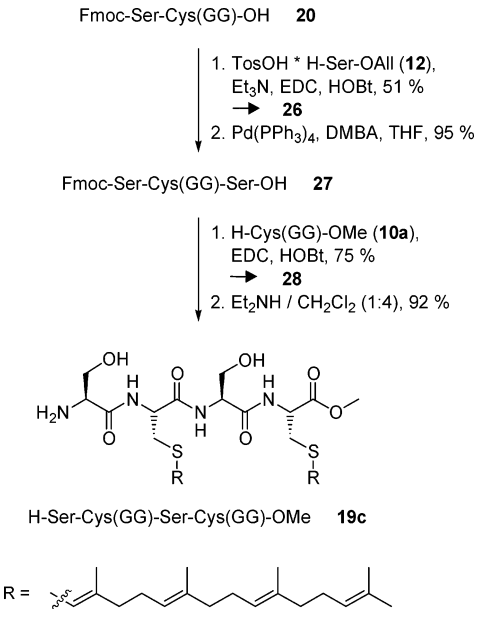
Scheme 4. Synthesis of the Monoprenylated Tetrapeptides **19a**,**b**



Scheme 5. Formation of the Diprenylated Tetrapeptide



complete and in most cases irreversible denaturation of proteins, we sought an alternative substance that could support the coupling of the prenylated peptides. To this end, a total of 76 different detergents were screened using the above-described SDS−PAGE-based assay, and five more detergents that supported the ligation reaction were identified (Figure 1). Interestingly, the extent to which the detergents supported the in vitro ligation reaction was not influenced by either the number of isoprenoids conjugated to the peptide or the nature of the isoprenoid group. Analysis of detergents facilitating in vitro ligation of prenylated peptides indicates that most of them share similar structural elements, yet it does not provide a direct clue to the possible mechanism of their involvement in the ligation reaction, thus calling for further investigations (Figure 1). For further experiments, we selected cetyltrimethylammonium bromide (CTAB), which appeared to be the most potent facilitator of the prenylation reaction.

Synthesis of Mono- and Diprenylated Rab7. To generate preparative amounts of fluorescent Rab7 proteins bearing geranylgeranyl moieties at different positions, we performed preparative in vitro ligation reactions as described in the Experimental Section. The reactions proceeded overnight, yielding typically 85% of coupled Rab protein. Depending on the conditions, such as detergent and salt concentration, the protein either remained in solution or precipitated. In both cases the protein was accompanied by a large amount of unligated peptide throughout subsequent purification steps. Further analysis indicated that the interaction was noncovalent and probably mediated by hydrophobic interactions (data not shown). To remove the noncovalently bound peptide, we extracted the ligation mixture with organic solvent (dichloromethane, methanol, ethyl acetate), which led to full precipitation of the protein and extraction of the lipidated peptide into the organic phase. The remaining insoluble pellet was dissolved in 6 M guanidinium chloride and then refolded by stepwise dilution into CHAPS containing buffer. Subsequently, REP-1 protein was added and the formed complex was further purified by dialysis

Scheme 6. Synthesis of the Target Peptides **30a**−**c**

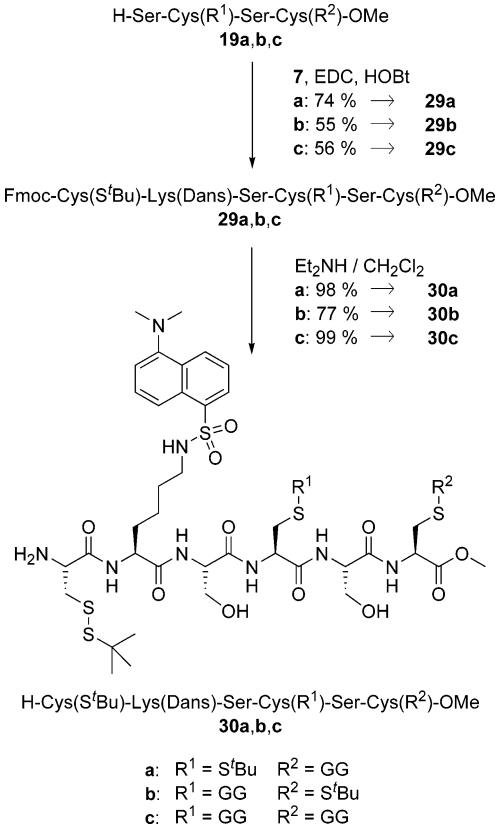


Table 1. Molecular Masses Observed for the Coupling Products of Lipopeptides **30a**−**c** and Rab7ΔC6 -MESNA-thioester[a]

protein[b]	$M_{calcd.}$	M_{obsd}	D
Rab7Δ6-CK(Dans)SCSC(GG)-OMe	23 939	23 951	+12
Rab7Δ6-CK(Dans)SC(GG)SC-OMe	23 939	23 929	−10
Rab7Δ6-CK(Dans)SC(GG)SC(GG)-OMe	24 212	24 227	+ 15

[a] Deviation of the observed molecular masses from the calculated values is within the error range of the mass spectrometer. [b] The molecular masses were calculated for proteins without the first N-terminal methionine.

and gel filtration as described before.[14] The developed procedure produced similar results regardless of the number of conjugated prenyl groups. The obtained semisynthetic protein complexes displayed the expected molecular masses (Figure 2D, Table 1). The approach yielded native, correctly folded Rab7 GTPases, as could be inferred by their interaction with REP-1 and GGTase-II as well as by their competence to accept another prenyl group in the second round of prenylation (see below). Moreover, the same procedure was recently used to prepare monoprenylated Ypt1 in complex with RabGDI. The 3D structure of this complex was solved by X-ray diffraction and indicated a native fold of both components.[15]

Interaction of RabGGTase with Mono- and Diprenylated Rab7:REP-1 Complex. The availability of fluorescent monoprenylated Rab7:REP-1 proteins allowed us to determine the affinity of RabGGTase for its monoprenylated reaction intermediate. The dansyl group integrated into the semisynthetic Rab7 molecules provides a convenient and sensitive reporter for monitoring of protein:protein interactions and conformational rearrangements. For our experiments we excited the dansyl group via fluorescent resonance energy transfer (FRET) employing tryptophan residues as fluorescent donors. Using this fluorescence signal we performed fluorescent titration experiments where ca. 100 nM of either the unprenylated or the mono- or diprenylated complex was titrated with increasing concentrations of RabGGTase (Figure 3). The results obtained are summarized in Table 2. The dissociation constants indicate that addition of an isoprenoid group to the C-terminus of the RabGTPase increases the affinity of its interaction with RabGGTase by approximately an order of magnitude. Unexpectedly, we found that prenylation of the Rab molecules on the distal (terminal) cysteine results in ca. 5-fold higher affinity for RabGGTase than when the protein was prenylated on the upstream residue. These findings can be rationalized by assuming that association of the conjugated isoprenoid with the active site of RabGGTase is less sterically favorable when it is in the upstream position due to the need for accommodation of the C-terminal Ser and Cys residues in the active site. This probably also explains the previously observed preference of RabGGTase for the distal cysteine of Rab7 in the prenylation reaction.[5]

To gain insight into the kinetics of the interactions, we performed displacement experiments in which the above characterized ternary complexes were mixed with a large excess of nonfluorescent diprenylated Rab7:REP-1 complex. This results in a decrease of fluorescence that reflects the off rate of the complex. The obtained constants are summarized in Table 2 and indicate that in the case of monoprenylated complexes the differences in affinity must be due to a difference in association rates, since the dissociation rate constants are similar. The apparent association rate constants can be calculated to be ca. 4×10^6 M^{-1} s^{-1} in the case of the distally prenylated Rab7 and 7×10^5 M^{-1} s^{-1} in the proximal case. Since these values are several orders of magnitude lower than that expected for diffusion-controlled reactions, the association is likely to be a complex (i.e., at least two-step) process, and the difference between the two situations could be in the rate constant for a specific isomerization step rather than in the initial encounter rate of the protein entities.

Development of a Fluorescent in Vitro Prenylation Assay Based on Semisynthetic Rab Proteins. One of the inherent problems encountered in efforts to elucidate Rab prenylation mechanisms relates to the lack of a time-resolved prenylation assay. Previous studies relied on either radioactive or HPLC-based assays.[5,19] Since the dansyl group is known to be environmentally sensitive, we tested whether the developed fluorescent probes could be used for monitoring Rab prenylation, thus enabling direct measurements of rates for incorporation of the second isoprenoid group into the monoprenylated protein. To this end, we excited the fluorescence of the Rab7ΔC6-CK-(Dans)SC(GG)SC-OMe:REP-1:RabGGTase complex via FRET and then supplied the reaction mixture with 10 μM GGPP. This led to a time-dependent decrease of fluorescence that could be fitted with a double-exponential function (Figure 4). The observed reaction indeed reflected Rab prenylation, since addition of an inhibitor of RabGGTase, NE10790,[20] led to retardation or at high concentrations total inhibition of the reaction. To additionally ascertain that the observed fluorescent change represented genuine prenylation, we performed a prenylation reaction with diprenylated Rab7 protein. As can be seen

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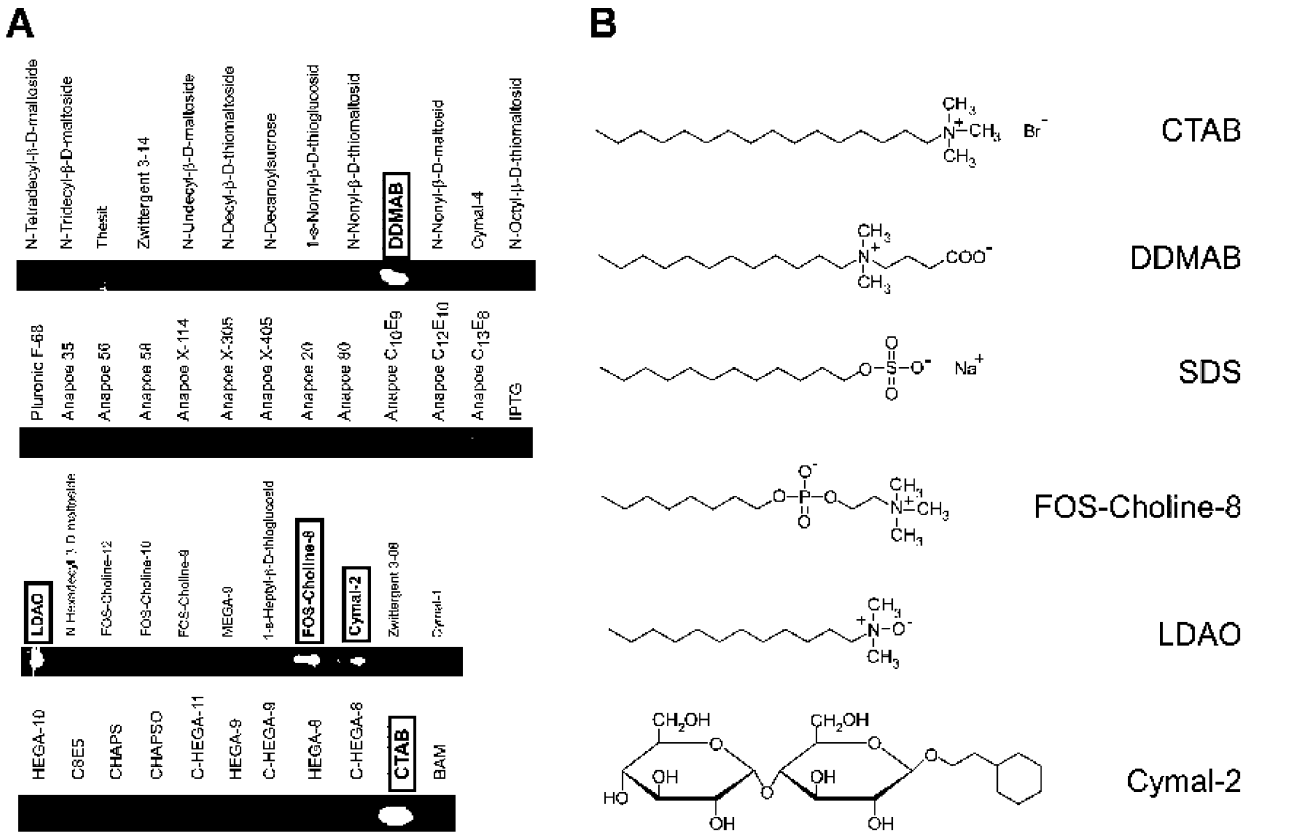

Figure 1. Identification of detergents that support in vitro ligation of prenylated peptides. Ligation of Rab7ΔC6-MESNA thioester to H-Cys-Lys(Dans)-Cys(GG)-OMe in the presence of detergents. (A) SDS−PAGE gels photographed in UV light. In the efficient ligation reactions a fluorescent protein band emerges at 24 kDa. (B) Structures of detergents able to support in vitro ligation of prenylated peptides: cetyltrimethylammoniumbromide (CTAB), lauryldimethylamine-*N*-oxide (LDAO), *N*-dodecyl-*N*,*N*-(dimethylammonio)butyrate (DDMAB), sodiumdodecyl sulfate (SDS), *n*-octyl-phosphocholine (FOS-Choline-8), and cyclohexyl-ethyl-β-D-maltoside (Cymal-2).

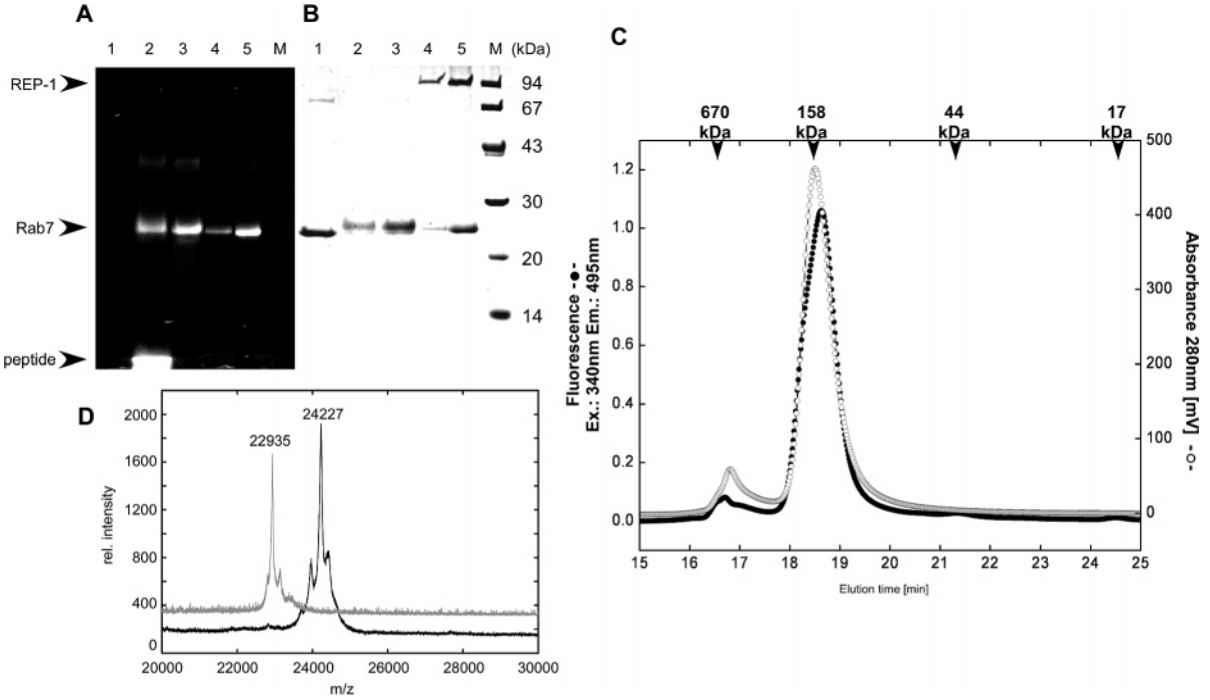

Figure 2. Characterization of the product obtained from the ligation reactions of Rab7ΔC6-MESNA with lipopeptides **30b**,**c**. (A,B) SDS−PAGE gel of Rab7ΔC6-MESNA thioester before (lane 1) and after incubation with peptide **30c** (lane 2), after removal of unligated peptide (lane 3), and after complex formation with REP-1 and Superdex-200 gel filtration purification (lane 4). Lane 5 represents the final purified Rab7:REP-1 complex as shown in lane 4, except that peptide **30b** was used for the ligation. The gel was photographed either in UV light (A) or visible light after Coomassie blue staining (B). (C) Analytical gel filtration of Rab7ΔC6-**30b**:REP-1 complex. Detection was based on absorbance at 280 nm (○) or fluorescence at 495 nm observed upon excitation at 280 nm (●). The elution position of standard molecular weight markers is indicated by arrows. (D) MALDI-TOF analysis of Rab7ΔC6-MESNA thioester (M_{calcd} = 22932 Da, gray) and Rab7 Rab7ΔC6-**30c** (M_{calcd} = 24212 Da).

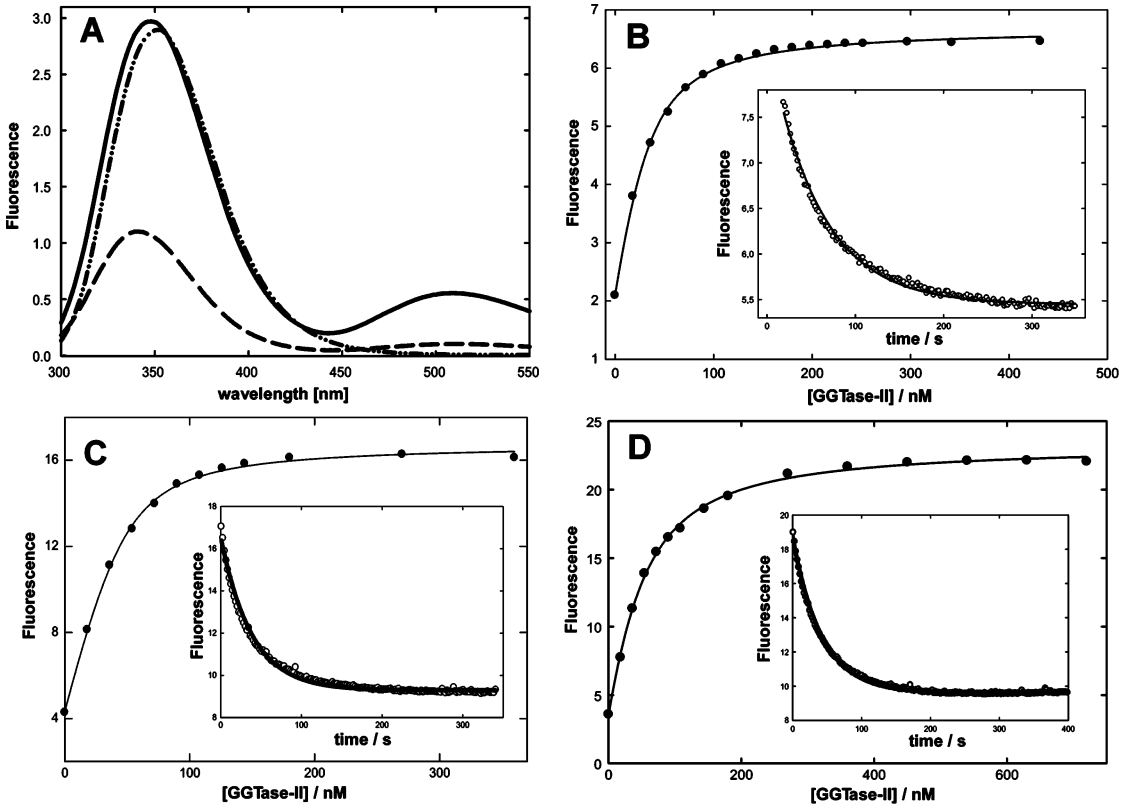

Figure 3. Interaction of RabGGTase with mono- and diprenylated Rab7. (A) Emission scans of RabGGTase (− · · −) and Rab7ΔC6-CK(Dans)SC(GG)-SC-OMe:REP-1 (- - -) alone and upon ternary complex formation (—). The concentration of each component was 200 nM. (B) Fluorescence titration of 100 nM Rab7ΔC6-CK(Dans)SC(GG)SC(GG)-OMe with RabGGTase. The solid line represents a fit to a K_d value of 14 ± 1 nM. The inset shows the displacement of the semisynthetic Rab7:REP-1 complex from RabGGTase induced by addition of 1 μM diprenylated nonfluorescent Rab7:REP-1. The solid line represents a fit to a single exponential with a k_{off} value of 0.018 ± 1 s^{-1}. (C) Fluorescence titration of 100 nM Rab7ΔC6-CK(Dans)SCSC(GG)-OMe with RabGGTase. The solid line represents a fit to a K_d value of 7 ± 2 nM. The inset shows the displacement of the semisynthetic Rab7:REP-1 complex from RabGGTase performed as in B. The solid line represents a fit to a single-exponential equation yielding a k_{off} value of 0.027 ± 1 s^{-1}. (D) Fluorescence titration of 150 nM Rab7ΔC6-CK(Dans)SC(GG)SC-OMe with RabGGTase leading to a K_d value of 35 ± 4 nM. The inset shows the determination of k_{off} of 0.024 ± 1 s^{-1} obtained as in B. The signal was based on FRET with excitation at 280 nm while collecting data at 495 nm.

Table 2. K_d and k_{off} Values of the Ternary Complexes of RabGGTase, REP-1, and Differently Geranylgeranylated Rab7 Proteins in Comparison with Unprenylated Wild-Type Rab7[a]

ternary complex	K_d [nM]	k_{off} [s^{-1}]
Rab7Δ6-CK(Dans)SCSC-OH: REP-1: RabGGTase[13]	111	
Rab7Δ6-CK(Dans)SCSC(GG)-OMe:REP-1: RabGGTase	7 ± 2	0.027 ± 0.001
Rab7Δ6-CK(Dans)SC(GG)SC-OMe:REP-1: RabGGTase	35 ± 4	0.024 ± 0.001
Rab7Δ6-CK(Dans)SC(GG)SC(GG)-OMe:REP-1: RabGGTase	13 ± 1	0.018 ± 0.001

[a] Values are the mean of at least three independent measurements.

in Figure 4A, only a minor change in fluorescence following the addition of GGPP to the Rab7ΔC6-CK(Dans)SC(GG)SC-(GG)-OMe:REP-1:RabGGTase complex was observed. The slight fluorescence decrease could potentially arise from low-efficiency incorporation of the geranylgeranyl moiety into the cysteine at the site of the ligation. The observation that the reaction is not single exponential is probably explained by the fact that the chemical step must be followed by a conformational change of the enzyme in which the diprenylated C-terminus is moved out of the active site and associates with the lipid binding site on the REP molecule. At the simplest level of interpretation, the two rate constants would correspond to the rates of these two steps, but further experiments and analysis will be required to allow definite conclusions to be drawn.

We used the developed assay to obtain the prenylation rates for both monoprenylated reaction intermediates. The obtained values are summarized in the caption of Figure 4 and indicate that the presence of the isoprenoid group on the amino terminal cysteine results in a nearly 4-fold reduction of the prenylation rate compared to the case when the C-terminal cysteine is prenylated first. This possibly reflects the obstruction of the active site of RabGGTase by a tightly bound N-terminally (upstream) coupled geranylgeranyl group. Thus, apparently, the tighter bound monoprenylated reaction intermediate is also converted faster to the diprenylated product (i.e., the intermediate that is prenylated on the terminal cysteine residue).

Discussion

Many previous studies in the field of lipoproteins have focused on lipidated model peptides, which have proven to be valuable tools in the studies of selective membrane targeting and association.[21,22] Efficient methods for the synthesis of differently lipidated peptides have been developed,[17,23−28]

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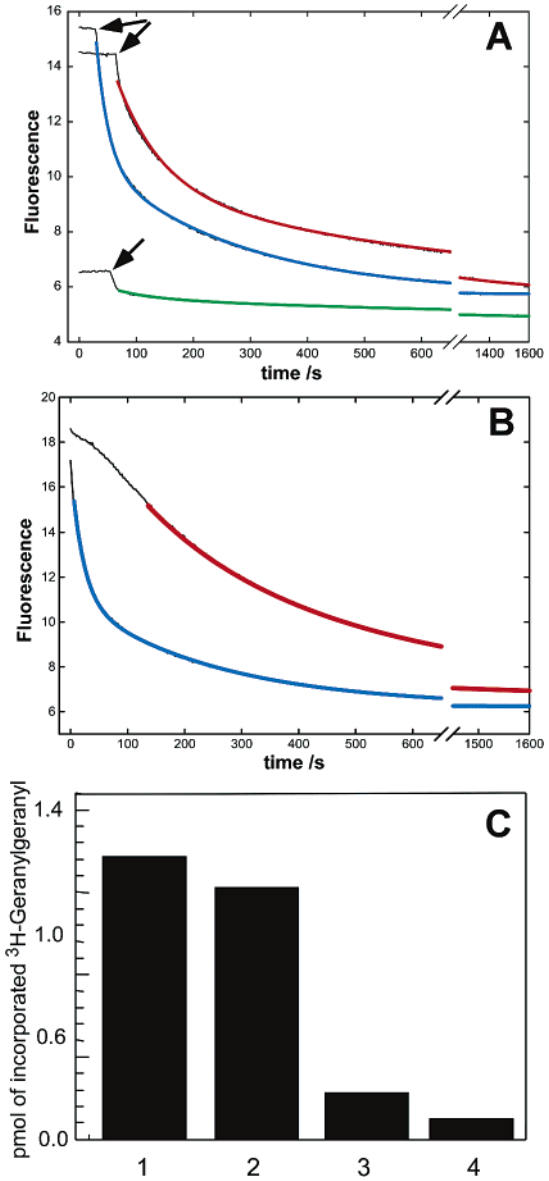

Figure 4. (A) Prenylation reactions of mono- and diprenylated semisynthetic Rab7 proteins; ca. 75 nM Rab:REP complex was incubated with 75 nM RabGGTase, and 10 μM GGpp was added at the point indicated by arrows. The solid lines represent fits to a double-exponential equation for Rab7ΔC6-CK(Dans)SC(GG)SC-OMe in red ($k_1 = 0.012$ s^{-1} and $k_2 = 0.002$ s^{-1}), Rab7ΔC6-CK(Dans)SCSC(GG)-OMe in blue ($k_1 = 0.047$ s^{-1} and $k_2 = 0.004$ s^{-1}), and Rab7ΔC6-CK(Dans)SC(GG)SC(GG)-OMe in green ($k_1 = 0.015$ s^{-1} and $k_2 = 0.001$ s^{-1}). (B) Prenylation of Rab7ΔC6-CK(Dans)-SCSC(GG)-OMe in the presence (red) or absence (blue) of 500 μM inhibitor NE10790. The solid lines represent fits to a double-exponential equation, yielding a k_1 of 0.047 (−, inhibitor) and 0.005 s^{-1} (+, inhibitor) and a k_2 of 0.004 and 0.002 s^{-1}, respectively. (C) Incorporation of [^{3}H]-geranylgeranyl by RabGGTase into semisynthetic Rab:REP complexes. (1) REP-1: Rab7ΔC6-CK(Dans)SCSC(GG)-OMe; (2) REP-1:Rab7ΔC6-CK(Dans)SC-(GG)SC-OMe; (3) REP-1:Rab7ΔC6-CK(Dans)SC(GG)SC(GG)-OMe, and (5) negative control (no RabGGTase).

although due to problems associated with the acid and base sensitivity of these compounds a sophisticated protecting-group strategy has to be employed. However, the use of model peptides can replace experiments with the native lipoprotein only in a limited number of cases, so that the need arises for the development of a generic strategy for construction of biologically active prenylated protein probes.[27,28] To construct fluorescently labeled mono- and diprenylated Rab proteins, we used a combination of chemical synthesis and in vitro protein ligation. We adopted a convergent peptide synthesis strategy that proved to be both flexible and efficient. Therefore, after introduction of the geranylgeranylated building blocks, which were generated in five steps starting from farnesyl bromide, the number of reaction steps could be reduced to a minimum. Likewise, using EDC/HOBt as mild coupling reagents the hydroxy groups of the serine side chains could be left unprotected during the coupling reactions and further deprotection steps could be avoided. Moreover, the convergent strategy allows simple introduction of a fluorescent marker according to experimental needs.

Unexpectedly, geranylgeranylated peptides could not be ligated to a thioester-tagged protein by in vitro protein ligation under the conditions typically used for this procedure with peptides not carrying lipid groups. After extensive investigation we identified a small subset of detergents that can support the in vitro ligation reaction with lipidated peptides. It is likely that prenylated peptides form higher order structures in aqueous solution that can be made accessible to the protein via formation of mixed detergent micelles. We speculate that both thioester-tagged protein and the peptide are incorporated into detergent micelles. This could result in high effective concentrations of the reactants in the micellar phase, which has been recognized as the main factor involved in the rate enhancement of bimolecular reactions in micellar solutions.[29] Furthermore, CTAB has been reported to decrease the pK_a of protein thiols, which at a given pH would increase the fraction of thiolate anions.[30] Since this species represents the actual nucleophile in the native chemical ligation reaction, this could explain the observed powerful effect of CTAB on such reactions. However, it remains unclear why some detergents are dramatically more efficient than others in this respect.

Using the approach described above we synthesized mono- and diprenylated fluorescent Rab7 GTPases that represent genuine intermediates of the RabGGTase-mediated Rab prenylation reaction. We analyzed the interaction of these intermediates with prenyltransferase and found that the C-terminally prenylated (Cys 207) form interacts with RabGGTase with severalfold higher affinity than Rab7 prenylated on Cys 205. The higher affinity of prenylated Rab proteins appears to arise from a difference in effective association rates. We propose that this is due to steric constraints imposed by the active site of RabGGTase on the monoprenylated C-terminus.

The availability of an environmentally sensitive fluorophore near the prenylation site allowed us to develop the first fluorescence-based Rab prenylation assay. Using this assay we determined the rates for conversion of the monoprenylated reaction intermediates into the diprenylated reaction product. The obtained rate is in excellent agreement with values recently reported for the Rab diprenylation reaction as determined by

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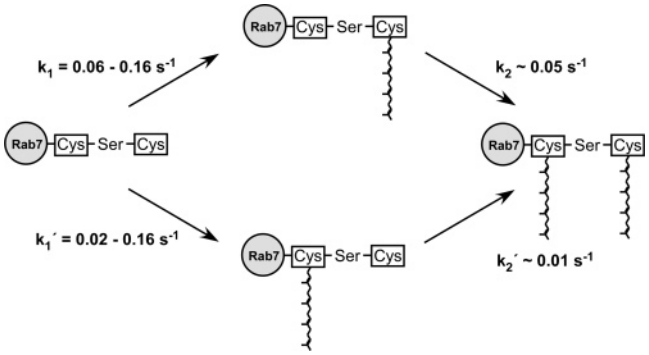

Figure 5. Schematic representation of Rab7 diprenylation reaction by RabGGTase. Rate constants were determined under single-turnover conditions and saturating concentrations of REP-1, RabGGTase, Rab7, and GGPP. The rate constants of the first prenyl transfer (k_1 and k_1') were determined by employing single cysteine mutants of Rab7 and represent the lower limit of the reaction rate. The upper limit was obtained by measuring the rate of monoprenylated species accumulation in prenylation of wild-type Rab7 due to $k_1 > k_2$.

other assays.[5] Our data indicate that the more tightly bound monoprenylated reaction intermediate is also more rapidly converted to the diprenylated product. The only structural feature that distinguishes these Rab7:REP-1 prenylation reaction intermediates is the location of the unprenylated cysteine residue and the prenyl group. We hypothesize that binding of the first lipid to RabGGTase influences the fixation of the unprenylated cysteine in the active site. A likely coordination site is the essential zinc ion, which is believed to activate the thiol group for the nucleophilic attack on the C1 of GGPP by stabilizing the thiolate form of the cysteine. It is conceivable that stronger stabilization (stronger binding, lower K_d value) results in faster activation of the cysteine (higher reactivity, faster prenyl transfer). This could explain the observation that in the cases examined the affinities of the reaction intermediates for RabGGTase are directly related to their ability to accept the second prenyl group. Thus, transfer of the first prenyl group onto the upstream cysteine results in an unfavorable orientation of the terminal cysteine, which is reflected by the larger dissociation constant of the interaction and the slower rate of the second prenyl transfer reaction. Conversely, accommodation of the isoprenoid attached to the C-terminal cysteine residue and coordination of the upstream cysteine residue to the zinc ion is preferred. These findings suggest that RabGGTase has evolved an active site that clearly prefers one of the two possible reaction intermediates for the second round of prenylation. The rate constants for the first and second prenylation reaction are summarized in Figure 5. We conclude that the majority of Rab7 proteins proceed to diprenylation by first acquiring prenylation on the C-terminal cysteine, followed by modification of the N-terminal cysteine.[5] However, due to the heterogeneity of Rab prenylation motifs, the sequence of events could be different for other Rab proteins.[4]

Experimental Section

Standard Peptide Coupling Conditions. Unless otherwise stated, all peptide coupling reactions were performed by means of the following standard procedure: To a 1:1 (molar) solution of each coupling partner dissolved in CH_2Cl_2 (5−40 mL) at 0 °C was added 1-hydroxybenzotriazole (HOBt) (1.5−2.0 equiv), followed by ethyl(dimethylamino)-propylcarbodiimide (EDC) (1.2−1.5 equiv). The reaction was warmed to room temperature and stirred for 18 h. The reaction mixture was then diluted with ethyl acetate (50−100 mL) and extracted with 0.5 N HCl (2 × 10 mL). The organic layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The product could then be isolated from the remaining residue by flash chromatography on silica gel using the solvent systems noted for each particular compound.

Standard Procedure for the Removal of Allyl Esters. To a solution of the protected peptide in dry THF (12−15 mL) was added dimethylbarbituric acid (DMAB) (0.55 equiv), followed by a catalytic amount of tetrakis(triphenylphosphine)palladium(0). The reaction was monitored by TLC and judged complete with the disappearance of starting material (generally 1.5−2 h). The solvent was then removed under reduced pressure. The product could be isolated from the remaining residue by the methods noted for each particular compound.

Standard Procedure for the Removal of Boc Groups. To a solution of the protected peptide in dry CH_2Cl_2 (4.0−6.0 mL) was added thioanisole (2.0 equiv) and at 0 °C trifluoroacetic acid (1.6−3.0 mL). The mixture was then left to stir for 15 min at 0 °C and 1.5−2.5 h at room temperature. The solvent was removed by coevaporation with toluene (2 × 30−50 mL) and chloroform (30−50 mL). The desired product was isolated from the remaining residue by the methods noted for each particular compound.

***S-tert*-Butylthio-l-cysteyl-*N*$^{(\epsilon)}$-5-(dimethylamino)-naphthalene-1-sulfonyl-l-l ysyl-l-seryl-*S-tert*-butylthio-l-cysteyl-l-seryl-*S*-geranylgeranyl-l-cysteine Methyl Ester (H-Cys(S^{t}Bu)-Lys(dans)-Ser-Cys(S^{t}Bu)-Ser-Cys(GG)-OMe) (30a).** At room temperature, to a solution of Fmoc-Cys(S^{t}Bu)-Lys(dansyl)-Ser-Cys(S^{t}Bu)-Ser-Cys(GG)-OMe 29a (20 mg, 13 μmol) in dry dichloromethane (0.08 mL) under an argon atmosphere was added a solution of dry dichloromethane/diethylamine (1:1; 0.11 mL). The mixture was left to stir for 3 h, and the solvent was removed by coevaporation with toluene (40 mL) and chloroform (40 mL). The compound was purified by flash chromatography on silica gel with dichloromethane/methanol (50:1) as eluent to yield 17 mg (98%) of the desired product 30a as a yellowish-green oil. $R_f = 0.58$ (dichloromethane/methanol (10:1)). mp = 170−171 °C. $[\alpha]^{20}_D = -65.8$ (c = 0.325, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ (10:1)): δ 8.53 (d, J = 8.4 Hz, 1H, CH-2 dansyl), 8.31 (d, J = 8.4 Hz, 1H, CH-8 dansyl), 8.19 (dd, J = 7.4, 1.0 Hz, 1H, CH-4 dansyl), 7.58 (dd, J = 8.6, 7.8 Hz, 1H, CH-7 dansyl), 7.53 (dd, J = 8.4, 7.6 Hz, 1H, CH-3 dansyl), 7.21 (d, J = 7.2 Hz, 1H, CH-6 dansyl), 5.19 (t, J = 7.8 Hz, 1H, C=C*H*−CH_2−S GG), 5.08−5.13 (m, 3H, 3CH GG), 4.73 (dd, J = 8.4, 4.8 Hz, 1H, α-CH Cys_{StBu}), 4.67 (dd, J = 7.8, 5.4 Hz, 1H, α-CH Cys(GG)), 4.51 (dd, J = 5.0 Hz, 1H, α-CH Ser), 4.41 (dd, J = 5.0 Hz, 1H, α-CH Ser′), 4.21 (dd, J = 7.8, 6.2 Hz, 1H, α-CH Lys), 3.91 (dd, J = 11.8, 5.4 Hz, 2H, 2β-CH_{2a} Ser), 3.76−3.81 (m, 2H, 2β-CH_{2b} Ser), 3.74 (s, 3H, OCH_3), 3.69 (dd, J = 8.4, 4.0 Hz, 1H, α-CH $Cys_{StBu'}$), 3.24 (dd, J = 13.8, 5.0 Hz, 1H, β-CH_{2a} Cys_{StBu}), 3.18−3.28 (m, 1H, CH_{2a}-S GG), 3.17 (dd, J = 13.4, 4.2 Hz, 1H, β-CH_{2a} $Cys_{StBu'}$), 3.07−3.14 (m, 2H, β-CH_{2b} Cys_{StBu}, CH_{2b}-S GG), 2.94 (dd, J = 14.0, 4.8 Hz, 1H, β-CH_{2a} Cys(GG)), 2.89 (s, 6H, $N(CH_3)_2$), 2.86−2.89 (m, 2H, ϵ-CH_2 Lys), 2.81 (dd, J = 13.6, 7.6 Hz, 1H, β-CH_{2b} Cys(GG)), 2.80 (dd, J = 13.4, 8.6 Hz, 1H, β-CH_{2b} $Cys_{StBu'}$), 1.95−2.12 (m, 12H, 6CH_2 GG), 1.71−1.80 (m, 1H, β-CH_{2a} Lys), 1.68 (s, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.60−1.68 (m, 1H, β-CH_{2b} Lys), 1.60 (s, 9H, 3CH_3), 1.37−1.49 (m, 4H, δ-, γ-CH_2 Lys), 1.35 (s, 9H, $C(CH_3)_3$), 1.33 (s, 9H, $C(CH_3)_3$). ^{13}C NMR (125.8 MHz, $CDCl_3/CD_3OD$ (10:1)): δ 172.7, 171.0, 170.8, 151.7, 139.8, 135.1, 134.7, 134.4, 131.1, 130.5, 129.6, 129.5, 128.4, 124.2, 123.3, 119.6, 119.1, 115.4, 61.9, 55.6, 55.4, 53.9, 53.8, 53.2, 52.5, 52.2, 48.2, 45.4, 45.3, 42.5, 40.8, 39.7, 32.7, 31.5, 29.9, 29.7, 28.5, 26.7, 25.7, 22.2, 17.7, 15.9. ESI−MS m/z: calcd for $(M + H)^+$ $C_{62}H_{101}N_8O_{11}S_6$, 1325.6; found, 1325.5 $(M + H)^+$.

***S-tert*-Butylthio-l-cysteyl-*N*$^{(\epsilon)}$-5-(dimethylamino)-naphthalene-1-sulfonyl-l-lysyl-l-seryl-*S*-geranylgeranyl-l-cysteyl-l-seryl-*S-tert*-butylthio-l-cysteine Methyl Ester (H-Cys(S^{t}Bu)-Lys(dans)-Ser-Cys-(GG)-Ser-Cys(S^{t}Bu)-OMe) (30b).** At room temperature, Fmoc-Cys(S^{t}Bu)-Lys(dansyl)-Ser-Cys(GG)-Ser-Cys(S^{t}Bu)-OMe 29b (8.5 mg, 5.5 μmol) was dissolved in dry dichloromethane/diethylamine (3:1; 1.0

mL) under an argon atmosphere. The mixture was left to stir for 1.25 h, and the solvent was removed by coevaporation with toluene (2 × 40 mL). The compound was purified by flash chromatography on silica gel. Beforehand, the silica gel was washed with dichloromethane/methanol (30:1) containing 1% dimethylethylamine, followed by dichloromethane/methanol (30:1), and finally pure dichloromethane. For purification of the product, dichloromethane/methanol (15:1) was used as eluent. The pure product was dissolved in dichloromethane (30 mL) and washed with brine (20 mL). The aqueous layer was back-extracted with dichloromethane (2 × 20 mL). The combined organic layers were dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure to yield 5.6 mg (77%) of the desired product 30b as a pale yellowish-green oil. $R_f = 0.38$ (dichloromethane/methanol (10:1)). $[\alpha]^{20}_D = -37.6$ ($c = 0.25$, CH_2Cl_2/CH_3OH (10:1)). ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ (10:1)): δ 8.54 (d, $J = 8.4$ Hz, 1H, CH-2 dansyl), 8.32 (d, $J = 8.4$ Hz, 1H, CH-8 dansyl), 8.19 (dd, $J = 7.2$, 1.2 Hz, 1H, CH-4 dansyl), 7.58 (dd, $J = 8.4$, 7.6 Hz, 1H, CH-7 dansyl), 7.54 (dd, $J = 8.4$, 7.2 Hz, 1H, CH-3 dansyl), 7.23 (d, $J = 7.2$ Hz, 1H, CH-6 dansyl), 5.23 (t, $J = 7.4$ Hz, 1H, C=C*H*−CH_2−S GG), 5.07−5.13 (m, 3H, 3CH GG), 4.78 (dd, $J = 7.4$, 5.0 Hz, 1H, α-CH Cys_{StBu}), 4.59 (dd, $J = 8.0$, 6.0 Hz, 1H, α-CH Cys(GG)), 4.51 (dd, $J = 5.2$ Hz, 1H, α-CH Ser), 4.43 (dd, $J = 5.2$ Hz, 1H, α-CH Ser′), 4.26 (dd, $J = 8.2$, 5.8 Hz, 1H, α-CH Lys), 3.87−3.93 (m, 2H, 2β-CH_{2a} Ser), 3.77−3.83 (m, 3H, 2β-CH_{2b} Ser, α-CH $Cys_{StBu'}$), 3.75 (s, 3H, OCH_3), 3.14−3.26 (m, 4H, CH_2−S GG, 2β-CH_{2a} Cys_{StBu}), 3.08 (dd, $J = 13.8$, 7.4 Hz, 1H, β-CH_{2b} Cys_{StBu}), 2.99−3.04 (m, 1H, β-CH_{2a} Cys(GG)), 2.90 (s, 6H, $N(CH_3)_2$), 2.84−2.90 (m, 4H, ϵ-CH_2 Lys, β-CH_{2b} Cys(GG), β-CH_{2b} $Cys_{StBu'}$), 1.95−2.13 (m, 12H, 6CH_2 GG), 1.71−1.83 (m, 2H, β-CH_2 Lys), 1.67 (s, 6H, 2CH_3), 1.60 (s, 9H, 3CH_3), 1.37−1.52 (m, 4H, δ-, γ-CH_2 Lys), 1.35 (s, 9H, $C(CH_3)_3$), 1.32 (s, 9H, $C(CH_3)_3$). ESI-MS *m*/*z*: calcd for $(M + H)^+$, $C_{62}H_{101}N_8O_{11}S_6$ 1325.6; found, 1325.7.

***S*-*tert*-Butylthio-l-cysteyl-*N*$^{(\epsilon)}$-5-(dimethylamino)-naphthalene-1-sulfonyl-l-lysyl-l-seryl-*S*-geranylgeranyl-l-cysteyl-l-seryl-*S*-geranylgeranyl-l-cysteine Methyl Ester (H-Cys(S^{t}Bu)-Lys(dans)-Ser-Cys(GG)-Ser-Cys(GG)-OMe) (30c).** At room temperature, Fmoc-Cys(S^{t}Bu)-Lys(dansyl)-Ser-Cys(GG)-Ser-Cys(GG)-OMe 29c (10.0 mg, 5.8 μmol) was dissolved in dry dichloromethane/diethylamine (2:1; 1.0 mL) under an argon atmosphere. The mixture was left to stir for 1.25 h, and the solvent was removed by coevaporation with toluene (2 × 40 mL). The compound was purified by flash chromatography on silica gel. Beforehand, the silica gel was washed with dichloromethane/methanol (30:1) containing 1% dimethylethylamine, followed by dichloromethane/methanol (30:1), and finally pure dichloromethane. For purification of the product, dichloromethane/methanol (15:1) was used as eluent. The pure product was dissolved in dichloromethane (30 mL) and washed with brine (20 mL). The aqueous layer was back-extracted with dichloromethane (2 × 20 mL). The combined organic layers were dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure to yield 8.6 mg (99%) of the desired product 30c as a pale yellowish-green oil. $R_f = 0.35$ (dichloromethane/methanol (20:1)). $[\alpha]^{20}_D = -31.4$ ($c = 0.22$, CH_2Cl_2/CH_3OH (10:1)). ^1H NMR (400 MHz, $CDCl_3/CD_3OD$ (10:1)): δ 8.53 (d, $J = 8.4$ Hz, 1H, CH-2 dansyl), 8.31 (d, $J = 8.8$ Hz, 1H, CH-8 dansyl), 8.19 (dd, $J = 7.2$, 1.6 Hz, 1H, CH-4 dansyl), 7.57 (dd, $J = 8.8$, 7.6 Hz, 1H, CH-7 dansyl), 7.53 (dd, $J = 8.4$, 7.2 Hz, 1H, CH-3 dansyl), 7.21 (dd, $J = 7.6$, 0.4 Hz, 1H, CH-6 dansyl), 5.22 (t, $J = 8.6$ Hz, 1H, C=C*H*-CH_2−S GG), 5.19 (t, $J = 9.0$ Hz, 1H, C=C*H*-CH_2−S GG′), 5.07−5.13 (m, 6H, 6CH GG), 4.66 (dd, $J = 8.0$, 5.2 Hz, 1H, α-CH Cys(GG)), 4.59 (dd, $J = 8.0$, 6.0 Hz, 1H, α-CH Cys(GG)′), 4.51 (dd, $J = 5.0$ Hz, 1H, α-CH Ser), 4.42 (dd, $J = 5.2$ Hz, 1H, α-CH Ser′), 4.24 (dd, $J = 8.0$, 6.0 Hz, 1H, α-CH Lys), 3.92 (dd, $J = 11.6$, 3.6 Hz, 1H, β-CH_{2a} Ser), 3.90 (dd, $J = 11.2$, 4.4 Hz, 1H, β-CH_{2a} Ser′), 3.75−3.81 (m, 3H, 2β-CH_{2b} Ser, α-CH Cys_{StBu}), 3.74 (s, 3H, OCH_3), 3.16−3.25 (m, 4H, CH_2−SGG, CH_{2a}-S GG′, β-CH_{2a} Cys_{StBu}), 3.11 (dd, $J = 13.4$, 7.0 Hz, 1H, CH_{2b}-S GG), 3.01 (dd, $J = 13.8$, 5.8 Hz, 1H, β-CH_{2a} Cys(GG)), 2.94 (dd, $J = 13.8$, 5.4 Hz, 1H, β-CH_{2a} Cys(GG)′), 2.89 (s, 6H, $N(CH_3)_2$), 2.83−2.89 (m, 4H, ϵ-CH_2 Lys, β-CH_{2b} Cys(GG), β-CH_{2b} Cys_{StBu}), 2.80 (dd, $J = 14.0$, 7.6 Hz, 1H, β-CH_{2b} Cys(GG)′), 1.95−2.13 (m, 24H, 12CH_2 GG), 1.63−1.78 (m, 2H, β-CH_2 Lys), 1.67−1.68 (4s, 12H, 4CH_3), 1.60 (s, 18H, 6CH_3), 1.39−1.48 (m, 4H, δ-, γ-CH_2 Lys), 1.35 (s, 9H, $C(CH_3)_3$). ESI-MS *m*/*z*: calcd for $(M + H)^+$ $C_{78}H_{125}N_8O_{11}S_5$ 1509.8, found 1510.0.

Cloning, Protein Expression, and Purification. REP-1 and RabGGTase were purified as described previously.[31,32] Briefly, RabGGTase was purified from *E. coli* cells coexpressing α- and β-subunits by a combination of metal-chelating and gel-filtration chromatography.[31] REP-1 was purified in a similar manner from yeast.[32] Diprenylated wild-type Rab7:REP-1 complex was prepared by in vitro prenylation as described.[31]

The coding region of the canine Rab7 gene truncated by six amino acids was amplified by PCR using pET3a Rab7 plasmid as a template[33] and the synthetic oligo-nucleotides 5′-ATTGGTACCCTTGGCAAAG-CATGAGGTCTTGGCCCGGTCGTTC-3′ and T7 promoter as primers. The PCR product was gel purified, digested with *Kpn*I and *Nde*I, and ligated into the pTYB1 (New England Biolabs) vector precut with the same enzymes. The resulting plasmid (pTYB1-Rab7ΔC6) was transformed into *E. coli* BL21(DE3) cells, and transformants were selected on ampicillin (50 mg/L) agar plates. A single colony was inoculated into 5 mL of LB medium containing 125 mg/L Ampicillin, and the culture was grown overnight at 37 °C. This preculture was used to seed 2 L of fresh LB medium (containing 125 mg/L Ampicillin), and the culture was incubated at 37 °C until the absorbance at 600 nm (OD_{600}) reached 0.5−0.7. IPTG was added to a final concentration of 0.5 mM, and overnight (or 10−12 h) induction was performed at 20 °C. Cells were harvested by centrifugation (5000*g*, 20 min, 4 °C) and washed once in wash buffer (10 mM Na−Phosphate, pH 7.2, 0.1 M NaCl). The bacterial pellet was resuspended in lysis buffer (25 mM Na−Phosphate, pH 7.5, 0.5 M NaCl, 0.5 mM PMSF, 2 mM $MgCl_2$ and 2 μM GDP), and cells were lysed by passing them twice through a Microfluidizer (Microfluidics). A fresh portion of 0.5 mM PMSF and Triton X-100 (1% final concentration) were added. The lysate was cleared by ultracentrifugation (30 000*g*, 40min, 4 °C). An appropriate amount of chitin beads, equilibrated with lysis buffer containing 1% Triton X-100, was added to the supernatant, and the mixture was incubated for 2 h on a rotating wheel at 4 °C. The beads were isolated by centrifugation (2500*g*, 5 min, 4 °C) and washed 4 times with lysis buffer containing 1% Triton X-100 followed by 4 times washing with buffer without the detergent. Cleavage of the fusion protein was induced by adding powdered MESNA to the beads suspension to a concentration of 0.5 M and overnight incubation at room temperature. The supernatant was collected by centrifugation and passed over a gel filtration column equilibrated with ligation buffer (10 mM Na−Phosphate, pH 7.5, 0.1 mM $MgCl_2$, 2 μM GDP). The pooled fractions were concentrated to at least 10 mg/mL and shock frozen in multiple aliquots using liquid nitrogen. The proteins could be stored at −80 °C for at least 2 years without loss of ligation efficiency. Yields typically ranged from 10 to 30 mg of Rab7 protein thioester per liter of bacterial culture.

Detergent Screen. The collection of 72 detergents was from Hampton detergent screen 1−3 (Hampton Research). A 40 μg (1.7 nmol) amount of Rab7Δ6-MESNA thioester was mixed with 12 μg (16 nmol) of peptide Cys-Lys(Dans)-Cys(GG)-OMe and the respective detergent. The final detergent concentration was 3.8 times above its respective critical micellar concentration. The mixture was incubated at 30 °C for 10 h with gentle agitation. An aliquot of the mixture was mixed with SDS−sample buffer and analyzed by SDS−PAGE.

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In Vitro Protein Ligation. A 500 μL amount of Rab−thioester protein (typically 20 mg/mL, ca. 500 nmol) in ligation buffer was supplemented with 50 mM CTAB and 125 mM MESNA (final concentrations). Ligation was initiated by adding 3.5−5 μmol of the respective peptide from a ca. 30 mM stock solution in dichloromethane/methanol (1:5). The reaction mixture was incubated overnight at 37 °C with vigorous agitation. The reaction mixture was centrifuged, and the supernatant was removed. The pellet was washed once with 1 mL of methanol, 4 times with 1 mL of dichloromethane, 4 times with 1 mL of methanol, and 4 times with 1 mL of Milli-Q water at room temperature in order to resolubilize contaminating peptide and unligated protein. The precipitate was dissolved in denaturation buffer (100 mM Tris−HCl, pH 8.0, 6 M guanidinium−HCl, 100 mM DTE, 1% CHAPS, 1 mM EDTA) to a final protein concentration of 0.5−1.0 mg/mL and incubated overnight at 4 °C with slight agitation. The solution was cleared by centrifugation or filtration. Protein was renatured by diluting it at least 25-fold dropwise into refolding buffer (50 mM Hepes pH 7.5, 2.5 mM DTE, 2 mM $MgCl_2$, 10 μM GDP, 1% CHAPS, 400 mM Arginine−HCl, 400 mM Trehalose, 0.5 mM PMSF, 1 mM EDTA) with gentle stirring at room temperature. The mixture was incubated 30 min at the same temperature and subsequently centrifuged to remove misfolded protein.

Complex Formation, Purification, and Analytics. An equimolar amount of REP-1 was added to the solution containing refolded protein, and the sample was incubated for 1 h on ice. The mixture was dialyzed overnight against two 5 L changes of dialysis buffer (25 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 2 μM GDP, 2.5 mM DTE, 100 mM $(NH_4)_2SO_4$, 10% glycerol, 0.5 mM PMSF, 1 mM EDTA). The dialyzed material was concentrated to a protein concentration of 2−5 mg/mL using size-exclusion concentrators (MWCO, 30 kDa) and loaded on a Superdex-200 gel filtration column (Pharmacia) equilibrated with gel filtration buffer (25 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 10 μM GDP, 2.5 mM DTE, 100 mM $(NH_4)_2SO_4$, 10% glycerol). The peak fractions containing the desired complex (judged by SDS−PAGE) were pooled, concentrated to approximately 10 mg/mL, and stored frozen at −80 °C in multiple aliquots. The typical recovery yield ranged from 10% to 30% with respect to starting Rab−thioester.

Fluorescence Measurements. Fluorescence measurements were performed with a Spex Fluoromax-3 spectrofluorometer (Jobin Yvon, Edison, NJ). Measurements were carried out in 1 mL quartz cuvettes (Hellma) with continuous stirring at 25 °C unless otherwise indicated. Steady-state fluorescence measurements for monitoring interactions between semisynthetic Rab7:REP-1 complexes with RabGGTase were followed in 50 mM Hepes, pH 7.2, 50 mM NaCl, and 5 mM DTE. Typically the dansyl-labeled Rab7:REP-1 complex was placed in a cuvette in 1 mL of buffer to give a final concentration of ca. 100−200 nM and incubated for 5 min at 25 °C. The excitation and emission monochromators were set to 280 and 495 nm, respectively. Small aliquots of RabGGTase (typically 10−20 nM at each step) were then added to the cuvette until the fluorescence signal was saturated or showed a continuous linear increase. The change in fluorescence was plotted as a function of the total RabGGTase concentration and corrected for unspecific fluorescence. The data were fitted to the following equation using GraFit 4.0 (Erithacus software): $F = F_{min} + [K_d + P_0 + L - [(K_d + P_0 + L)^2 - 4P_0L]^{1/2}](F_{max} - F_{min})/2P_0$, where F is the observed fluorescence after each step of titrator addition, F_{min} is the initial value at $[L]_0 = 0$, F_{max} is the final value at saturation, $[L]_0$ is the total (cumulative) concentration of RabGGTase, $[P]_0$ is the Rab7:REP-1 complex concentration, and K_d is the equilibrium constant, which is to be determined.

For determination of k_{off}, the dansyl-labeled semisynthetic Rab7:REP-1 complex (100 nM) was incubated with an equal amount of RabGGTase (100 nM) at 25 °C in 1 mL of buffer (50 mM Hepes, pH 7.2, 50 mM NaCl and 5 mM DTE) for 5 min. Excitation was set to 280 nm, while data were collected at 495 nm. The fluorescently labeled complex was displaced from RabGGTase by addition of an at least 10-fold excess of diprenylated wt Rab7:REP-1 complex (1−1.5 μM final concentration). The obtained displacement curve was fitted to a single-exponential equation, yielding a rate constant which under the employed conditions corresponds to k_{off} for dissociation of the semisynthetic Rab7:REP-1 complex from RabGGTase.

For real-time monitoring of prenylation reactions, typically 50−100 nM of dansyl-labeled semisynthetic Rab7:REP-1 complex was mixed with an equal amount of RabGGTase in a cuvette containing 1 mL of buffer (50 mM Hepes, pH 7.2; 50 mM NaCl, 5 mM DTE, 2 mM $MgCl_2$, 100 μM GDP). Following a 5 min incubation at 25 °C, the reaction was initiated by adding geranylgeranyl-pyrophosphate to a final concentration of 10 μM. Excitation and emission monochromators were adjusted to 280 and 510 nm, respectively. Data were fitted to a double-exponential equation using GraFit 4.0 (Erithacus software).

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Supporting Information Available: Synthesis and characterization details containing experimental ^1H and ^{13}C NMR spectra, ESI-MS, and MALDI-TOF of the synthesized peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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