Allosteric Regulation of Substrate Binding and Product Release in Geranylgeranyltransferase Type II†

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ABSTRACT: GTPases of the Rab family are key components of vesicular transport in eukaryotic cells. Posttranslational attachment of geranylgeranyl moieties is essential for Rab function. Geranylgeranyltransferase type II (GGTase-II) catalyzes the modification of Rab proteins once they are in complex with their escort protein (REP). Upon completion of prenylation, REP and modified Rab leave the enzyme, enabling a new round of catalysis. We have studied the mechanism underlying substrate binding and product release in the geranylgeranylation of Rab proteins. Binding of the Rab7:REP-1 complex to GGTase-II was found to be strongly modulated by geranylgeranyl pyrophosphate (GGpp). The affinity of GGTase-II for the Rab7:REP-1 complex increases from ca. 120 nM to ca. 2 nM in the presence of GGpp. To study the effect of GGpp on interaction of the enzyme with its product, we generated semisynthetic doubly prenylated Rab7 bearing a fluorescent reporter group. Using this novel compound, we demonstrated that the affinity of doubly prenylated Rab7:REP-1 complex for GGTase-II was 2 and 18 nM in the absence and presence of GGpp, respectively. The difference in affinities originates mainly from a difference in the dissociation rates. Thus, binding of the new isoprenoid substrate molecule facilitates the product release by GGTase-II. The affinity of GGpp for the prenylated Rab7:REP-1:GGTase-II was $K_d = 22$ nM, with one molecule of GGpp binding per molecule of prenylated ternary complex. We interpreted this finding as an indication that the geranylgeranyl moieties transferred to Rab protein do not occupy the GGpp binding site of the GGTase-II. In summary, these results demonstrate that GGpp acts as an allosteric activator that stabilizes the Rab7:REP-1:GGTase-II complex and triggers product release upon prenylation, preventing product inhibition of the enzyme.

The family of Rab GTPases regulates intracellular vesicular transport in eukaryotic cells by controlling membrane docking and fusion events (*1, 2*). The ability to reversibly associate with intracellular membranes is critical for Rab protein activity (*3, 4*). Such association is made possible by posttranslational attachment of one or two geranylgeranyl isoprenoids onto the C-terminus of Rab protein (*5*). The enzyme responsible for this modification is geranylgeranyltransferase type II (GGTase-II)[1] and belongs to the family of prenyl transferases together with geranylgeranyltransferase type I (GGTase-I) and farnesyltransferase (FTase). All three enzymes recruit phosphoisoprenoids and transfer isoprenoid moieties onto the C-terminus of a diverse group of proteins ranging from small GTPases to heterotrimeric G proteins and nuclear lamins. Interest in this modification markedly increased after the discovery of the importance of FTase activity for sustaining the transformed phenotype of proto-oncogenes of the Ras family (*6*). FTase and GGTase-I are heterodimers composed of tightly associated subunits that recognize a four amino acid C-terminal sequence motif on their target proteins. This motif is often referred to as the CAAX box (C, cysteine; A, aliphatic residue; and X, serine/methionine/alanine) in which cysteine is subjected to post-translational modification by isoprenoids. The choice between farnesylation and geranylgeranylation is determined by the hydrophobicity of the X residue [see (*7*) for a review]. In the case of FTase, an ordered sequential mechanism operates with lipid substrate binding first, increasing the enzyme's affinity for the protein substrate (*8, 9*). The reaction product remains tightly associated to the enzyme until binding of a new protein or lipid substrate weakens the affinity and triggers product release (*10*).

GGTase-II differs from the FTase and GGTase-I in both enzyme structure and reaction mechanism. An accessory factor termed Rab escort protein (REP) recruits Rab proteins preferentially in the GDP-bound form and presents them to the catalytic heterodimer, forming a tight ternary complex (*11, 13*). No C-terminal consensus motif could be defined,

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[1] Abbreviations: dRab7GG, Rab7A202C E203L(dansyl) C205 (GG) C207 (GG); Fpp, farnesyl pyrophosphate; FRET, fluorescence resonance energy transfer; GG, geranylgeranyl; GGpp, geranylgeranyl pyrophosphate; mant, *N*-methylanthraniloyl; mantGDP, *N*-methylanthraniloyl-GDP; MESNA, 2-mercaptoethanesulfonic acid; FTase, protein farnesyltransferase; REP-1, Rab Escort Protein-1; GGTase-I, protein geranylgeranyltransferase type I; GGTase-II, protein geranylgeranyltransferase type II; Rab7SS, Rab7 C205S and C207S.

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and Rab proteins with typical CAAX box sequences were shown to be modified by GGTase-II in vivo (*14*). Prenylated Rab proteins are then chaperoned to their destined vesicular compartment by REP which is subsequently released into the cytosol (*15, 16*).

To date, the mechanisms governing Rab:REP interaction with the GGTase-II:GGpp complex and release of the prenylated product are unknown. In particular, investigation of product release was hindered by the absence of an adequate assay system. Most of the previously available data originated from the steady-state prenylation assays based on radioactive phosphoisoprenoids (*17, 18*). This approach has limited applicability for studies on the individual steps of the prenylation reaction. In the case of FTase or GGTase-I, lipidated peptides bearing a fluorescent group were generated by total chemical synthesis (*19*), allowing spectroscopic analysis to be carried out. This methodology could not be applied directly to GGTase-II and Rab due to the large size of the protein substrate. We circumvented this problem by using recently developed intein-mediated protein ligation methodology [see (*20*) for review] to generate fluorescent prenylated Rab7. Using this novel tool, we characterized interaction of GGTase-II with its product. Our results indicate that GGpp acts as allosteric regulator of GGTase-II that controls both protein substrate binding and product release.

MATERIALS AND METHODS

Protein Expression and Purification. Expression of rat GGTase-II and REP-1 in SF21 cells and subsequent purification were performed as described (*13, 21*). Vector construction and expression of GST-tagged GGTase-II in *Escherichia coli* will be described elsewhere (Kalinin and Alexandrov, unpublished experiments). The GDP-bound forms of Rab7 wild type, Rab7C205SC207S mutant, and Rab7C205SC207S:REP-1 complex were prepared as described (*13*). Expression and purification of Rab7ΔC6-thioester is described elsewhere (*22*). Briefly, 1 L of *E. coli* BL21 cells transformed with pTYB1Rab7ΔC6 was grown to mid-log phase in Luria−Bertani medium and induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside at 20 °C for 10 h. After centrifugation, cells were resuspended in 60 mL of lysis buffer (25 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, 300 mM NaCl, 1 mM $MgCI_2$, 10 μM GDP, 1.0 mM phenylmethylsulfonyl fluoride) and lysed using a fluidizer (Microfluidics Corp.). After lysis, Triton X-100 was added to a final concentration of 1%. The lysate was clarified by ultracentrifugation and incubated with 10 mL of chitin beads (New England Biolabs) for 2 h at 4 °C. The beads were washed extensively with the lysis buffer and incubated for 14 h at room temperature with 40 mL of the cleavage buffer (25 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, 300 mM NaCl, 1 mM $MgCI_2$, 10 μM GDP, 500 mM MESNA). The supernatant containing Rab7ΔC6-thioester was concentrated using Centripreps 10 (Amicon) to a final concentration of 200 μM and stored frozen at −80 °C.

Protein Ligation. Peptide C-L(dans)-S-C-S-C was synthesized and HPLC-purified to more then 90% purity by Interactiva (Ulm, Germany). Peptide was dissolved to a final concentration of 50 mM in 25 mM Tris, pH 7.2, and 5% CHAPS. In the ligation reaction, the thioester-tagged Rab7 was mixed with the peptide in a buffer containing 25 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, 300 mM NaCl, 500 mM MESNA, 1 mM $MgCl_2$, 5% CHAPS, and 100 μM GDP and allowed to react overnight at room temperature. Nonreacted peptide and detergent were removed by passing the reaction mixture over a PD-10 desalting column (Pharmacia). The extent of ligation was determined by SDS−PAGE gel electrophoresis and mass spectrometry. Imaging was performed with a Fluro-S fluorescent imager (BioRad).

In Vitro Prenylation and Purification of dRabGG:REP-1 and Wild-Type Rab7GG:REP-1 Complex, Rab7 Labeling. Protein complex formation and in vitro prenylation were performed in 1 mL of 40 mM Hepes, pH 7.2, 150 mM NaCl, 5 mM DTE, 3 mM $MgCl_2$, and 0.3% CHAPS. Depending on the experiment, the 1 mL mixture contained 50 μM REP-1, 55 μM Rab7 or Rab7A202C E203Ldans, 60 μM GST-GGTase-II, and 500 μM GGpp. The sample was mixed, incubated at 37 °C for 3 min, and diluted to 3 mL with the same buffer containing 5% CHAPS, and 500 μL of glutathione−Sepharose (Pharmacia) was added. The sample was incubated at 4 °C for 1 h on a rotation wheel. After the indicated period, supernatant was separated from the beads and concentrated on a Centricon 30 (Amicon) to a final volume of 300 μL. The sample was centrifuged in a benchtop centrifuge for 5 min at 4 °C and loaded onto a 10/20 Superdex 200 gel filtration column (Pharmacia) driven by an FPLC system. The flow rate was 0.8 mL/min, and fractions of 1 mL were collected and analyzed by SDS−PAGE followed by fluorescent scanning and Coomassie-Blue staining. Fractions containing binary dRab7GG:REP-1 complex were pooled, concentrated, and stored in multiple aliquots at −80 °C. Labeling of Rab7 with tetramethyl rhodamine and preparation of *N*-methylanthraniloyl-GDP (mantGDP)-bound Rab7 were performed as previously described (*23, 24*).

Fluorescence Measurements. Fluorescence spectra and long time base fluorescence measurements were performed with an Aminco SLM 8100 spectrophotometer (Aminco, Silver Spring, MD). All reactions were followed at 25 °C in 25 mM Hepes, pH 7.2, 40 mM NaCl, 2 mM $MgCl_2$, and 2 mM DTE in 1 mL volume unless otherwise indicated. The fluorescence of dansyl-labeled Rab7 was excited at 333 nm, and data were collected at 440 nm. Stopped-flow experiments using dansyl-labeled Rab7 were performed in a High-Tech Scientific SF61 apparatus (Salisbury, England). The dansyl fluorescence was excited at 290 or 333 nm and detected through a 389 nm cutoff filter. For measurement of the direct fluorescence signal, the rhodamine-labeled Rab7 was excited at 570 nm, and data were collected at 590 nm. Data collection and primary analysis of rate constants were performed with the package from High Tech Scientific; the secondary analysis was performed with the Grafit 3.0 program (Erithacus software).

Electrospray Mass Spectrometry. MALDI/TOF measurements were carried out with a Perkin-Elmer Voyager TM station with the instrument set in positive ion mode.

RESULTS

Regulation of substrate binding as well as product release is important in the function of any enzyme. GGTase-II has previously been shown to bind its lipid substrate GGpp with an affinity of around 4 nM (*25*) and the protein substrate Rab7:REP-1 with an affinity of approximately 120 nM (*13*).

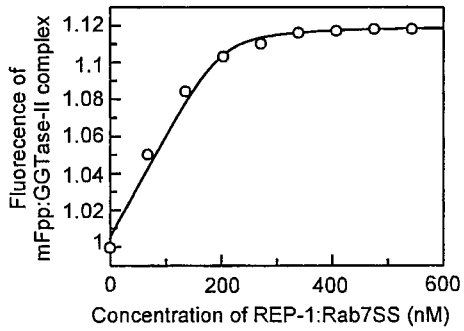


FIGURE 1: Fluorescence titration of mFpp:GGTase-II complex (200 nM) versus increasing concentrations of the Rab7SS:REP-1 complex. The experimental curve was fitted, resulting in a K_d value of ca. 2 nM. By inspection and from the fitting procedure, a stoichiometry of 1:1 (mFpp:GGTase-II to Rab7SS:REP-1) was deduced.

The fact that both lipid and protein substrates are bound to GGTase-II very tightly poses the question of how the lipidated product is released from GGTase-II once these two molecules are joined. We therefore investigated the processes underlying substrate binding and product release in GGTase-II. We examined whether Rab7:REP-1 binding to GGTase-II is affected by the presence of GGpp. Second, we characterized the affinity relationships underlying product release in Rab geranylgeranylation. These aspects have previously not been addressed, and the results presented here provide insights into the molecular mechanism of the prenylation reaction catalyzed by GGTase-II.

Determination of the Affinity of the Rab7:REP-1 Complex for GGTase-II in Complex with GGpp. To determine the affinity of Rab7:REP-1 toward GGpp-bound GGTase, we took advantage of the fluorescent analogue of *N*-methylanthraniloyl farnesyl pyrophosphate (mFpp). This fluorescent phosphoisoprenoid had previously been shown to have properties very similar to those of GGpp (*25*, *26*). On interaction of the complex between this GGpp analogue and GGTase-II with the Rab:REP complex, there is an increase in the FRET signal. Figure 1 shows the titration of a 1:1 complex between GGTase-II and mFpp with increasing concentrations of the Rab7SS:REP-1 complex. This mutant of Rab7 does not undergo prenylation and thus can be used in equilibrium titrations. The observed titration curve was saturable, and data were fitted to a K_d of ca. 2 nM using the quadratic equation (Figure 1). This value is not very well determined because of the high concentrations of Rab:REP complex used. The affinity obtained is much higher than that in the absence of GGpp (120 nM), indicating that the presence of lipid substrate dramatically increases the affinity of GGTase-II for its protein substrate.

To confirm that Rab7 is still associated with the complex under the experimental conditions used, we performed a displacement experiment. For this, a 200 nM sample of Rab7SS mutant preloaded with the fluorescent nucleotide analogue mantGDP was mixed with equimolar amounts of REP-1, GGTase-II, and 2 μM GGpp. The fluorescence of the mant group was excited by fluorescence energy transfer (FRET) from tryptophan at 295 nm, and data were collected at 440 nm. A 20-fold molar excess of unlabeled Rab7SS was added to the cuvette. This resulted in a slow fluorescent decrease corresponding to dissociation of mGDP-bound Rab7 from the complex. As expected, the obtained dissociation rate was in the range of 0.01 s^{-1}, similar to that obtained for dissociation of the Rab7:REP-1 complex (*23*) (data not shown). This result suggests that mGDP−Rab7 is still complexed with REP-1 under prevailing concentrations. Based on the equilibrium titration and stopped-flow experiments, we conclude that the presence of GGpp increases the affinity of Rab7:REP-1 for GGTase-II by about 20-fold.

Construction and Purification of Fluorescently Labeled Rab7GG:REP-1 Complex. Having found that GGpp tightens the interaction between GGTase-II and its protein substrate, we continued to investigate whether GGpp was also involved in post-prenylational events. To address this point, we sought to generate a fluorescent prenylated Rab7:REP-1 complex for spectroscopic studies by combining in vitro protein ligation and in vitro prenylation techniques. In vitro protein ligation methodology was essential for construction of such a fluorescent probe since standard methods of protein labeling result either in incorporation of multiple groups at random positions or in modification of reactive C-terminal cysteines (*23*). To circumvent these problems, we generated a thioester-tagged Rab7 truncated by six amino acids (*22*). A dansyl-bearing synthetic peptide mimicking the last six amino acids of Rab7 was ligated to it as described under Materials and Methods. Subsequently, geranylgeranyl moieties were added to the semi-synthetic protein by in vitro prenylation as summarized in Figure 2. For in vitro prenylation, Rab7A202C E203Ldans was mixed with equimolar amounts of REP-1, GST-tagged GGTase-II (Kalinin and Alexandrov, unpublished experiments), and an approximately 10-fold molar excess of GGpp. Upon completion of the reaction, GST-tagged GGTase-II was separated from the reaction mixture by precipitation with glutathione−Sepharose beads in the presence of 6% CHAPS. The presence of CHAPS is critical for this process, since it disrupts the interaction between GGTase-II and prenylated Rab7:REP-1 complex (Alexandrov, unpublished results). To ensure homogeneity of the obtained complex as well as to separate it from detergent, gel filtration chromatography was performed. The doubly prenylated dansRab7A202C E203L:REP-1 complex will be referred to as dRab7GG:REP-1. As can be seen in Figure 3, dRab7GG:REP-1 eluted with a molecular mass of around 150 kDa and was clearly separated from the minor peak of the ternary complex. The peak migrating with an apparent molecular mass of over 1000 kDa probably represents protein aggregates. Fractions 19−21 (Figure 3) that contain only the prenylated dRab7GG:REP-1 complex were pooled and concentrated. The molecular weight and purity of the sample were determined by MALDI-TOF mass spectroscopy. The observed molecular mass of 24 191 Da matched closely the calculated mass of doubly geranylgeranylated dRab7A202C E203L (24 170 Da) (data not shown).

Equilibrium Titration of dRab7GG:REP-1 with GGTase-II. To study the interaction of prenylated dRab7:REP-1 complex with GGTase-II, a spectroscopic assay was developed based on the changes in dansyl fluorescence upon GGTase binding. Excitation and emission scans of a solution of dRab7GG:REP-1 revealed that the fluorescence had an excitation maximum at 340 nm while the emission maximum was at 545 nm (Figure 4). To assess whether the prenylated complex interacts with GGTase-II, 70 nM dRab7GG:REP-1 was mixed with 300 nM GGTase-II. As can be seen in Figure 4, the addition resulted in an approximately 10% increase

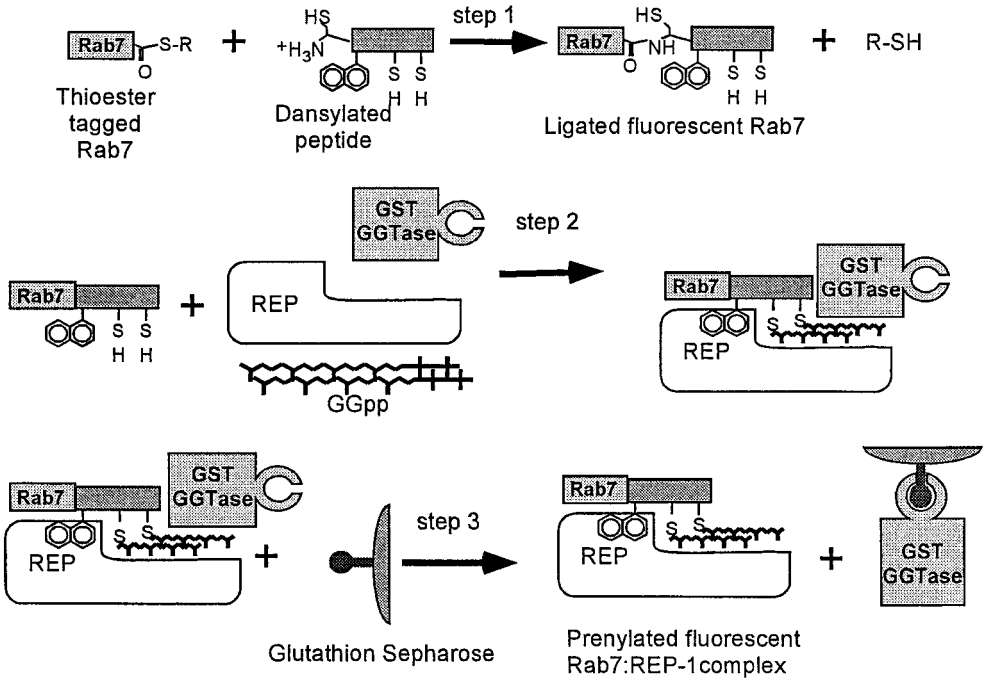


FIGURE 2: Schematic representation of the in vitro ligation and prenylation procedure for generation of the dRab7GG:REP-1 complex. Step 1: ligation of the dansyl-containing peptide onto the thioester-tagged Rab7. Step 2: formation of dRab7GG:REP-1 complex and in vitro prenylation with GST-GGTase-II. Step 3: separation of GST-GGTase-II with glutathione−Sepharose.

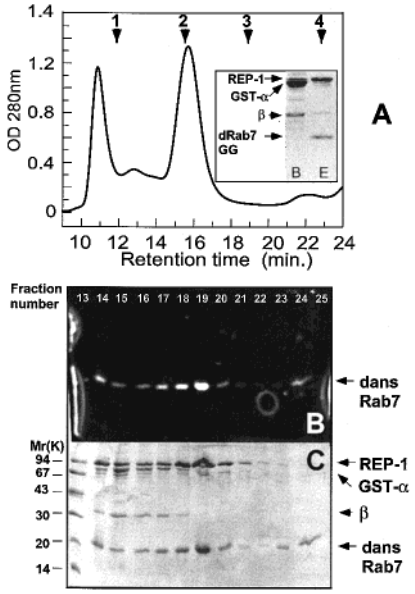


FIGURE 3: Purification of the dRab7GG:REP-1 complex on a Superdex 200 10/20 column. Run conditions are described under Materials and Methods. Molecular masses of peaks were determined by comparison with protein standards of known molecular mass (1, 670 kDa; 2, 158 kDa; 3, 44 kDa; 4, 17 kDa) that are shown as arrowheads on panel A). The inset of panel A shows the protein distribution between the glutathione beads (B) and the eluate (E) in the loaded sample preparation. The collected fractions were subjected to SDS gel electrophoresis on 15% mini gels, and proteins were visualized by fluorescent scanning (B) and Coomassie Blue staining (C). Horizontal arrows denote the positions of migration of REP-1, the α and *β* subunits of GGTase-II, and Rab7 (right side), and the molecular mass markers (left side). Fractions 19−21 were pooled and used for further experiments.

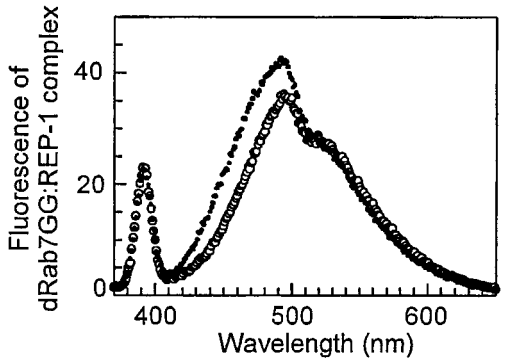


FIGURE 4: Emission scans of dRab7GG:REP-1 complex in the absence (open circles) and presence (closed circles) of GGTase-II. The excitation wavelength used was 340 nm, and 70 nM dansRab7GG:REP-1 was used in both experiments. The concentration of GGTase-II was 300 nM.

in fluorescence. To determine the affinity of GGTase-II toward its product, 70 nM dRab7GG:REP-1 was titrated with GGTase-II. The obtained data were fitted using a quadratic equation yielding a K_d of ca. 2 nM. The stoichiometry (obtained initially manually as shown, but also verified by allowing the concentration of the complex to vary in the fit procedure) indicated that that 1 mol of dRab7GG:REP-1 bound to 1 mol of GGTase-II (Figure 5A). We chose to test if whether the observed low K_d for product binding is at least partially due to the presence of the dansyl fluorophore in the prenylated dRab7:REP-1 complex. Using the in vitro prenylation protocol outlined above, we prepared prenylated wild-type Rab7:REP-1 complex (Rab7GG:REP-1). The correct molecular weight of Rab7GG was confirmed by mass spectrometry (data not shown). In the experiment, 100 nM prenylated Rab7:REP-1 was mixed with 200 nM rhodamine−Rab7:REP-1 complex (*13*). The resulting protein mixture was titrated with increasing concentrations of GGTase-II. The curve obtained was sigmoidal, indicating that GGTase-II bound more tightly to Rab7GG:REP-1 than to rhodamine−Rab7:REP-1 (Figure 6). In such a case, the GGTase-II will, at low concentrations, preferentially bind to Rab7GG:REP-1, giving rise to an initial 'lag'. However,

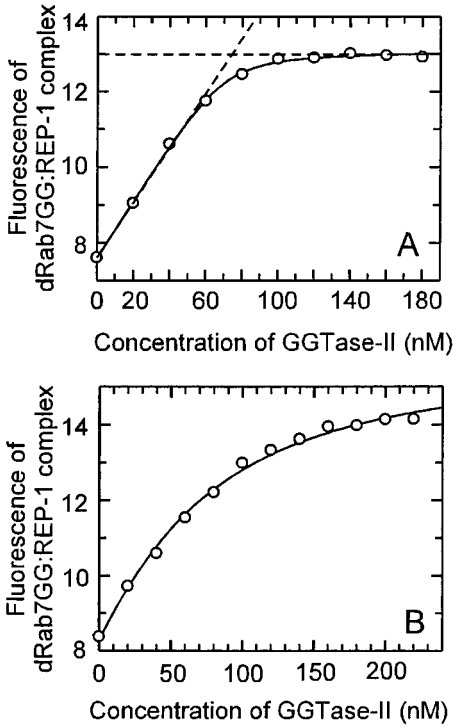


FIGURE 5: Titration of GGTase-II to a nominal concentration of 70 nM dRab7GG:REP-1 using direct fluorescence as a signal for binding (excitation wavelength 338 nm, emission 490 nm) in the absence (A) and in the presence (B) of GGpp. It can be inferred from the intersection of straight dashed lines and from the fitting procedure that 1 mol of dRab7GG:REP-1 bound 1 mol of GGTase-II. The solid lines show the quadratic fit giving values of 2 and 18 nM for the K_d, respectively.

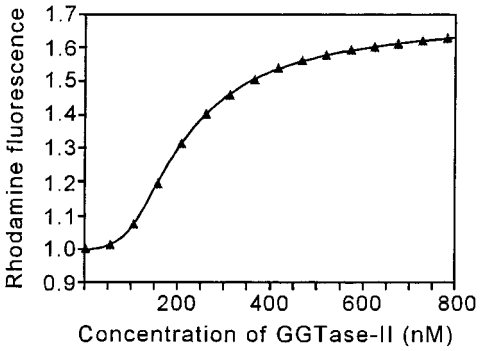


FIGURE 6: Spectrofluorometric competition titration of rhodamine Rab7:REP-1 fluorescence by GGTase-II in the presence of wild-type Rab7GG:REP-1 complex. The rhodamine Rab7:REP-1 complex concentration was 100 nM; wt Rab7GG:REP-1 concentration was 200 nM. Data were fitted using the program Scientist 2.0 and led to a K_d value of 0.7 nM for the interaction of GGTase-II with the wild-type Rab7GG:REP-1 complex.

at higher concentration of GGTase-II, when Rab7GG:REP-1 is mostly saturated, the enzyme will also bind to rhodamine−Rab7:REP-1, giving rise to an increase in fluorescence. The data obtained were fitted using the program Scientist as described under Materials and Methods and in (*25*). Keeping the K_d for the fluorescent rhodamine−Rab7:REP-1 constant at the independently determined value of 100 nM (*13*), a K_d of 0.7 nM was obtained for the affinity of wild-type Rab7GG:REP-1 toward GGTase-II (Figure 6). Thus, the dansylated Rab7GG:REP-1 complex binds to the GGTase-II with similar affinity to the wild-type.

Transient Kinetic Experiments on dRab7GG:REP-1 Association with GGTase-II. To confirm data obtained using an independent method as well as to gain insight into the mechanism of the reaction, we performed transient kinetic experiments employing the stopped-flow technique. Rapid mixing of dRab7GG:REP-1 with GGTase-II resulted in an increase of fluorescence when exciting at 295 nm, monitoring fluorescence through a 398 nm cutoff filter (Figure 7A). The stopped-flow traces could be fitted to a single-exponential function. There was a linear dependence of the observed rate versus concentration of GGTase-II (Figure 7B). Based on the slope of a linear fit, an association rate constant (k_{on}) of 0.0086 nM^{-1} s^{-1} was obtained. The dissociation rate constant, k_{off}, as inferred from the intercept with the ordinate was 0.01 s^{-1}. To determine the dissociation rate directly, displacement experiments were performed. To this end, 70 nM dRab7GG:REP-1 preincubated with 70 nM GGTase-II was mixed with a 10-fold molar excess of wild-type Rab7GG:REP complex. The resulting time course was fitted to a single-exponential function, resulting in an observed dissociation rate of 0.017 s^{-1} (Figure 7C). The K_d, based on an association rate of 0.0086 nM^{-1} s^{-1} and an independently determined dissociation rate of 0.017 s^{-1}, is 1.98 nM, which is in good agreement with previous equilibrium measurements.

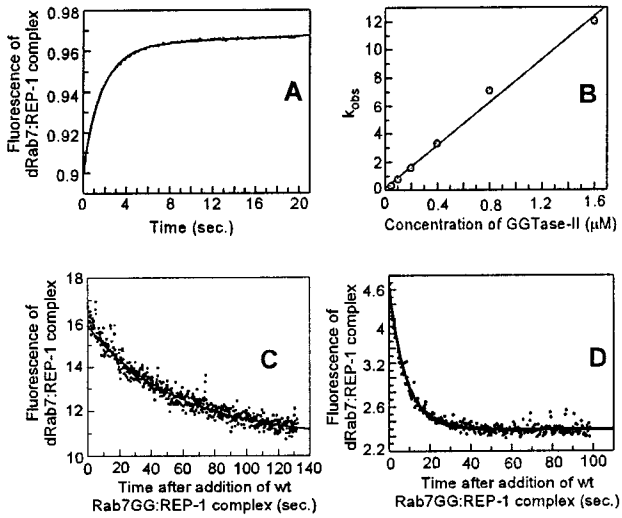


FIGURE 7: (A) Time course of the fluorescent energy transfer signal change seen on mixing dRab7GG:REP-1 complex (10 nM) with GGTase-II (50 nM) in the stopped-flow machine. Excitation was at 289 nm, and emission was detected through a 389 nm cutoff filter. The shown fit is to a single-exponential equation with a rate constant (k_{ass}) of 0.57 s^{-1}. Panel B shows the secondary plot of data from six experiments of the above-described type. Circles represent k_{obs} values plotted against the concentration of GGTase-II. Time course of the direct fluorescence signal change seen on mixing dRab7GG:REP-1:GGTase-II complex (70 nM) with wild-type Rab7GG:REP-1 complex (700 nM). The shown fit is to a single-exponential equation with a rate constant (k_{off}) of 0.017 s^{-1}. (D) Same as panel C, but the reaction was supplemented with 2 mM GGpp, leading to an acceleration of the observed dissociation rate to 0.14 s^{-1}.

Given that the affinity of the substrate, unprenylated Rab7, in complex with REP-1, is ca. 2 nM, this tight product binding ($K_d \approx 1$ nM) would result in severe product inhibition of the enzymatic reaction. We therefore assessed whether the presence of phosphoisoprenoid substrate has an effect on the affinities governing product release.

Equilibrium Titration of dRab7GG:REP-1 versus GGTase-II in the Presence of GGpp. To determine the effect of lipid substrate on product release, 70 nM dRab7GG:REP-1 was titrated with increasing concentrations of GGTase-II in the

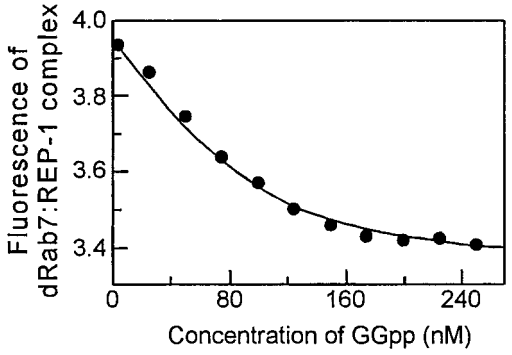


FIGURE 8: Titration of 100 nM dRab7GG:REP-1:GGTase-II complex with GGpp using the direct fluorescence signal, exciting at 335 nm and measuring the emission at 490 nm. The solid line shows the quadratic fit to the data giving a value of 22 nM for the K_d.

presence of 2 μM GGpp. Fitting of the observed binding isotherm to a quadratic equation led to a K_d of ca. 18 nM for the interaction of the dRab7GG:REP-1 complex with GGTase-II (Figure 5B). This result indicates a large decrease of binding affinity due to the presence of GGpp.

Transient Kinetic Experiments on dRab7GG:REP-1 Association with GGTase-II in the Presence of GGpp. To determine what effect the presence of GGpp had on individual rate constants we repeated the stopped-flow experiments described above in the presence of 2 μM GGpp. The observed k_{on} was slightly faster (0.013 nM^{-1} s^{-1}) and could not explain for the difference in affinities observed in the equilibrium titration experiments (Figure 7B). However, when the dissociation experiment was repeated in the presence of GGpp, the reaction proceeded much faster with an observed rate of 0.14 s^{-1} (Figure 7D). These results clearly demonstrate that binding of an additional molecule of lipid substrate to prenylated ternary complex weakens its affinity by increasing the dissociation rate by a factor of approximately 10-fold.

Determination of the Affinity of GGpp versus the Prenylated dRabGG:REP-1:GGTase-II Complex. Further fluorescence titrations were conducted to determine the affinity and the stoichiometry of GGpp binding to GGTase-II. 300 nM dRabGG:REP-1:GGTase-II complex was titrated against increasing concentration of GGpp (Figure 8).

A fit to the data using a quadratic equation resulted in a K_d of 22 nM for the interaction, with 1 mol of GGpp binding per mole of prenylated ternary complex. This value is close to that observed previously for interaction of GGpp with unprenylated ternary complex (13 nM) (*25*). These data indicate that both protein-bound and free isoprenoid could be accommodated by the ternary complex, thus suggesting that the GGpp binding site on the GGTase-II is not blocked by prenyl groups attached to Rab protein.

DISCUSSION

Processes such as substrate binding and product release are of fundamental importance for enzymatic activity. A paradox that a good enzymatic catalyst has to solve is to bind the substrate reasonably tightly, while at the same time being able to release the product. The product, however, has chemical properties very similar to the substrate. This conflict is especially pronounced for hydrophobic substrates which are bound to buried hydrophobic binding sites on the enzyme. In this work we addressed the question of how substrate binding and product release in GGTase-II, which transfers two hydrophobic geranylgeranyl moieties onto Rabs, occur. It has been previously shown that GGTase-II binds its lipid and protein substrates very tightly, exhibiting K_d values of 8 and 100 nM, respectively (*13*, *25*). However, it is not known how the doubly prenylated Rab:REP complex is released from GGTase-II.

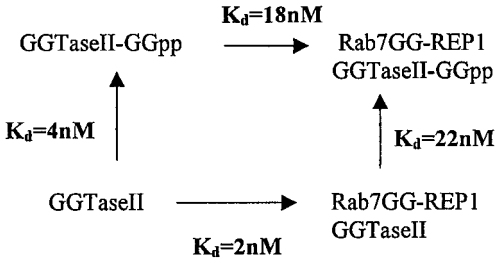
We addressed these points in two stages: first, we determined the affinity of the substrate Rab7:REP-1 complex for GGTase, followed by a comparison of the observed affinities with those of product release. Titrating GGTase-II complexed to a fluorescent analogue of GGpp with Rab7SS:REP-1 complex, we determined a K_d value of ca. 2 nM for this interaction—compared with a K_d of 120 nM for this interaction in the absence of GGpp (*13*). We see two possible explanations for this phenomenon. First, it was shown that GGTase-II has a weak (micromolar) affinity for Rab proteins (*22*), and this interaction is likely to involve the flexible C-terminus of Rab protein. Upon association of GGpp with GGTase-II, this tail might become fixed in the active site, leading to the affinity increase. An increase of affinity for peptide substrate in the presence of Fpp was observed in the case of FTase (*9*). This 70-fold increase of affinity was attributed to coordination of the peptide sulfur and formation of a bound thiolate. Another possibility is that a conformational change in the GGTase-II:GGpp complex could increase its affinity for REP and subsequently for the Rab:REP complex. To establish which mechanism governs the changes observed in the case of GGTase-II further experiments will be necessary.

Studies addressing the affinities and the kinetics underlying product release by GGTase-II have been held back by technical difficulties in preparation of the doubly prenylated Rab7:REP-1 complex and the lack of appropriate assays for monitoring its interaction with other molecules. In vitro ligation has proved to be a powerful and convenient tool for incorporating fluorescent groups into specific positions of the C-terminus of Rab7. The subsequent development of an enzymatic prenylation strategy using tagged GGTase-II on glutathione beads has led to production of fluorescently labeled, doubly geranylgeranylated Rab7:REP-1 complex in preparative amounts. The combination of chemical synthesis with in vitro prenylation has great potential, especially for studies of Rab protein interactions with other molecules and membranes in in vivo and in vitro assays.

Using the fluorescent prenylated Rab7:REP-1 complex, we were able to measure the affinity of GGTase-II for the doubly prenylated Rab7:REP-1 complex. Based on equilibrium titrations and kinetic experiments, we found that the affinity of GGTase-II toward its product is around 2 nM in the absence of GGpp. In the presence of GGpp, however, the interaction between the product complex and the enzyme was determined to have a K_d of ca. 18 nM. Therefore, in contrast to substrate binding, the presence of phosphoisoprenoid weakens binding of the product by approximately 10-fold. From this perspective, GGTase-II functions similarly to FTase, where binding of a new round of phosphoisoprenoid is necessary for product release (*10*).

The lipid-induced decrease in affinity for the product, on the one hand, and the increase in affinity for the substrate,

Scheme 1



on the other, are crucial for catalytic function of GGTase-II. In the absence of excess phosphoisoprenoid, Rab7:REP-1 is bound with an affinity of 120 nM. Also in the absence of excess GGpp, the prenylated product is attached to the enzyme with a K_d of ca. 1 nM. In this case, the enzyme would suffer from severe product inhibition. However, the presence of GGpp increases the affinity of GGTaseII for substrate (K_d of 2 nM), while decreasing its affinity for product (K_d of 18 nM) (Scheme 1). It is worth noting, however, that the substrate binding experiments were performed with the Rab7SS mutant, whose C-terminus has quite different chemical characteristics from the SH groups containing wild-type protein. By analogy with farnesyl transferase, it is possible that GGTase-II binds its wild-type substrate even tighter due to the formation of bound thiolates of the C-terminal cysteines *(9)*.

Transient kinetic measurements reveal the mechanism of the GGpp influence on product binding and release. The observed differences in affinity originate primarily from differences in the dissociation rates (0.017 s^{-1} in the absence and 0.14 s^{-1} in the presence of GGpp). Examination of the steady-state parameters previously determined for GGTase-II indicates a k_{cat} of approximately 0.02 s^{-1} (*17*). In the presence of GGpp, the dissociation rate is sufficiently fast not to become rate-limiting. It is presently unclear what the rate-determining step of the prenylation reaction is. Overall it appears that GGpp binding serves three different roles in the catalytic cycle of GGTase-II. First, it functions as an allosteric activator, enabling tighter substrate binding. Second, it acts as the phosphoisoprenoid donor in the prenylation reaction, and third, it senses the completion of catalysis and concomitantly triggers substrate release by increasing the dissociation rate of the product.

Finally we addressed the interaction of GGpp with the prenylated ternary complex. The results indicate that GGpp binds to the prenylated ternary complex with a K_d value of 22 nM and 1:1 stoichiometry. The implication of this finding is that the ternary complex has to possess at least two binding sites for prenyl groups: one for the two geranylgeranyl moieties attached to Rab7, and one for the geranylgeranyl pyrophosphate added in the titration. It is likely that protein-conjugated geranylgeranyl moieties are shifted to REP, which must have a docking site for them. Moreover, these results suggest that Rab-linked geranylgeranyl moieties are removed from the active site of GGTase-II upon completion of catalysis.

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