Semi-synthetic Rab proteins as tools for studying intermolecular interactions

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Abstract Rab GTPases play a key role in the regulation of membrane traffic. Posttranslational geranylgeranylation is critical for their biological activity and is conferred by a Rab geranylgeranyl transferase (RabGGTase). To study the interactions between Rab proteins and RabGGTase, we used in vitro ligation methodology to generate a fluorescent semi-synthetic Rab7 protein. The obtained protein was functionally active and was used to demonstrate a micromolar affinity interaction of Rab7 with the RabGGTase in the absence of Rab escort protein (REP). This finding is consistent with an earlier proposed model according to which RabGGTase possesses two independent weak binding sites for REP and Rab proteins.

Key words: Intein; Protein ligation; RabGGTase; Rab escort protein; Rab7

1. Introduction

Rab GTPases belong to a family of Ras-like proteins that play key roles in the secretory and endocytic pathways [1]. They are located on distinct membrane-bound compartments, and are believed to control events in membrane docking and fusion in eukaryotic cells [2,3].

Most Rabs are doubly geranylgeranylated at the C-termini, which leads to their membrane association. This postranslational modification is crucial for their biological activity and is catalyzed by a Rab geranylgeranyl transferase (RabGGTase) [4]. RabGGTase belongs to the family of protein prenyl transferases together with geranylgeranyl transferase-I (PGGT) and protein farnesyl transferase (PFT). A characteristic feature of PFT and PGGT is their ability to bind and prenylate cysteine containing peptides as short as four amino acids [5]. RabGGTase possesses several features that set it apart from PFT and PGGT. First it transfers the geranylgeranyl moiety onto two C-terminal cysteines of Rab proteins in a broad context of amino acids. Secondly, RabGGTase does not recognize its substrate directly but exerts its function in concert with another protein termed REP (Rab escort protein). Despite a number of related reports, the molecular details of the prenylation reaction remain largely unknown. One of the reasons for this is the lack of quantitative time-resolved studies. In the case of PFT, detailed kinetic studies were performed using fluorescently labeled substrate peptides [6,7]. This approach allowed the kinetic dissection of the substrate binding and prenyl transfer reactions. The same approach could not be applied directly to RabGGTase due to the much larger size of the protein substrate. In this work we have used in vitro protein ligation methods to generate semi-synthetic Rab7 protein in which a fluorescent group could be incorporated into any part of the C-terminus. Using the fluorescently labeled ligation product, we have investigated the interaction of Rab7 with RabGGTase and demonstrated a direct interaction with micromolar affinity.

2. Materials and methods

2.1. Construction of intein-Rab7 expression vectors

In order to generate C-terminal fusion of the Rab7ΔC6 mutant with intein the coding sequence of the former was amplified by PCR with expand polymerase (Roche) using 1 ng of pET3-Rab7 plasmid as template [8]. The following primers were used: 5′-ATTGGTACCCTTGGCAAAGCATGAGGTCTTGGCCCGGTCGTTC-3′ containing a *Kpn*I site and the T7 promoter. The *Nde*I and *Kpn*I digested PCR product was cloned into the pTYB1 expression vector (New England Biolabs) pre-cut with the same enzymes. The integrity of the reading frame and the coding sequence was determined by nucleotide sequencing.

2.2. Protein expression and purification

RabGGTase and REP-1 were expressed and purified as described elsewhere [9]. To purify the chitin binding domain (CBD)-intein-Rab7ΔC6 fusion protein, 1 l of *Escherichia coli* BL21 cells transformed with pTYB1Rab7ΔC6 were grown to mid-log phase in Luria-Bertani medium and induced with 0.3 mM isopropyl-1-thio-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 $MgCl_2$, 10 μM GDP, 1.0 mM phenylmethylsulfonyl fluoride) and lysed using a fluidizer (Microfluidics Corporation). After lysis, Triton X-100 was added to a final concentration of 1%. The lysate was clarified by ultracentrifugation and incubated with 9 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 $MgCl_2$, 10 μM GDP, 500 mM 2-mercaptoethanesulfonic acid (MESNA)). The supernatant was analyzed by SDS–PAGE and mass spectrometry. The Rab7ΔC6 thioester was concentrated using Centripreps 10 (Amicon) to a final concentration of 200 μM and stored frozen at −80°C.

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Abbreviations: Rab, ras-like protein from rat brain; RabGGTase, Rab geranylgeranyl transferase; REP, Rab escort protein

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2.3. Peptide synthesis and protein ligation

Peptide C-L(dans)-S-C-S-C was synthesized and HPLC purified to more than 90% purity by Interactiva (Ulm, Germany). The 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. The final concentrations were 240 μM and 2 mM for Rab7 and peptide respectively. Unreacted peptide and detergent were removed by passing the reaction mixture over a PD-10 desalting column (Pharmacia) equilibrated with 25 mM HEPES pH 7.2, 40 mM NaCl, 2 mM $MgCl_2$, 100 μM GDP and 2 mM DTE. The extent of ligation was determined by SDS–PAGE gel electrophoresis and mass spectrometry. For visualization of ligated fluorescent product reaction mixture was separated on a 15% SDS–PAGE gel. The acetic acid fixed gels were viewed in unfiltered UV light. Imaging was performed with a Fluro-S fluorescent imager (BioRad).

2.4. Fluorescence measurements

Fluorescence spectra and long time base fluorescence measurements were performed with an Aminco SLM 8100 spectrophotometer (Aminco, Silver Spring, MD, USA). All reactions were followed at 25°C in 25 mM HEPES pH 7.2, 40 mM NaCl, 2 mM $MgCl_2$, 100 μM GDP and 2 mM DTE. 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 using a High-Tech Scientific SF61 apparatus (Salisbury, England). The dansyl fluorescence was excited via fluorescent resonance energy transfer (FRET) at 290 or directly at 333 nm and detected through a 389 nm cutoff filter. Data collection and primary analysis of rate constants were performed with the package from High Tech Scientific, secondary analysis with the program Grafit 3.0 (Erithacus software).

2.5. In vitro prenylation assay

Rab GGTase activity was determined by measuring the incorporation of [H]GG pyrophosphate (American Radiolabeled Chemicals, St. Louis, MO, USA) to Rab proteins expressed in *E. coli*, as described previously [10].

2.6. GTPase measurements

GTP hydrolysis was assayed as a function of time by HPLC on a C_{18} reversed phase column in the presence of tetrabutylammonium bromide under isocratic conditions as described elsewhere [11].

3. Results and discussion

In order to study the interaction of Rab7 with RabGGTase and other interacting molecules we have devised a strategy for incorporating fluorescent groups into specific positions of the C-terminus (Fig. 1). To this end Rab7 C-terminally truncated by six amino acids was fused with an intein domain that allows in vitro coupling of virtually any peptide to Rab7. The resulting fusion protein was expressed in *E. coli*, purified using chitin agarose and cleaved as described in Section 2. The cleavage product was analyzed by SDS–PAGE gel and mass

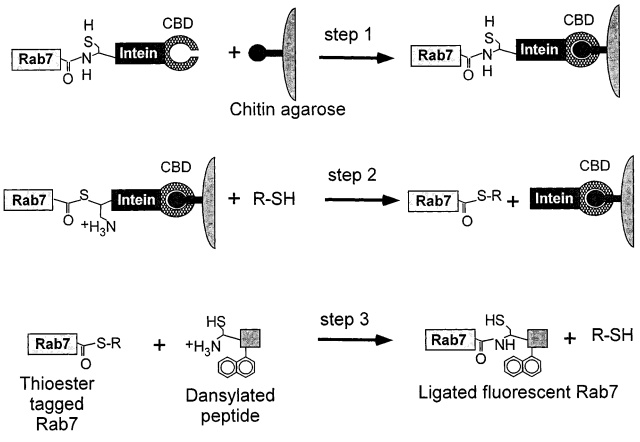

Fig. 1. Schematic representation of the purification and ligation procedure for generation of the semi-synthetic Rab7. Step 1, isolation of the Rab7ΔC6-intein-chitin binding domain (CBD) fusion protein on chitin agarose beads. Step 2, thiol induced cleavage of Rab7ΔC6 and its separation from the intein-CBD fusion domain. Step 3, ligation of the dansyl containing peptide onto the thioester tagged Rab7.

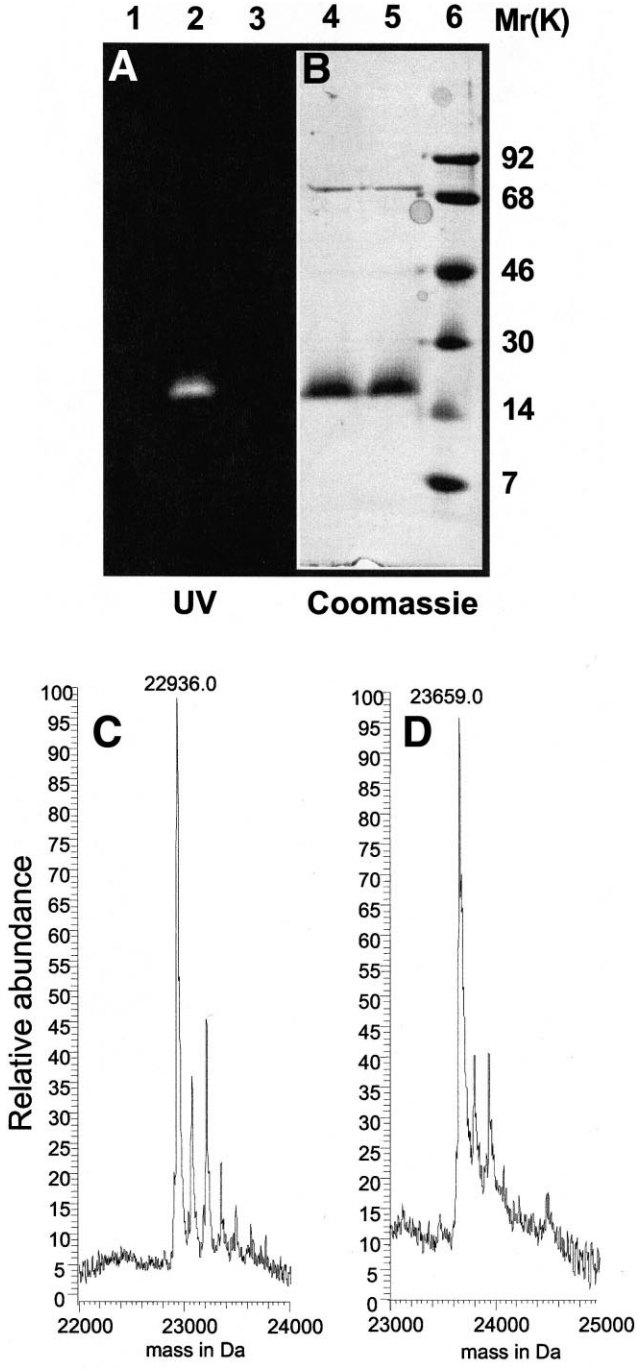

Fig. 2. SDS–PAGE gel of thioester tagged Rab7Δ6C before (lane 2) and after ligation to a dansylated peptide (lane 5) photographed either in the UV light (A) or visible light after Coomassie blue staining (B). ESI-MS spectrum of thioester tagged Rab7ΔC6 (expected mass 22 924 Da) (C) and Rab7A202CE203Ldans ligation product (D).

spectroscopy (Fig. 2B, lane 4 and Fig. 2C and A). Full length Rab7 was restored by coupling to the thiol activated C-terminal residue a fluorescently labeled peptide with the sequence Cys-Lys(dans)-Ser-Cys-Ser-Cys making use of the ligation chemistry developed by Kent and coworkers [12]. The resulting ligation product deviated from the wild-type in two positions: alanine 202 was substituted by cysteine and glutamic acid 203 was changed to a dansyl labeled lysine. Ligated protein was then subjected to SDS–PAGE. A distinct fluorescent band was observed at the position corresponding to Rab7 when the SDS–PAGE gel was viewed in UV light (Fig. 2A). Ligation was independently confirmed by MS analysis of the ligation products. Fig. 2D reveals that the main protein peak had a mass of 23 659 Da (expected mass 23 669 Da). Based on the mass spectrometry, gel quantification and fluorescent yield measurements (data not shown) it appears that 90% of the

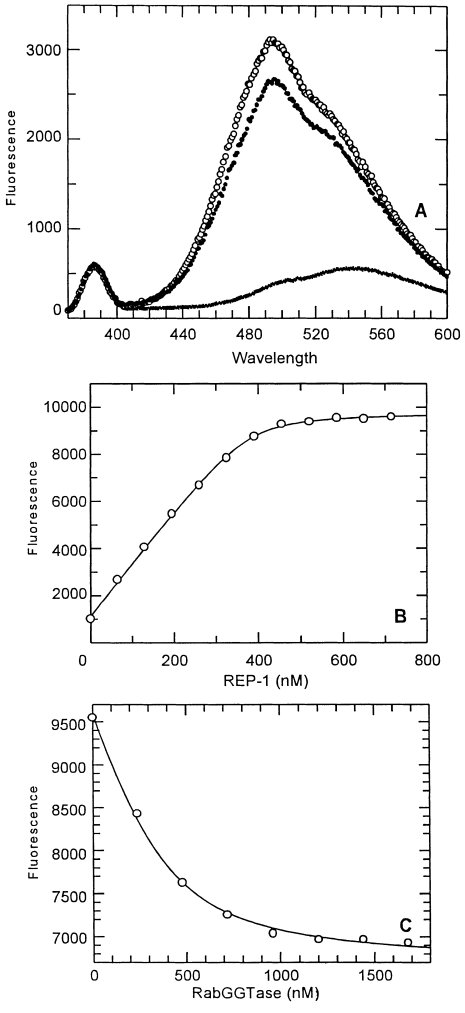

Fig. 3. A: Emission spectra of 380 nM of Rab7A202CE203Ldans alone (solid line), upon addition of 500 nM REP-1 (white circles) or upon further addition of 1 μM of RabGGTase (black circles). Excitation wavelength used was 338 nm. B: Titration of REP-1 to a nominal concentration of 380 nM Rab7A202CE203Ldans using direct fluorescence as a signal for binding (excitation wavelength 338 nm, emission 490 nm). The solid line shows the quadratic fit to a giving value of 4 nM for the K_d and an effective REP-1 concentration of 391 nM. C: Spectrofluorimetric titration of Rab7A202CE203Ldans:REP-1 complex (380 nM) with RabGGTase under conditions described above. The K_d for the Rab7A202CE203Ldans:REP-1:RabGGTase is 111 nM.

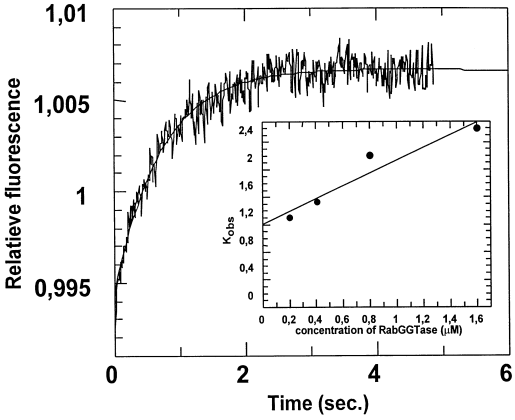

Fig. 4. Time course of the fluorescent energy transfer signal change seen on mixing Rab7A202CE203Ldans (120 nM) with RabGGTase (200 nM) in the stopped flow machine. Excitation was at 289 nm, and emission was detected through a 389 nm cutoff filter. The shown fit is to a single exponential equation with a rate constants (k_{ass}) of 1.1 s^{-1}. The inset shows the secondary plot of data from four experiments of the above described type. Closed circles represent k_{obs} values plotted against the concentration of RabGGTase.

Rab7 protein ligated to the dansylated peptide and no further purification was necessary. We designated the resulting protein Rab7A202CE203Ldans. Measurements of its intrinsic GTP hydrolysis rate (1.6×10^{-6} s^{-1}) showed that it was not significantly different compared to the wild-type protein (9×10^{-5} s^{-1}) (data not shown).

Excitation/emission scans of a solution of Rab7A202CE203Ldans revealed that, as expected from the reporter group used, the fluorescence had an excitation maximum at 340 nm (data not shown) and emission at 545 nm (Fig. 3A). Binding of Rab7 to its native substrate REP-1 was used to assess whether semi-synthetic Rab7 was functionally active. REP-1 was added to a cuvette containing 380 nM of Rab7A202CE203Ldans to the final concentration of 500 nM. This addition resulted in a 6-fold increase of fluorescence (Fig. 3A). The emission maximum was strongly blue shifted and had a maximum at 493 nm. Further additions of REP-1 did not result in an increase of fluorescence, indicating the formation of a stoichiometric Rab7A202CE203Ldans:REP-1 complex. This fluorescent signal change was used to determine the affinity of Rab7A202CE203Ldans for REP-1. We titrated 380 nM of Rab7A202CE203Ldans with REP-1. The obtained data were fit using a quadratic equation yielding a K_d of 4 nM and were consistent with a 1:1 stoichiometry (Fig. 3B). This is in reasonable agreement with the previously determined K_d of 1 nM for Rab7:REP-1 complex thus indicating that incorporation of a dansyl group at the position 202 of Rab7 does not significantly perturb its interaction with REP-1 [13].

Next we examined whether introduction of the fluorescent group influenced interaction of the Rab7A202CE203Ldans:REP-1 complex with RabGGTase. Addition of RabGGTase to a cuvette containing 380 nM of the Rab7A202CE203Ldans:REP-1 complex resulted in a dose dependent and saturable fluorescence decrease by about 30% (Fig. 3A). This observation indicates that formation of the ternary Rab7:REP-1:RabGGTase complex results in further environmental changes at the Rab7's C-terminus. In the following experiment we titrated 380 nM of d_Rab7:REP-1 complex with

RabGGTase and processed the data as in the previous case. The K_d value was 111 nM (Fig. 3C). Previously we determined a K_d of 120 nM for interaction of Rab7:REP-1 with RabGGTase [13]. Therefore we concluded that introduction of a fluorescent group in position −5 of the Rab7 C-terminus did not influence the interaction of Rab7 with the subunits of RabGGTase. Moreover, Rab7A202CE203Ldans protein was geranylgeranylated by RabGGTase in the in vitro prenylation assay with the same efficiency as the wild-type Rab7 (data not shown).

Since the in vitro ligation methodology enabled us to generate Rab7 molecules with different C-termini we then determined whether the fluorescent signal would be influenced by the position of the dansyl group. The same methodology was used to generate a Rab7 molecule containing an extra alanine residue inserted between recombinant protein and the dansylated peptide. Compared with the previous ligation product, in this molecule the dansylated residue was shifted by one amino acid. Emission scans of this molecule revealed that in this case formation of the d_Rab7:REP-1 complex led to only a 2-fold increase in fluorescence while formation of the ternary complex resulted in a further 5-fold increase (data not shown). This observation shows that the position of the dansyl label strongly influences the changes in fluorescent yield and thus can be used to tailor the fluorescent assay according to experimental requirements.

We previously proposed a model postulating relatively low affinity binding sites on RabGGTase for both Rab and REP proteins (probably in the micromolar range) [13]. These sites would then lead to a cooperatively enhanced binding to RabGGTase with the observed affinity of about 100 nM. The fluorescent assay we have developed possesses sufficient sensitivity to reveal such weak interactions. Since the direct titration approach becomes technically difficult in this affinity range we applied the stopped flow methodology to detect the direct interaction of Rab7 with RabGGTase. The fluorescence resonance transfer signal observed on interaction of the fluorescent Rab7 with RabGGTase showed a much larger change than the direct fluorescence signal and therefore was used for transient kinetic measurements. As shown in Fig. 4, mixing of d_Rab7 with RabGGTase resulted in an increase of fluorescence when exciting at 296 nm. The overall fluorescent change was about 1.5% and the data could be fitted by a single exponential term. The same experiment was repeated with increasing concentrations of RabGGTase led to a linear increase of the observed pseudo-first order rate constant. Fitting the obtained rates using linear regression led to a value of ca. 0.9×10^6 M^{-1} s^{-1} for the association rate constant (k_{ass}). These data also give an indication of the dissociation rate constant k_{off} from the intercept with y-axis (Fig. 4). This value was 1.2 s^{-1}, thus allowing calculation of the affinity of the Rab7:RabGGTase interaction. Based on the k_{off}/k_{on} ratio, K_d was estimated to be 1.3 μM. Thus Rab can interact directly with RabGGTase with micromolar affinity.

In summary, we have used the in vitro ligation approach to generate semi-synthetic Rab7 protein containing a fluorescent group close to the C-terminus. The obtained protein was functionally active as determined by its near native intrinsic GTP hydrolysis rate and its affinity for the subunits of RabGGTase. Moreover, the ligated protein was efficiently geranylgeranylated by RabGGTase in vitro. The semi-synthetic Rab7 protein, therefore, represents a powerful tool for studies of the prenylation reaction and postprenylational events. The proposed methodology has general applicability for studies of GTPases and other molecules. It has also been applied to YPT7 and YPT1 (Alexandrov, unpublished). We expect that the proposed approach will be broadly used for studying interaction of Rab proteins with their interacting partners at the molecular as well as at the organelle level. This application is especially promising since the C-terminus of Rab proteins largely determines their intracellular localization and is likely to undergo conformational changes during membrane association [14].

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