[4] Application of Protein Semisynthesis for the Construction of Functionalized Posttranslationally Modified Rab GTPases

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Abstract

Rab GTPases represent a family of key membrane traffic regulators in eukaryotic cells. To exert their function, Rab proteins must be modified with one or two geranylgeranyl moieties. This modification enables them to reversibly associate with intracellular membranes. *In vivo* the newly synthesized Rab proteins are recruited by Rab escort protein (REP) that presents them to the Rab geranylgeranyl transferase (Rab GGTase), which transfers one or two geranylgeranyl moieties to the C-terminal cysteines. Detailed understanding of the mechanism of prenylation reaction and subsequent membrane delivery of Rab proteins to the target membranes were hampered by lack of efficient technologies for the generation of preparative amounts of prenylated Rab GTPases. To circumvent this problem, we developed an approach that combines recombinant protein production, chemical synthesis of lipidated peptides with precisely designed and readily alterable structures, and a technique for peptide-to-protein ligation. Using this approach, we generated a number of semisynthetic prenylated Rab GTPases. Some of the proteins were also supplemented with fluorophores, which enabled us to develop a fluorescence-based *in vitro* prenylation assay. The approach described allows production of preparative amounts of prenylated GTPases, which was demonstrated by generation and crystallization of a monoprenylated YPT1:Rab GDI complex.

Introduction

Elucidation of protein function typically requires a combination of *in vivo* and *in vitro* approaches. In both cases only a fraction of the required information can be extracted using proteins in their native form. Commonly, modification with new functionalities is required to provide a detectable readout for the protein activity under study. Traditionally such modifications involve attachment of isotopic, fluorescent, affinity, or other functional groups to protein molecules. Although over the past century a very

extensive toolbox of functional entities suitable for biological studies was developed, their targeted conjugation with proteins remains problematic. The problem becomes particularly acute when the desired functionalities have to be attached with a high degree of control to a protein in a site-specific manner, for instance, in the proximity of an active center or an interaction surface. These problems are related to the inapplicability of the otherwise powerful methods of organic chemistry to such large and diversely reactive molecules as proteins. The situation becomes even more dramatic when the proteins of interest undergo posttranslational modifications that are incommensurate with other, desired protein modifications or engineering since the modified proteins would not be recognizable by natural modification pathways. This situation applies to GTPases of the Rab family that function as key regulators of intracellular vesicular transport. Like most GTPases, Rab proteins are posttranslationally modified by geranylgeranyl isoprenoids covalently attached to their C-terminal cysteine(s) via a thioether linkage. This modification is essential for the ability of Rab proteins to associate with their target membranes and to exert their biological function (Pfeffer, 2001). Since geranylgeranylated GTPases are insoluble in water due to their high hydrophobicity, their production and engineering have been challenging. This chapter describes the construction of several variants of prenylated Rab GTPases either in the native form or modified with functional groups. These proteins have been used for structural studies and for the development of a novel *in vitro* prenylation assay.

Use of Expressed Protein Ligation for the Construction of Functionalized Semisynthetic GTPases

Principle

To generate preparative amounts of native or engineered monoprenylated or diprenylated Rab GTPases, we used a combination of chemical synthesis and expressed protein ligation (EPL) (Muir, 2003). Central to this method is the ability of certain protein domains (inteins) to cleave from an N-terminally fused protein of choice by combination of an N→S(O) acyl shift and a transthioesterification reaction with an added thiol reagent, thus leaving a thioester group attached to the C-terminus of the desired N-terminal protein fragment. This thioester group can then be used to couple essentially any polypeptide to the thioester-tagged protein by restoring a peptide bond in a reaction known as native chemical ligation, which is essentially a reversal of the cleavage steps (Dawson *et al.*, 1994). The only requirement for the ligation reaction is the presence of an N-terminal cysteine on the peptide or protein to be ligated.

Methods

Vector Construction, Protein Expression, Purification, and Ligation. We generated several expression vectors for C-terminal fusion of Rab7 Δ C6, Rab7 Δ C3, Rab3A Δ C3, Rab27A Δ C3, Ypt1 Δ C2, Sec4 Δ C2, etc. with inteins by polymerase chain reaction (PCR) amplifying the coding sequence of the GTPases with 3' oligonucleotides designed in such a way that the resulting cDNA encoded a truncated GTPase protein that could be fused to the N-terminus of the intein, which in turn was attached to a chitin-binding domain (CBD). The PCR products were subcloned into the pTYB1, pTWIN-1, or pTWIN-2 expression vectors (New England Biolabs) (Xu and Evans, 2001).

To obtain the GTPase-intein-CBD fusion protein, 1 liter of *Escherichia coli* BL21 cells transformed with the corresponding expression plasmid were grown to mid-log phase in Luria-Bertani medium and induced with 0.3 mM isopropyl-1-thio-D-galactopyranoside at 20° for 12 h. After centrifugation, cells were resuspended in 60 ml of lysis buffer (25 mM Na₂HPO₄/NaH₂PO₄, pH 7.2, 300 mM NaCl, 1 mM MgCl₂, 10 μ M guanosine diphosphate [GDP], 1.0 mM phenylmethylsulfonyl fluoride [PMSF]) and lysed using a fluidizer (Microfluidics Corporation). After lysis, Triton X-100 was added to a final concentration of 1% (v/v). The lysate was clarified by ultracentrifugation and incubated with 9 ml of chitin beads (New England Biolabs) for 2 h at 4°. 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 Na₂HPO₄/NaH₂PO₄, pH 7.2, 300 mM NaCl, 1 mM MgCl₂, 10 μ M GDP, and, depending on the GTPases, 50–500 mM 2-mercaptoethanesulfonic acid (MESNA)]. The supernatant containing GTPase-thioester was concentrated to a final concentration of 200 μ M using Centrprep 10 (Amicon) and stored frozen at –80° until needed.

REP-1 was purified from baculovirus-infected insect cell lines as described (Alexandrov *et al.*, 1999). Yeast Rab GDI and Rab GGTase were purified as described previously (Kalinin *et al.*, 2001; Rak *et al.*, 2003).

Peptide Synthesis. Peptides C-K[5-dimethylaminonaphthalene-1-sulfonyl chloride) (Dans)]-S-C-S-C and C-K-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-C were synthesized and high-performance liquid chromatography (HPLC) purified to more than 90% purity by Thermo Hybaid (Ulm, Germany). The geranylgeranylated dipeptide H-Cys(StBu)-Cys(GerGer)-OH was synthesized using solution phase peptide chemistry (Rak *et al.*, 2003). Fmoc-Cys(S^tBu)-OH and geranylgeranylated cysteine were coupled under standard conditions employing *N*-hydroxysuccinimide and dicyclohexylcarbodiimide as coupling reagents to yield Fmoc-Cys(S^tBu)-Cys(GerGer)-OH (Brown *et al.*, 1991). Final Fmoc deprotection was performed by treatment with

diethylamine/methylenechloride (1:4) to obtain the final dipeptide H₂N-Cys(S'Bu)-Cys(GerGer)-OH in 70% yield. The N-terminal cysteine side chain remained protected until the ligation reaction, during which *in situ* deprotection occurred due to an excess of thiol reagent (MESNA).

For the construction of the prenylated peptides C-K(Dans)-S-C-S-C(GG), C-K(Dans)-S-C(GG)-S-C, and C-K(Dans)-S-C-(GG)-S-C(GG), we chose a block condensation strategy due to flexibility considerations (Alexandrov *et al.*, 2002; Durek *et al.*, 2004). For example, for construction of the C-K(Dans)-S-C-S-C(GG) peptide, tripeptide, Fmoc-Ser-Cys(S'Bu)-Ser-OAll was first deprotected at the C-terminus utilizing tetrakis(triphenylphosphine)palladium(0) with *N,N'*-dimethylbarbituric acid as a scavenger and coupled with the prenylated cysteine methyl ester, which was accessible via alkylation of cysteine methyl ester with geranylgeranylchloride in 2 *N* NH₃ (MeOH) (Brown *et al.*, 1991). Subsequent removal of the Fmoc-protecting group afforded tetrapeptide H-Ser-Cys(S'Bu)-Ser-Cys(GG)-OMe. This was then condensed with the Fmoc-Cys(S'Bu)-Lys(dan)-OH building block, and Fmoc removal with diethylamine finally resulted in the monogeranylgeranylated fluorescently labeled hexapeptide C-K(Dans)-S-C-S-C(GG)-OMe. A similar strategy was applied for the other peptides. Alternative approaches for the synthesis of the prenylated peptides (such as solid-phase techniques) were reported recently (Brunsveld *et al.*, 2005).

Before ligation, unprenylated peptides were dissolved to a final concentration of 50 mM in 25 mM Tris, pH 7.2, and 5% 3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonate (CHAPS). The prenylated peptides were dissolved in dichloromethane/methanol (1:5) to a final concentration of ca. 50 mM.

Protein Ligation

UNPRENYLATED PEPTIDES. In the ligation reaction the thioester-activated Rab7 was mixed with the peptides in a buffer containing 25 mM Na₂HPO₄/NaH₂PO₄, pH 7.2, 300 mM NaCl, 500 mM MESNA, 1 mM MgCl₂, 5% CHAPS, and 100 μM GDP and allowed to react overnight at room temperature. The final concentrations were 200 μM and 2 mM for Rab7 and peptide, respectively. Unreacted peptide and detergent were removed by gel filtration of the reaction mixture on a Superdex-75 column (Pharmacia) equilibrated with 25 mM HEPES, pH 7.2, 40 mM NaCl, 2 mM MgCl₂, 10 μM GDP, and 2 mM 1,4-dithioerythritol (DTE). The extent of ligation was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry. For visualization of ligated fluorescent product, the reaction mixture was separated on a 15% SDS-PAGE gel and the gels were viewed in unfiltered UV light.

Prenylated Peptides. 500 μ l of Rab-thioester protein (typically 20 mg/ml, ca. 500 nmol) in 25 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2, 300 mM NaCl, 1 mM MgCl_2 , and 100 μ M GDP was supplemented with 50 mM cetyltrimethylammonium bromide (CTAB) and 125 mM MESNA (final concentrations). Ligation is initiated by adding 3.5–5 μ mol of the respective peptide from a ca. 30 mM stock solution in dichloromethane/methanol (1:5). The reaction mixture was incubated overnight at 37° with vigorous agitation. It was then centrifuged and the supernatant removed. The pellet was washed once with 1 ml methanol, four times with 1 ml methylene chloride, four times with 1 ml methanol, and four times with 1 ml Milli-Q water at room temperature to resolubilize the 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 ethylenediaminetetraacetate [EDTA] to a final protein concentration of 0.5–1.0 mg/ml and incubated overnight at 4° with slight agitation. The solution was cleared by centrifugation or filtration. Protein was renatured by diluting it at least 25-fold drop wise into refolding buffer (50 mM HEPES, pH 7.5, 2.5 mM DTE, 2 mM MgCl_2 , 10 μ M 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 for 30 min at the same temperature and was subsequently centrifuged to remove insoluble misfolded protein.

Complexation of Prenylated Rab GTPase with Rab Escort Protein-1 (REP-1) and Rab GDP Dissociation Inhibitor (Rab GDI) and Isolation of the Complexes. An equimolar amount of REP-1 or Rab GDI were added to the solution containing refolded protein, and the sample was incubated for 1 h on ice. The mixture was dialyzed over night against two 5-liter charges of dialysis buffer (25 mM HEPES, pH 7.5, 2 mM MgCl_2 , 2 μ M GDP, 2.5 mM DTE, 100 mM $[\text{NH}_4]_2\text{SO}_4$, 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 (molecular weight cutoff: 30 kDa) and loaded onto a Superdex-200 gel filtration column (Pharmacia) equilibrated with gel filtration buffer (25 mM HEPES, pH 7.5, 2 mM MgCl_2 , 10 μ M GDP, 2.5 mM DTE, 100 mM $[\text{NH}_4]_2\text{SO}_4$, 10% glycerol). Glycerol was omitted from this buffer for protein complexes intended for protein crystallization. The peak fractions containing the desired complex (judged by SDS-PAGE) were pooled, concentrated to approximately 10 mg/ml, and stored frozen at –80° in multiple aliquots. The typical recovery was 10–30% with respect to the starting Rab-thioester.

In Vitro Prenylation of Rab7C-K(Dans)-S-C-S-C and Purification of the Rab7C-K(Dans)-S-C(GG)-S-C(GG): REP-1 Complex. 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 MgCl₂, and 0.3% CHAPS. A 1 ml mixture contained 50 μ M REP-1, 55 μ M Rab7C-K (Dans)-S-C(GG)-S-C(GG), 60 μ M GST-Rab GGTase, and 500 μ M geranylgeranyl pyrophosphate (GGpp). The sample was mixed, incubated at 37° for 3 min, diluted to 3 ml with the same buffer containing 5% CHAPS, and 500 μ l of glutathione-Sepharose beads (Pharmacia) was added. The sample was incubated at 4° for 1 h on a rotating wheel. After the indicated period the supernatant was separated from the beads and concentrated in a Centricon 30 (Amicon) to a final volume of 300 μ l. The sample was centrifuged in a bench top centrifuge for 5 min at 4° 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 the binary Rab7C-K(Dans)-S-C(GG)-S-C(GG):REP-1 complex were pooled, concentrated, and stored in multiple aliquots at -80°.

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 (Hellman) with continuous stirring at 25°. 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 Rab GGTase in a cuvette, containing 1 ml of buffer (50 mM HEPES, pH 7.2, 50 mM NaCl, 5 mM DTE, 2 mM MgCl₂, 100 μ M GDP). Following a 5-min incubation at 25°, the reaction was initiated by adding GGpp to a final concentration of 10 μ M. Excitation and emission monochromators were set to 280 nm and 510 nm, respectively. Data were fitted to a double exponential equation using GraFit 4.0 (Erithacus software).

Results

Choice of Expression Vector and Generation of Thioester Tagged Rab GTPases

For generation of C-terminally thioester tagged Rab GTPases, we tested pTYB-1, pTWIN-1, and pTWIN-2 vectors. In our experience, pTWIN-1 showed the best performance in terms of protein yield, protein solubility, and cleavage efficiency. In this vector a fusion protein consisting of the desired Rab GTPase and an intein-chitin binding domain assembly,

fused in this order, is generated. The fusion GTPase is then isolated with chitin beads and the GTPase is cleaved off by addition of thiol reagent (MESNA in our case), which promotes the thiolysis of the polypeptide bond between the GTPase and the intein. The released protein has a thioester group at its C-terminus that can be used for the ligation reaction. Some GTPases precipitate at high concentrations of MESNA, so optimal conditions must be established experimentally for each protein.

We found that terminal Cys, Gln, and Asn residues located directly at the Rab C-terminus (former cleavage site) may lead to formation of byproducts arising from intramolecular side reactions between the thioester and the side chains of the mentioned amino acids (T. Durek, Y. Wu, and K. Alexandrov, unpublished). Therefore, mass spectrometric analysis of the protein to ascertain the presence of the intact thioester is advisable at this stage. The resulting thioester-tagged proteins can be stored for years at -80° .

*Construction of Fluorescent Rab7CK(Dans)SC(GG)SC(GG):REP-1
Complex Using a Combination of Protein Ligation and
In Vitro Prenylation*

Construction of fluorescent, geranylgeranylated GTPases requires synthesis of prenylated peptides bearing the desired fluorophores and their subsequent coupling with the thioester-tagged protein. Although a very powerful approach, this has the drawback that it requires a high degree of chemical expertise and significant resources. Therefore, initially we chose a two-step procedure where first a nonprenylated fluorescent Rab GTPase was constructed using EPL and was then subjected to *in vitro* prenylation (Fig. 1A).

We used a fluorescently labeled peptide mimicking the truncated six amino acids of Rab7 to restore a full-length protein (Iakovenko *et al.*, 2000). For *in vitro* prenylation, Rab7CK(Dans)SCSC was mixed with equimolar amounts of REP-1, GST-tagged Rab GGTase, and an approximately 10-fold molar excess of GGpp (Kalinin *et al.*, 2001). Upon completion of the reaction, GST-tagged Rab GGTase 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 Rab GGTase and the prenylated Rab7:REP-1 complex (Thoma *et al.*, 2001a). To ensure the homogeneity of the obtained complex, as well as to separate it from the detergent, gel filtration chromatography was performed. As can be seen in Fig. 1B, Rab7CK(Dans)SC(GG)SC(GG):REP-1 eluted with a molecular mass of around 150 kDa

FIG. 1. (A) Schematic representation of the *in vitro* ligation and prenylation procedure for the generation of the Rab7CK(Dans)SC(GG)SC(GG):REP-1 complex. Step 1—ligation of the dansyl-containing peptide onto thioester-tagged Rab7. Step 2—*in vitro* prenylation of Rab7CK(Dans)SCSC with GST-Rab GGTase. Step 3—separation of GST-Rab GGTase using glutathione Sepharose. (B) Purification of the Rab7CK(Dans)SC(GG)SC(GG):REP-1 complex on a Superdex-200 10/20 column. Run conditions are described in Methods. Molecular weights of peaks were determined by comparison with protein standards of known molecular weight (1–670 kDa, 2–158 kDa, 3–44 kDa, 4–17 kDa) that are shown as arrowheads. The inset 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% minigels, and proteins were visualized by fluorescent scanning (D) and Coomassie blue staining (C). Horizontal arrows denote the position of migration of REP-1, α and β subunits of Rab GGTase and Rab7 (right side), and the molecular mass markers (left side).

and was clearly separated from the minor peak of the ternary complex. The molecular weight and purity of the sample were monitored by MALDI-TOF mass spectroscopy. The observed molecular weight of 24191 Da closely matched the calculated mass of doubly geranylgeranylated Rab7CK(Dans)SCSC (24170 Da) (data not shown).

Construction of Monoprenylated YPT1 GTPase, Its Complexation to Yeast GDI, and Complex Crystallization

Attempts to determine the structure of the Rab:Rab GDI complex and related protein complexes have been hampered by failure to produce sufficiently large amounts of prenylated Rab GTPases, which precluded the generation of a sufficient diversity of Rab:GDI complexes necessary for successful crystallization trials. To obtain preparative amounts of prenylated Rab GTPase, we ligated the Ypt1 protein truncated by two amino acids to a synthetic dipeptide, Cys-Cys(geranylgeranyl)-OH. This resulted in formation of native monoprenylated YPT1 GTPase. The reaction was carried out in the presence of 50 mM of the detergent CTAB, which for unknown reasons greatly facilitates the ligation of prenylated peptides (Durek *et al.*, 2004). Upon completion of the reaction, the detergent and the excess peptide have to be separated from the ligated protein. For this purpose, the reaction mixture was treated with dichloromethane, which results in complete protein precipitation and extraction of unligated peptide into the organic phase. The protein pellet was treated first with methanol and then with water and dissolved in 6 M guanidinium-HCl. The semisynthetic protein was refolded by diluting it in a buffer containing CHAPS, arginine-HCl, and trehalose, a procedure that is known to facilitate the refolding process (De Bernardes *et al.*, 1999). At this stage Rab GDI was added in equimolar amounts and the complex was concentrated by ultrafiltration and further purified by gel filtration (Fig. 2). The complex between YPT1(GG):GDI eluting at the position corresponding to 50 kDa was concentrated to ca. 10 mg/ml and used for crystallization trials. Crystals were obtained at 20° using the vapor diffusion method in hanging drop setups (Rak *et al.*, 2003).

Construction of Fluorescent Monoprenylated and Diprenylated Rab7 GTPase and Development of a Fluorescence-Based Rab Prenylation Assay

Double prenylation of Rab GTPase by Rab GGTase is a multistep process that was proposed to follow a random sequential mechanism where one cysteine is somewhat preferred for the first prenylation (Shen and

FIG. 2. *In vitro* ligation of Ypt1ΔC2 with a geranylgeranylated dipeptide and assembly of the Ypt1GG:Rab GDI complex. (A) Thioester-tagged Ypt1, yeast Rab GDI, and *in vitro* assembled Ypt1GG:Rab GDI complex resolved on an SDS-PAGE gel stained with Coomassie blue. (B) LC-ESI-MS spectrum of $^{1-204}$ Ypt1Δ2-MESNA thioester ($M_{\text{calc}} = 23,131$ Da) and of (C) $^{1-204}$ Ypt1Δ2-CC(GG) ($M_{\text{calc}} = 23,486$ Da). (D) MALDI-TOF-MS of the semisynthetic complex. The theoretical molecular mass of yRabGDI is 51,401 Da. The discrepancy of the determined values for the Ypt1Δ2-CC(GG) protein measured by ESI-MS (C, 23,484 Da) and MALDI-MS (D, 23,521 Da) is due to the inaccuracy of the MALDI spectrometer.

Seabra, 1996). However, in all cases monocysteine mutants of Rab proteins were used that provide only an approximation of the native situation. Moreover, the available reports dispute the exact sequence of isoprenoid addition (Shen and Seabra, 1996; Thoma *et al.*, 2001b). To clarify this point

it would be necessary to generate two monoprenylated reaction intermediates and analyze the rates of their prenylation. Since *in vitro* prenylation is not a viable approach for generating these intermediates, we used EPL for generating the reaction intermediates and the double prenylated product, all carrying a dansyl label attached to the side chain of lysine 205. To this end we used a block condensation strategy for the synthesis of the three peptides: CK(Dans)SCSC(GG)-OMe, CK(Dans)SC(GG)SC-OMe, and CK(Dans)SC(GG)SC(GG)-OMe. The peptides were ligated to thioester tagged Rab7 truncated C-terminally by six amino acids ($\Delta C6$) and the resulting protein was separated from excess peptide and detergent by extraction with organic solvents as described in the Methods section (Fig. 3A). The resulting protein was denatured in guanidinium-HCl and refolded in the presence of REP-1 protein added in stoichiometric amounts. This procedure resulted in formation of binary Rab:REP complexes that were further purified by gel filtration (Fig. 3D). MALDI-MS analysis of the resulting protein complexes revealed the expected molecular masses corresponding to monoprenylated (Fig. 3D) or diprenylated Rab7:REP-1 complexes (data not shown). We used an established *in vitro* incorporation of [^3H]geranylgeranyl to confirm that the complexes obtained were indeed intermediates of the prenylation reaction (Seabra and James, 1998). As expected, both monoprenylated complexes could be further modified with one molecule of geranylgeranyl per molecule of Rab7 while the doubly prenylated complex showed only background incorporation of isoprenoid (Alexandrov *et al.*, 2002; Durek *et al.*, 2004).

The competence of the single prenylated semisynthetic Rab proteins to accept another isoprenoid group by Rab GGTase catalysis encouraged us to test whether the transfer of the prenyl group can be observed by fluorescence changes of the dansyl reporter group. When the single prenylated Rab7:REP-1 complexes ($\sim 75 \text{ nM}$) were mixed with equal amounts of transferase, formation of the ternary complex could be inferred from the strong fluorescence increase observed at 510 nm upon excitation at 280 nm (not shown). Addition of GGpp ($10 \mu\text{M}$) resulted in a time-dependent decline of the fluorescence signal. The observed reaction was essentially completed within 10 min and the time traces obtained could be fitted to a double exponential equation (Fig. 3C). Under the same conditions, the observed changes of the fluorescence amplitude of the singly prenylated Rab substrates were significantly larger than for the doubly prenylated Rab protein (Fig. 3C). The small fluorescence change observed in the latter case possibly represents low efficiency transfer of a third prenyl group onto the ligation site cysteine. The observed rate constants are in excellent agreement with values previously obtained using other assays (Thoma *et al.*, 2000).

FIG. 3. Preparation of fluorescent monoprenylated Rab7. (A) Ligation of Rab7Δ6-MESNA thioester with CK(Dans)SCSC(GG)-OMe and purification of the semisynthetic Rab:REP-1 complex. SDS-PAGE gel loaded with thioester-tagged Rab7 (lane A), mixed with peptide (lane B), and following an incubation (lane C). Excess peptide was removed by washing with organic solvents (lane D). The protein was renatured, complexed to REP-1, and further purified by gel filtration (lane E). The gel was photographed while exposed to UV light (right) prior to Coomassie blue staining (left). (B) MALDI-MS analysis of $^{2-201}$ Rab7Δ6-MESNA thioester (before ligation, $M_{\text{calc}} = 22,932$ Da) and the semisynthetic Rab:REP-1 protein complex [$^{2-201}$ Rab7Δ6-CK(Dans)SCSC(GG)-OMe ($M_{\text{calc}} = 23,939$ Da)]. The inset shows the range from 60 to 80 kDa and the signal corresponding to REP-1 ($M_{\text{calc}} = 73,475$ Da). (C) Fluorescent *in vitro* prenylation assay: 75 nM of either Rab7Δ6-CK(Dans)SCSC(GG)-OMe, Rab7Δ6-CK(Dans)SC(GG)SC-OMe, or Rab7Δ6-CK(Dans)SC(GG)SC(66)-OMe in complex with REP-1 was incubated with 75 nM Rab GGTase. At the moment indicated by arrows 10 μM GGpp was added. (D) Preparative gel filtration of the Rab7Δ6-CK(Dans)SCSC(GG)-OMe:REP-1 complex using a Superdex-200 (26/60) column. The elution position of standard molecular weight markers is indicated by arrows. The analysis of the individual fractions by SDS-PAGE is shown below. The lower part (UV) represents the gel region corresponding to 20–30 kDa photographed in UV light prior to Coomassie blue staining (Coom).

Conclusions

Several lines of evidence suggest the physiological importance of Rab proteins in intracellular membrane transport. Nevertheless, the biochemistry of their function as well as the mechanism of their interaction with other components of the docking and fusion machinery remain largely unknown. The elucidation of Rab function requires the dissection of such interactions at the molecular level. This stresses the need for the development of sensitive biochemical assays for the study of such interactions and development of methods for production of Rab proteins in prenylated form. The semisynthesis-based methods of Rab GTPase engineering described in this chapter provide researchers with a number of tools for studying the intercommunications of Rab proteins with subunits of Rab GGTase and other molecules and provide a methodological platform for construction of fluorescent GTPases for *in vivo* experiments.

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