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Intein-Mediated Synthesis of Geranylgeranylated Rab7 Protein in Vitro

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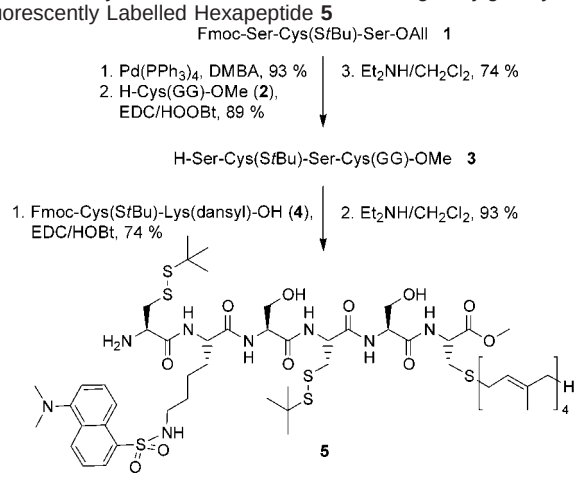
Production of recombinant proteins is a key technology in life sciences. However, progress in generation of authentic or engineered polypeptides has been largely limited to proteins that do not undergo posttranslational modification. This imbalance is primarily due to the intricacy of protein modification pathways as well as the lack of methodologies for their manipulation. The situation is even more complex when the protein in question is modified at multiple sites. This applies to Rab proteins—a sub-family of the ras-like GTPase superfamily with more than 60 members. Rab GTPases control a broad array of membrane docking and fusion events operating in processes ranging from fertilization to synaptic transmission.[1,2] For their function, Rab proteins require double modification with geranylgeranyl isoprenoids, which allows them to reversibly associate with membranes in a tightly controlled fashion. Covalent attachment of geranylgeranyl groups to one or two C-terminal cysteines is catalyzed by Rab geranylgeranyltransferase (RabGGTase). RabGGTase consists of a heterodimer of α and β subunits that function in concert with a 75 kDa protein chaperone termed REP (Rab escort protein).[3]

Studies of Rab protein function as well as the mechanism of Rab prenylation require methods that provide preparative amounts of prenylated Rab proteins with new functionalities such as fluorescence, photoreactivity, spin-labeled groups, or isoprenoid groups at non-native positions. The recently developed in vitro protein ligation method provides the necessary platform for combining large recombinant protein scaffolds with peptides generated by organic synthesis.[4,5] In this work we aimed to generate an intermediate of the Rab prenylation reaction—a Rab7 protein prenylated on only one of the two C-terminal reactive cysteines. Such intermediates cannot be isolated by enzymatic prenylation, since the RabGGTase utilizes a random sequential mechanism and generates a short-lived mixed population of monoprenylated Rab proteins.[6] The availability of such intermediates in pure form would greatly facilitate both mechanistic as well as structural studies of RabGGTase.

To make the semisynthetic proteins amenable to spectroscopic characterization, we chose to incorporate a fluorescent group in the proximity of the geranylgeranylated cysteine. As a reporter group, one of the smallest known fluorescent markers, the dansyl (5-(dimethylamino)-1-naphthalene sulfonyl) moiety, was chosen. Therefore, the amino acid following the N-terminal cysteine essential for in vitro protein ligation was changed from glutamic acid (Rab7) to lysine. This substitution is known to have no impact on the interaction of Rab7 with the subunits of RabGGTase.[7] A sulfonamide bond at the ϵ-amino group is expected to be stable under various reaction conditions, and this more general approach allows a direct attachment at a neutral site avoiding possible linker groups.[8] The reductively cleavable *S-tert*-butyl moiety was chosen as the protecting group of both the N-terminal and internal cysteines. This strategy prevents the exposure of the labile geranylgeranyl residue and deprotected sulfhydryl groups to unfavorable acidic and oxidative conditions. Moreover, the deprotection can even be carried out in situ during protein ligation with an excess of thiol agent.[5]

For peptide construction we chose a block condensation strategy due to flexibility considerations (Scheme 1). Therefore, tripeptide **1** was first deprotected at the C terminus, utilizing tetrakistriphenylphosphine palladium(0) with dimethylbarbituric acid (DMBA) as a scavenger and coupled with the prenylated cysteine methyl ester **2**, which was accessible via alkylation of cysteine methyl ester with geranylgeranyl chloride in 2 N NH_3 (MeOH).[9] Subsequent removal of the Fmoc protecting group afforded tetrapeptide **3**. Condensation of **3** with the N-terminal dansyl-labeled building block **4** and Fmoc removal with diethylamine finally resulted in the monogeranylgeranylated fluorescently labeled hexapeptide **5**.

Scheme 1. Synthesis of the C-Terminal Monogeranylgeranylated Fluorescently Labelled Hexapeptide **5**



The peptide was purified by flash chromatography and analyzed by NMR and mass spectrometry ($C_{62}H_{100}N_8O_{11}S_6$; ESI-MS; *m*/*z*: 1325.5 $[M + H]^+$).

For construction of the semisynthetic Rab7 protein we used a modification of a procedure described earlier.[7] The extent of ligation was determined by SDS-PAGE gel electrophoresis (Figure 1A,B). Unlike in other reported cases,[5,7] progression of the ligation reaction was strictly dependent on the presence of CTAB above the CMC concentration. Although native protein could be purified from the soluble fraction, it was accompanied by the unligated peptide through the following purification steps (T. Durek et al., *unpublished*). To circumvent this problem, we chose to conduct ligation reactions under high salt conditions and recover the protein from the pellet. To extract unligated peptide, the pellet was resuspended in dichloromethane and re-pelleted by centrifugation. The resulting

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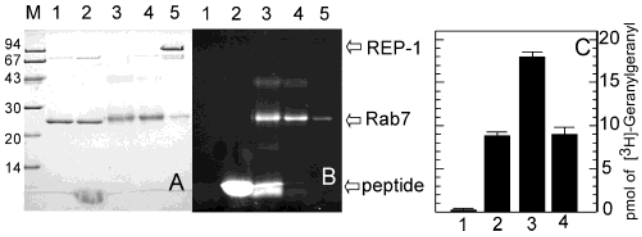

Figure 1. (A, B) SDS-PAGE gel of thioester-tagged Rab7Δ6C alone (lane 1), mixed with dansylated monolipidated peptide (lane 2), after separation of ligated protein pellet (lane 3), after peptide extraction and resolubilization (lane 4), after complex formation with REP-1 and Superdex 200 purification (lane 5) photographed either in UV light (B) or visible light after Coomassie blue staining (A). Horizontal arrows denote the position of migration of REP-1, Rab7, and free peptide (right side), and the molecular mass markers (left side). (C) In vitro prenylation assay using [^{3}H] labeled GGPP. Incorporation of [^{3}H]-geranylgeranyl into monoprenylated dansylated Rab7 (2), wild-type Rab7 protein (3) and Rab7C205S (4) catalyzed by mammalian RabGGTase. The reactions contained 50 pmol [^{3}H]-labeled GGPP (15000 cpm/pmol), 11 pmol of Rab7:REP-1 complex and 10 pmol of mammalian RabGGTase. RabGGtase was omitted in the control reaction 1 containing wt Rab7.

protein pellet was redissolved in a buffer containing 6 M guanidinium hydrochloride and then diluted ca. 20-fold with a buffer containing CHAPS (see Supporting Information). To stabilize the protein after removal of the chaotropic agent and the detergent, an equimolar amount of Rab escort protein-1 (REP-1) was added to the solution, and the resulting mixture was dialyzed.

The protein complex was concentrated and loaded onto a Superdex 200 gel filtration column equilibrated with the dialysis buffer. A single protein-containing peak eluted from the column at a position corresponding to approximately 110 kDa that contained both Rab7 and REP-1 proteins (Supporting Information). The elution position of the proteins indicated that they associated with a 1:1 stoichiometry which corresponds well with previous observations on the Rab:REP complex.[10,11] The obtained Rab7:REP-1 complex was homogeneously monoprenylated since Rab7 eluted as a single peak at the position corresponding to the monoprenylated reaction intermediate from a reversed phase matrix (not shown).[6] The mass of semisynthetic Rab7 was found to be 23928 Da as determined by MALDI-TOF which is in good agreement with the calculated mass of 23909 within the resolution of the method.

We wanted to address the ability of the generated Rab protein to function as a prenyl acceptor in the prenylation reaction meditated by RabGGTase. We measured the amount of [^{3}H]-geranylgeranyl that could be incorporated by RabGGTase into the semisynthetic protein as compared with the unprenylated full length Rab7 and to a mutant of Rab7 lacking one of two prenylatable cysteines (Rab7C205S). Semisynthetic Rab7 was prenylated to the same extent as the Rab7C205S mutant, demonstrating that the unprenylated cysteine was fully accessible and could function as a prenyl acceptor. The amount of radioactivity incorporated into the wild-type Rab7 was almost exactly double the amount incorporated into the monoprenylated Rab7, indicating that more than 90% of the ligated protein was active (Figure 1C).

Next, we took advantage of the changes in fluorescence of the C-terminal dansyl group upon interaction of the Rab7:REP-1 complex with RabGGTase. Addition of Rab7:REP-1 to a cuvette containing 70 nM solution of RabGGTase resulted in a dose-dependent and saturable increase of fluorescence when the dansyl group was excited at 280 nm via FRET from tryptophan. The data from this fluorescent titration experiment was fitted to solution of a quadratic equation yielding a K_d value of 4 ± 1.2 nM (Supporting Information), indicating that the intermediate of the prenylation reaction has high affinity for the enzyme. The double prenylation reaction must use one of two possible mechanisms: either the reaction intermediate remains bound to the RabGGTase until it is fully prenylated, or it must dissociate in the course of double prenylation reaction to bind a new GGPP-loaded transferase molecule. The choice between both scenarios depends on the relationship between the rate for the second prenylation step and the dissociation rate of the REP-1:Rab7GG:RabGGTase complex. Using the stopped flow technique, we characterized the kinetics of the interaction and concluded that the dissociation rate for the monoprenylated complex proceeded at a rate 0.01 s^{-1}. Since the second prenylation step is known to proceed at a rate of $k_2 = 0.04$ s^{-1}, these data indicate that dissociation of the reaction intermediate is not required for double prenylation of Rab proteins (for data see Supporting Information).

In summary, using a combination of organic synthesis, heterologous protein expression, and in vitro protein ligation, we have prepared a fluorescent monoprenylated Rab7 protein. The obtained protein was functionally active and displayed characteristics of the genuine intermediate of the Rab double prenylation reaction. This procedure is the first example of the in vitro generation of semisynthetic lipidated proteins using the native chemical ligation method. It is generally applicable to the production of lipidated proteins in preparative amounts for a variety of applications. In addition to the obvious applications for in vitro studies and in vivo imaging, the developed methodology provides a nonenzymatic source of posttranslationally modified GTPases in amounts suitable for crystallization. This is a particularly exciting development since progress in this direction has been hampered by the lack of methods for producing large amounts of pure prenylated proteins.

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Supporting Information Available: Experimental details and data on the kinetics of the interaction (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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