Mono Q Chromatography

Eluates from the Ni^{2+}–NTA–agarose columns are concentrated and dialyzed against 50 vol of Mono Q buffer. The sample is diluted to 10 ml, filtered through a 0.22-μm Durapore membrane (Millipore), and loaded onto an FPLC Mono Q HR5/5 column (Pharmacia) equilibrated with Mono Q buffer (flow rate: 1 ml/min). After washing the column with 20 ml of Mono Q buffer, it is developed with a linear gradient of 0–0.25 *M* NaCl in Mono Q buffer over 20 min and 1-ml fractions are collected. His_6-Rab1GG, which elutes in the range of 50–100 m*M* NaCl, is identified by analyzing aliquots of the fractions by SDS–PAGE and Coomassie blue staining. The fractions are pooled, concentrated, dialyzed against 25/125 containing 0.6% CHAPS, and stored in aliquots at −80°.

Note: We routinely recover ~0.2–0.5 mg of >95% pure His_6-Rab1GG from each liter of infected cells. The proportion of isoprenylated protein is >90%, as estimated by phase separation in Triton X-114 solution.[8]

Comments

His_6-Rab1GG prepared from Sf9 membranes and recombinant GDP dissociation inhibitor (GDI) isolated from *E. coli* can be used to reconstitute a soluble GDI–Rab1 complex *in vitro* (see [10] in this volume). This complex has been shown to serve as a functional source of Rab1 for vesicular transport between the ER and the Golgi complex *in vitro*.[4]

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[2] Purification of Posttranslationally Modified and Unmodified Rab5 Protein Expressed in *Spodoptera frugiperda* Cells

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Introduction

Rab proteins are posttranslationally modified at their C termini by addition of the 20-carbon isoprenoid, geranylgeranyl, mediated by Rab

geranylgeranyltransferase (Rab GGTase).[1] Although Rab proteins expressed in *Escherichia coli* do not undergo this modification, they are active in guanine nucleotide binding and GTP hydrolysis. Factors that modulate GDP/GTP exchange and GTP hydrolysis have been searched using these proteins.[2] However, geranylgeranylation has been shown to be essential for the function of Rab proteins *in vivo*[3] and to interact with one regulatory protein, Rab–GDP dissociation inhibitor (GDI), *in vitro*. Rab–GDI forms a complex with, and inhibits GDP dissociation from, several Rab proteins.[4–6] Furthermore, Rab–GDI modulates the membrane association of Rab proteins and is required for their function.[7] Therefore, it is important to obtain posttranslationally modified Rab proteins in order to study the mechanism of their membrane association and function.

Rab5 is a 25-kDa GTP-binding protein localized to the plasma membrane, clathrin-coated vesicles, and early endosomes, and functions as a regulatory factor of endocytosis.[8–10] As for other Rab proteins, Rab5 is geranylgeranylated at its C terminus[6] and this modification is essential for its function.[10] In order to obtain Rab5 in the isoprenylated form, we have made use of a baculovirus expression system. This chapter describes a method to purify both posttranslationally modified and unmodified Rab5 from *Spodoptera frugiperda* (Sf9) insect cells overexpressing the protein. Purified posttranslationally modified and unmodified Rab5 protein efficiently bind GTP and GDP. However, as expected, Rab–GDI is active only on modified Rab5. When modified Rab5 complexed with Rab–GDI is introduced into permeabilized cells, Rab5 is localized to its correct site of function and induces the formation of enlarged early endosomes as previously observed *in vivo*,[10] indicating that it is functionally active.[11]

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Purification of Posttranslationally Modified and Unmodified Rab5 from Sf9 Cells

Construction and Selection of Rab5-Containing Baculovirus

A full-length cDNA-encoding canine Rab5[12] is cloned in the *Bam*HI site downstream of the polyhedrin promoter in the baculovirus transfer vector pVL1393.[13] A Rab5 recombinant *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) is constructed by homologous recombination.[14] Briefly, 1 μg of linear AcMNPV DNA[15] (Invitrogen) is mixed in a polypropylene tube with 5 μg of the transfer vector containing the cDNA encoding Rab5 in 120 μl of a buffer [20 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, and 150 m*M* NaCl]. In a separate tube, 60 μl of the transfection reagent DOTAP (Boehringer-Mannheim) is added to 60 μl of the same buffer. Both solutions are mixed and incubated at room temperature for 15 min. Three milliliters of serum-free Grace's medium (GIBCO, Grand Island, NY) is then added to the transfection tube. Sf9 cells (2.0×10^6 cells), seeded in a 25-cm^2 flask 1 hr before to be allowed to attach to the substratum, are washed twice with the serum-free medium and then the transfection solution is added. After 7 hr, 3 ml of Grace's medium supplemented with 20% heat-inactivated fetal calf serum (FCS) is added and the cells are further incubated at 27°. After a week the medium is collected and used at different dilutions (10^{-1}–10^{-6}) to infect Sf9 cells plated 1 hr before at a density of 10^6/25-cm^2 flask. After 1 hr of infection, a plaque assay is performed as previously described[14] and cells are left at 27°. After 6–8 days, plaques containing putative recombinant virus are selected. The virus is eluted in the medium and is used for another plaque purification assay. Recombinant plaques are identified for the absence of occlusions that are normally formed on expression of the polyhedrin protein.

Expression of Rab5 in Sf9 Cells

Sf9 cells are grown in 165-cm^2 tissue culture flasks (Greiner) in Grace's medium supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 27°. A virus stock is prepared by infecting Sf9 cells with the recombinant virus. On the 5th day after infection, the medium is collected and centrifuged at 1000*g* for 10 min at 4° to remove

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floating cells. The supernatant containing the virus is stored at 4° as a virus stock. For producing Rab5 protein, subconfluent Sf9 cells grown on three 24.5 × 24.5-cm tissue culture plates (Nunc) are infected with 7.5 ml of the virus stock per plate in 75 ml of Grace's media supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin and are incubated for 3 days at 27°. The cells are harvested and pelleted by centrifugation at 1000*g* for 10 min at 4°. After one wash with 50 ml of phosphate-buffered saline, cells are centrifuged again and the pellet (3 ml) is stored at −80° until use. Subsequently, the cell pellet is fractionated into a high-speed pellet (membrane fraction) and a supernatant (cytosol fraction). The posttranslationally modified Rab5 is purified from the membrane fraction, whereas the cytosol fraction contains large amount of unmodified Rab5.

Preparation of Cytosol and Membrane Fractions from Sf9 Cells

The pellet of Rab5-expressing Sf9 cells is resuspended in 20 ml of ice-cold buffer A [20 m*M* HEPES/KOH, pH 7.2, 2 m*M* ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol] containing 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride and 100 m*M* KCl. This suspension is sonicated on ice 10 times each for 30 sec with 30-sec intervals to break the cells. Postnuclear supernatant (PNS) is obtained by centrifugation of the homogenate at 1000*g* for 5 min at 4°. The PNS is then centrifuged at 160,000*g* (Beckman SW40 rotor, 30,000 rpm) for 30 min at 4°. About 10% of Rab5 is recovered in the pellet and 90% in the supernatant, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie blue (Fig. 1B, lanes 1–3) and by Western blot analysis using anti-Rab5 monoclonal antibody (data not shown) (see [27] in this volume). The main band of Rab5 in the pellet migrates slightly faster than that in the supernatant on SDS–PAGE. This is an indication that Rab5 in the pellet is posttranslationally modified while the protein in the supernatant is not.[16] A further criterion to distinguish between the two forms is the interaction with Rab–GDI (see below). The reason why most of the Rab5 is recovered in cytosol may be due to limitations of the Rab GGTase and/or the substrate.

Purification of Posttranslationally Modified Rab5 from Membrane of Sf9 Cells

For purification of modified Rab5, the pellet (membrane fraction) is resuspended in 4 ml of ice-cold buffer A containing 0.6% (w/v) 3-[(3-cholam-

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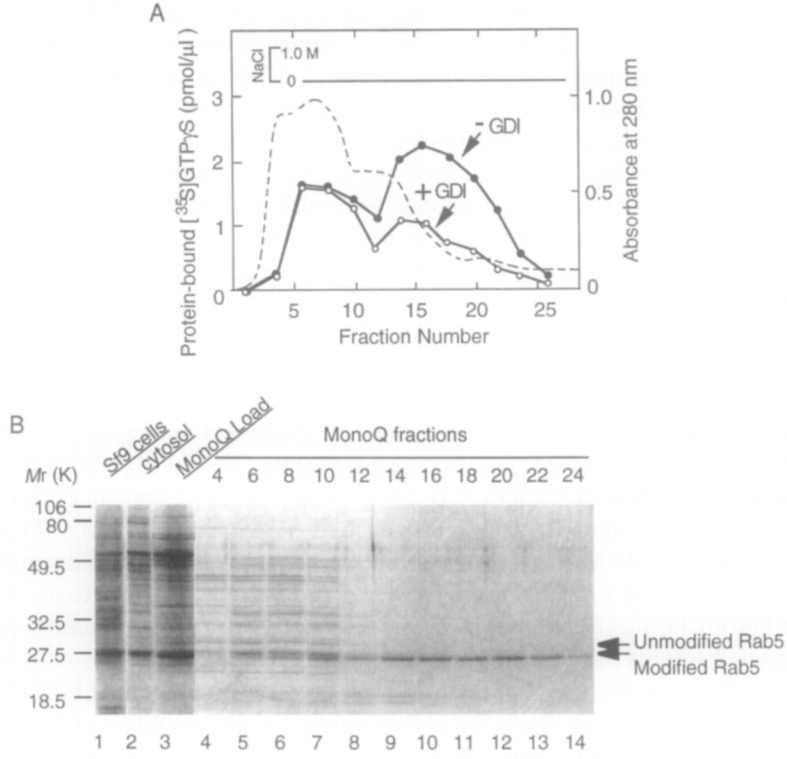

FIG. 1. Purification of posttranslationally modified Rab5 by Mono Q column chromatography. (A) Chromatography profile and [^{35}S]GTPγS binding activity in the presence (○) and in the absence (●) of Rab–GDI. ---, absorbance at 280 nm. (B) Analysis by SDS–PAGE (12% acrylamide gel) stained with Coomassie blue showing the starting homogenate of Sf9 cells expressing Rab5 (lane 1), 2 μl out of 20 ml of the cytosol fraction of the cells (lane 2), 5 μl out of 4 ml of the sample loaded onto a Mono Q column (lane 3), and a 10-μl aliquot of fractions 4–24 (lanes 4–14). For details, see text.

idopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) (Sigma) with sonication for 10 sec on ice and is incubated for 1 hr at 4° on a rotating wheel. The suspension is centrifuged at 160,000*g* for 30 min at 4°, and the supernatant (4 ml, 10 mg protein) is loaded onto a Mono Q HR5/5 column (Pharmacia) equilibrated with degassed buffer A containing 0.6% (w/v) CHAPS (Fig. 1A). After washing the column with 12 ml of the same buffer, proteins are eluted with buffer A containing 0.6% CHAPS and 1 *M* NaCl. Fractions (0.5 ml) are collected and analyzed by SDS–PAGE stained with Coomassie blue (Fig. 1B) and immunoblotting using anti-Rab5 monoclonal antibody (data not shown) (see [27] in this volume). Most of Rab5 is detected in two peaks. The first consists of the flow-through fractions (frac-

tions 4–11; about 20% recovery) and the second consists of the washing fractions (fractions 12–24, about 40% recovery), where Rab5 migrates slightly faster compared to the protein contained in the first peak on SDS–PAGE. These fractions are further characterized for the presence of posttranslationally modified Rab5. Since Rab–GDI has been shown to be active only on posttranslationally modified but not on unmodified Rab proteins,[5] we tested each fraction for Rab–GDI to inhibit GDP/GTP exchange as deduced by the binding of radiolabeled GTPγS[4] (Fig. 1A). An aliquot (2 μl) of each fraction is incubated in the presence or in the absence of 5 μ*M* Rab–GDI, purified from overexpressing *E. coli* as a His_6-tagged protein (see [27] in this volume), in a buffer (20 μl) containing 20 m*M* HEPES/KOH (pH 7.2), 10 m*M* EDTA, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and 1 μ*M* [^{35}S]GTPγS (20,000 cpm/pmol, DuPont-NEN) for 10 min at 30°. Protein-bound [^{35}S]GTPγS is measured by passing the reaction mixture through a nitrocellulose filter (0.45-μm pore size, 2.5 cm diameter, BA85, Schleicher & Schuell) immediately after adding 3 ml of filtration buffer [20 m*M* tris[hydroxymethyl]aminomethane hydrochloride (pH 7.5), 100 m*M* NaCl, and 25 m*M* $MgCl_2$]. After three washes with 3 ml filtration buffer, the filter is dried and the radioactivity is measured in 5 ml Ready Safe scintillation liquid (Beckman) using a Beckman LS 6000SC type scintillation counter. Proteins in these fractions effectively bind [^{35}S]GTPγS. Although Rab–GDI does not effect [^{35}S]GTPγS binding to the proteins of fractions 4–11, it effectively inhibits [^{35}S]GTPγS binding to the proteins of fractions 12–24, thus indicating that the second peak (fractions 12–24) contains posttranslationally modified Rab5. The Rab5 protein recovered in fractions 4–11 may come from the contaminating cytosol and/or aggregated cytosol Rab5. The samples are analyzed by SDS–PAGE (12% acrylamide gel) stained with Coomassie blue (Fig. 1B, lanes 4–14). Typically, about 200 μg of highly purified posttranslationally modified Rab5 is obtained in fractions 12–24.

Purification of Posttranslationally Unmodified Rab5 from Cytosol of Sf9 Cells

The posttranslationally unmodified Rab5 is purified from the cytosol of Rab5-expressing Sf9 cells by a one-step procedure using hydroxyapatite column chromatography. Hydroxyapatite (Seikagakukogyo, Tokyo, Japan) is swollen in distilled water and the fine particles are removed by changing the water every 30 min until the supernatant is clear. Then, 1 ml of hydroxyapatite is transferred onto a Poly-Prep chromatography column (Bio-Rad), followed by equilibration with buffer B (20 m*M* HEPES/KOH, pH 7.2, 5 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol). The cytosol (1 ml, 5 mg of protein)

is loaded onto the column. After washing the column with 5 ml of buffer B, the column is eluted with buffer B containing 0.6% CHAPS. Fractions (0.5 ml) are collected, and 150 μg of unmodified Rab5 is eluted in fractions 2–8. Because of the high level of expression and the particular property of Rab5 to be eluted by CHAPS, the purity is over 90%. Purified unmodified Rab5 efficiently binds GTP and GDP but, as expected, Rab–GDI does not inhibit [^{35}S]GTPγS binding in the same assay mentioned earlier. In this simple procedure, 3 mg of highly purified unmodified Rab5 can be expected from one preparation of the cytosol (20 ml).

[3] Expression of Rab9 Protein in *Escherichia coli*: Purification and Isoprenylation *in Vitro*

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Introduction

This chapter describes the purification of canine Rab9 after expression in *Escherichia coli*, and the small-scale and preparative-scale isoprenylation of Rab9 *in vitro*. *Escherichia coli*-expressed Rab proteins are valuable reagents in analyzing the biochemical properties, structural features, and functional activities of individual rab proteins. In addition, characterization of purified mutant forms of Rab proteins can provide valuable information to complement functional studies of Rab proteins in *in vitro* systems or in living cells.

The pET expression system developed by Studier *et al.*[1] is invaluable for the production of milligram quantities of specific proteins in *E. coli*. Rab9 cDNA was subcloned into the pET8c plasmid, which places the cDNA under the control of a T7 RNA polymerase promoter. The resulting expression vector, pET8c–Rab9, is transformed into the *E. coli* strain BL21 (DE3), which expresses the T7 RNA polymerase gene under the control of the *lacZ* promoter. The addition of isopropyl-β-D-thiogalactoside (IPTG) induces the synthesis of T7 RNA polymerase, which, when present at high levels, produces large amounts of Rab9 mRNA and thus large amounts of Rab9 protein.

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