Expression of Rab9 protein in Escherichia coli: purification and isoprenylation in vitro

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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 Rab9 to be eluted by CHAPS, the purity is over 90%. Purified unmodified Rab9 efficiently binds GTP and GDP but, as expected, Rab9-GDI does not inhibit \([^{35}S]GTPyS\) binding in the same assay mentioned earlier. In this simple procedure, 3 ng of highly purified unmodified Rab9 can be expected from one preparation of the cytosol (20 ml).

### Expression of Rab9 Protein in *Escherichia coli*:

**Purification and Isoprenylation in Vitro**

*By Markus A. Riederer, Thievery Soldati, Barbara Drag-Svistun, and Suzanne J. Piepho*

#### 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 vivo systems or in living cells.

The pET expression system developed by Studier et al. is invaluable for the production of milligram quantities of specific proteins in *E. coli*. Rab9 cDNA was subcloned into the pET3c 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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F. Studier, A. Rosenberg, J. Dunn, and J. Duda, this series. Vol. 185, p. 68.
Materials

PTG, ampicillin, geranylgeranyl pyrophosphate, and [H]geranylgeranyl pyrophosphate (GGPP and [H]GGPP, American Radiolabeled Chemicals).

Plasmids

E. coli expression plasmid pET8c-Rab9wt, pET8c-Rab9N21

Cells

E. coli strain BL21(DE3) [F−, ompT, r−, m−a−]

Equipment/Columns

Pressure filtration cell (Amicon)
Q-Sepharose Fast Flow column (Pharmacia)
Sephacyr S-100 column (Pharmacia)

Buffers

Lysis buffer: 64 mM Tris–HCl (pH 8.0), 8 mM MgCl₂, 2 mM EDTA, 0.5 mM dithiothreitol (DTT), 10 μM GTP, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine, 10 μg/mL leupeptin, 1 μM pepstatin, 3 μg/mL aprotinin, and 1 mM NαN
S-100 buffer: 64 mM Tris–HCl (pH 8.0), 100 mM NaCl, 8 mM MgCl₂, 2 mM EDTA, 0.2 mM DTT, 10 μM GDP, 1 mM PMSF, 10 mM benzamidine, and 1 mM NaF

Procedures

Expression and Purification of Rab9 Protein

The procedure was optimized for Rab9 purification based on a previously described method of Tucker et al.1

1. The cDNA of rab9 was cloned into the E. coli expression vector, pET8c.2 The pET8c plasmid was linearized with BamHI, filled in using the Klenow fragment of DNA polymerase I, and cut with NcoI. Both restriction enzyme sites are located in the polylinker of pET8c. A pGEM1–Rab9


Results

1. Introduction

After incubation for 3.5 hr, the 24+ h-Rad19 cell lysate was fractionated by a centrifugation method to obtain the nuclear fraction. The distribution of Rad19 protein was determined by Western blotting using a rabbit polyclonal antibody against Rad19. The results showed that Rad19 protein was distributed in both the nuclear and cytoplasmic fractions. The nuclear fraction was then subjected to SDS-PAGE and analyzed by Western blotting using the same antibody. The results showed that a single band, corresponding to Rad19 protein, was detected in the nuclear fraction. This indicates that Rad19 protein is localized in the nucleus.

2. Purification of Rad19 Protein

Rad19 protein was purified from the nuclear fraction using a combination of macrotip and column chromatography. The purified Rad19 protein was then eluted with a linear gradient of sodium chloride. The elution profile showed a single peak at a sodium chloride concentration of 0.5 M, corresponding to Rad19 protein. The purified Rad19 protein was then dialyzed against buffer containing 25 mM Tris-HCl, pH 7.5, and 10 mM MgCl₂.

3. Characterization of Rad19 Protein

The purified Rad19 protein was characterized by Western blotting using the rabbit polyclonal antibody against Rad19. The results showed that the purified Rad19 protein was identical to the protein present in the nuclear fraction. The purified Rad19 protein was also subjected to SDS-PAGE and analyzed by Western blotting using a goat polyclonal antibody against Rad19. The results showed that a single band, corresponding to Rad19 protein, was detected, confirming the identity of the purified protein.

Table 1: Purification of Rad19 Protein

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (μg/mg)</th>
<th>Specific Activity (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5</td>
<td>0.8</td>
</tr>
<tr>
<td>2</td>
<td>1.8</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Note: The purification was performed using a Q-Sepharose column, followed by a size-exclusion chromatography column.
to TGN transport\(^1\) 22 mM HEPES-KOH, pH 7.2, 20 mM Tris-HCl, 116 mM KCl, 4.3 mM magnesium acetate, 5 mM EDTA, and 0.2 mM GDP, plus a protease inhibitor cocktail and an ATP-regenerating system. After incubation at 37\(^\circ\)C for 2 hr, the prenylation reactions are clarified by ultracentrifugation at 300,000g for 10 min in a TLA-100.2 rotor (Beckman) and analyzed by SDS-PAGE and anti-Rab immunoblotting. Alternatively, no molecular size shift is expected, or for precise quantitation analysis, small-scale reactions should include 1 \(\mu\)M GDP and 0.1 \(\mu\)M \(^{[3]}\)HGGPP, and be followed by SDS-PAGE and fluorography.

Another small-scale prenylation assay used to assess prenylatability of a construct is based on the cell-free translation of an in vitro-transcribed Rab cDNA. Commercially available rabbit reticulocyte lysate (e.g., Promega) is gel filtered and therefore does contain enough endogenous GGPP to ensure prenylation of newly translated proteins. Efficient prenylation can be achieved by adding 10 \(\mu\)M GGPP in the in vitro translation reaction containing \(^{[3]}\)Smethionine (as judged then by a molecular size shift after SDS-PAGE and autoradiography analysis) or 1 \(\mu\)M GGPP and 0.1 \(\mu\)M \(^{[3]}\)HGGPP in reactions carried out with unlabeled amino acids (as judged by incorporation of radioactivity in the translation product analyzed by SDS-PAGE and fluorography).

**Preparative in Vitro Prenylation**

In a standard 0.5-mL reaction, 1 \(\mu\)g of purified Rab9 (100 \(\mu\)M) is prenylated in the presence of 5.6 mg/mL of crude Chinese hamster ovary (CHO) cytosol (prepared as described in Goda and Pfeffer\(^1\)) and 10 \(\mu\)M of geranylgeranyl pyrophosphate (GGPP, American Radiolabeled Chemicals, Inc) gel filtered and therefore does contain enough endogenous GGPP to ensure prenylation of newly translated proteins. Efficient prenylation can be achieved by adding 10 \(\mu\)M GGPP in the in vitro translation reaction containing \(^{[3]}\)Smethionine (as judged then by a molecular size shift after SDS-PAGE and autoradiography analysis) or 1 \(\mu\)M GGPP and 0.1 \(\mu\)M \(^{[3]}\)HGGPP in reactions carried out with unlabeled amino acids (as judged by incorporation of radioactivity in the translation product analyzed by SDS-PAGE and fluorography).

**Gel Filtration Chromatography and Fraction Analysis.** Samples are analyzed on a 30-mL Sephacryl S-100 (Pharmacia) column equilibrated and eluted in 0.1 M Tris-HCl, pH 8, 100 mM NaCl, and 0.1 M MgCl\(_2\), 2 mM EDTA, and 0.2 mM DTT, 10 \(\mu\)M GDP, and 1 \(\mu\)M PMSF. Forty 0.4 mL fractions are collected; alternate fractions are subjected to 12.5% SDS-PAGE and conventional immunoblotting. Rab9 protein is detected using rabbit or mouse antibodies raised against native, recombinant Rab9 protein.\(^6\) Detection of GDI is carried out using affinity-purified antibodies raised against purified Rab9A-GDI.\(^7\) Secondary antibodies are either goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad). All antibodies are used at 1:1000 dilution; antigen-antibody complexes are detected by enhanced chemiluminescence (ECL, Amer sham). Quantitation of ECL signals on X-ray films (Kodak) is carried out using a densitometric scanner (Model 300 A, Molecular Dynamics) or a Phosphorimager system (Molecular Dynamics).

**Characterization of Yeast Type-II Geranylgeranytransferase**

By YU JIANG, GUINDALINA ROSSI, and SUSAN FERRO-NOVICK

**Introduction**

Members of the Rab GTP-binding protein family are involved in the regulation of different exocytic and endocytic transport processes.\(^1\) They are localized to diverse intracellular compartments and participate in various steps of vesicular traffic.\(^1\) In yeast, two Rab GTPases, Sec4p and Ypt1p, have been shown to play a role on the exocytic pathway.\(^2\) They are significantly homologous to each other, but function at distinct stages of the pathway. Although Ypt1p is involved in mediating the transport of vesicles from the endoplasmic reticulum (ER) to the Golgi complex,\(^2\) Sec4p is required for membrane traffic from the Golgi to the plasma membrane.\(^2\) Like most small GTP-binding proteins, Ypt1p and Sec4p are synthesized in the cytosol, but become membrane bound after undergoing posttranslational modification. Mutations that block the membrane attachment of these proteins result in a block in secretion.\(^2\) Thus, the membrane association of Ypt1p and Sec4p is crucial for their function.

The ability of small GTP-binding proteins to bind to membranes is conferred by the addition of geranylgeranyl, a 20-carbon isoprenoid derivat...