Rab5 Affinity Chromatography without Nonhydrolyzable GTP Analogues

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Rab5 is an important small GTPase involved in endocytosis and membrane trafficking. Rab5-binding proteins can be identified using Rab5 affinity chromatography with nonhydrolyzable GTP analogues such as GTPγS or GppNHp. However, this method requires significant quantities of the GTP analogue and is thus time-consuming and expensive. In the present report we show a faster and more cost-effective method that does not use a GTP analogue but uses constitutively the active Rab5 mutant (Rab5Q79L) as a ligand. To validate this method, the binding of EEA-1 was confirmed and several novel Rab5-binding proteins were also identified by 2-dimensional electrophoresis and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Key words: GTPγS, GppNHp, Rab5 Affinity Chromatography

Introduction

Rab5, one of the small GTP-binding proteins, is a key regulator both in endocytosis and following membrane-trafficking events, such as clathrin-mediated endocytosis (Christoforidis and Zerial, 2000, 2001; Zhu *et al.*, 2007). Many Rab5-binding proteins have been identified and have provided important clues as to how intracellular vesicle transportation during endocytosis by Rab5 is regulated. Tuberin (TSC2) is one of the tumour suppressor proteins and we have identified Rab5 in a tuberin-enriched-organelle fraction (Yamamoto *et al.*, 2002). Therefore, to identify novel Rab5-binding proteins we provide data to understand the function of Rab5 on tumour suppressing through tuberin. Previous reports demonstrated a method to purify Rab5-binding proteins by Rab5 affinity chromatography through nucleotide exchange reaction (NE reaction) and nucleotide stabilization reaction (NS reaction) using wild-type bacterially expressed Rab5 with a nonhydrolyzable GTP analogue such as guanosine 5'-3-*O*-(thio)triphosphate (GTPγS) or guanyl-5'yl-imidodiphosphate (GppNHp) (Christoforidis *et al.*, 1999; Christoforidis and Zerial, 2000, 2001) (Fig. 1A). In this method, NE and NS reactions were developed to obtain the activated form of Rab5 for Rab5 affinity chromatography. The NE reaction is based on the use of EDTA to strip magnesium ions (Mg^{2+}) and to exchange GDP for the nonhydrolyzable GTP analogue. Following the NE reaction, Rab5 is stabilized in the activated form in the presence of excess nonhydrolyzable GTP analogue or, following the NS reaction, Mg^{2+} in the absence of EDTA. One problem with the conventional protocol is that it consumes not only significant experimental time but is an expensive experiment when using the nonhydrolyzable GTP analogue.

A GTP hydrolysis defective mutant, Rab5Q79L, and a GTP-binding defective mutant, Rab5S34N, have been well characterized to regulate endosome fusion. Moreover, Rab5-binding proteins have been analyzed using bacterially expressed Rab5Q79L in the presence of a nonhydrolyzable GTP analogue (Zhu *et al.*, 2007). However, whether the presence of the GTP analogue is necessary to analyze Rab5-binding proteins remained unclear. In this study, we modified this method and developed a Rab5 affinity chromatography with-

Abbreviations: GTPγS, guanosine 5'-3-*O*-(thio)triphosphate; GppNHp, guanyl-5'yl-imidodiphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry.

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out using either of the nonhydrolyzable GTP analogues but with only bacterially expressed Rab5 mutants (Rab5Q79L, Rab5S34N) (Fig. 1B).

Material and Methods

Antibodies

Antibodies were obtained from the following sources; anti-EEA1 from BD Bioscience (Franklin Lakes, NJ, USA) and anti-mouse IgG from Sigma-Aldrich Japan (Tokyo, Japan).

Preparation of bovine spleen cytosol

Bovine spleen was obtained from Tokyo Shibaura Zoki Co., Inc (Tokyo, Japan) to prepare cytosol. Bovine spleen homogenate was prepared using homogenization buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5] containing 6 μg/ml chymostatin, 0.5 μg/ml leupeptin, 10 μg/ml antipain hydrochloride, 2 μg/ml aprotinin, 0.7 μg/ml peptatin A, 10 μg/ml PMSF. The homogenate was centrifuged at 4,200 × *g* for 20 min at 4 ºC, and then the postnuclear supernatant was further centrifuged at 105,000 × *g* for 60 min at 4 ºC. The supernatant was dialyzed against homogenization buffer. Furthermore, potential aggregates as a result from dialysis were removed by preclearing the cytosol at 105,000 × *g* for 60 min at 4 ºC. The cytosol was stored at –80 ºC until usage.

Rab5Q79L affinity chromatography

Rab5Q79L and Rab5S34N mutants were subcloned into the pGEX-2T vector (Amersham, Tokyo, Japan) to obtain bacterially expressing GST-Rab5. GST-Rab5Q79L and GST-Rab5S34N vectors were transformed into BL21-CodonPlus (DE3)-RIL (STRATAGENE, Tokyo, Japan). *E. coli* was precultured overnight with 50 ml LB medium containing 30 μg/ml ampicillin and 50 μg/ml chloramphenicol. The medium was added to 1.0 l LB medium containing 30 μg/ml ampicillin and 50 μg/ml chloramphenicol, and then cultured to an OD_{600} of 0.5 at 37 ºC. The culture was induced with 1 mM isopropylthiogalactoside (IPTG) at 37 ºC for 3 h. The medium was centrifuged at 10,000 × *g* for 15 min at 4 ºC. The *E. coli*-expressing GST-Rab5Q79L was added to the lysis buffer [phosphate-buffered saline (PBS) containing 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 μg/ml RNase, 5 μg/ml DNase, 6 μg/ml chymostatin, 0.5 μg/ml leupeptin, 10 μg/ml antipain hydrochloride, 2 μg/ml aprotinin, 0.7 μg/ml peptatin A, and 10 μg/ml PMSF]. The suspension was treated by supersonic wave, centrifuged at 15,000 × *g* for 20 min at 4 ºC, and the supernatant passed through a single-use syringe filter (0.2 μm) (Sartorius, Goettingen, Germany). The lysate was incubated with 1 ml glutathione-Sepharose 4 Fast Flow (Pharmacia) for 2 h at 4 ºC under slow stirring and then washed with 10 ml PBS containing 5 mM 2-mercaptoethanol and 5 mM $MgCl_2$. Furthermore, the beads were washed with 10 ml homogenization buffer. 100 ml of the bovine spleen cytosolic fraction were successively added to GST-Rsb5Q79L and GST-Rab5S34N beads and incubated for 2 h at 4 ºC under very slow stirring. The cytosolic fraction was drained and passed through again. The beads were washed first with 10 ml wash buffer A (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5), second with 10 ml wash buffer B (20 mM HEPES, 250 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5), and third with 2 ml wash buffer C (20 mM HEPES, 250 mM NaCl, 1 mM DTT, pH 7.5). Then 1 ml elution buffer (20 mM HEPES, 2.0 M NaCl, 20 mM EDTA, 1 mM DTT, pH 7.5) was added to the beads, which were incubated overnight at 4 ºC under stirring, and the eluate was collected. Finally, 0.7 ml new elution buffer was passed again through the beads and combined with the previous eluate. Then the eluted samples were subjected to 2-dimensional electrophoresis by using an Ettan IPGphor instrument (Amersham, San Francisco, CA, USA), stained with Sypro Ruby (Bio-RAD, San Diego, CA, USA), and the proteins were visualized by a molecular imager FX® (Bio-RAD).

Result and Discussion

In the present study, to identify novel Rab5-binding proteins, we first analyzed the Rab5 expression levels of several rat organs such as brain, spleen and liver. The spleen had a relatively high expression of Rab5 (data not shown). Therefore, we used bovine spleen as a sample to identify Rab5-binding proteins. Previously, EEA1 had been identified from bovine brain cytosol using Rab5 affinity chromatography (Christoforidis *et al.*, 1999; Christoforidis and Zerial, 2000, 2001). This protein is required for fusion between early endosomes and is an essential component of the endosome membrane-docking machinery

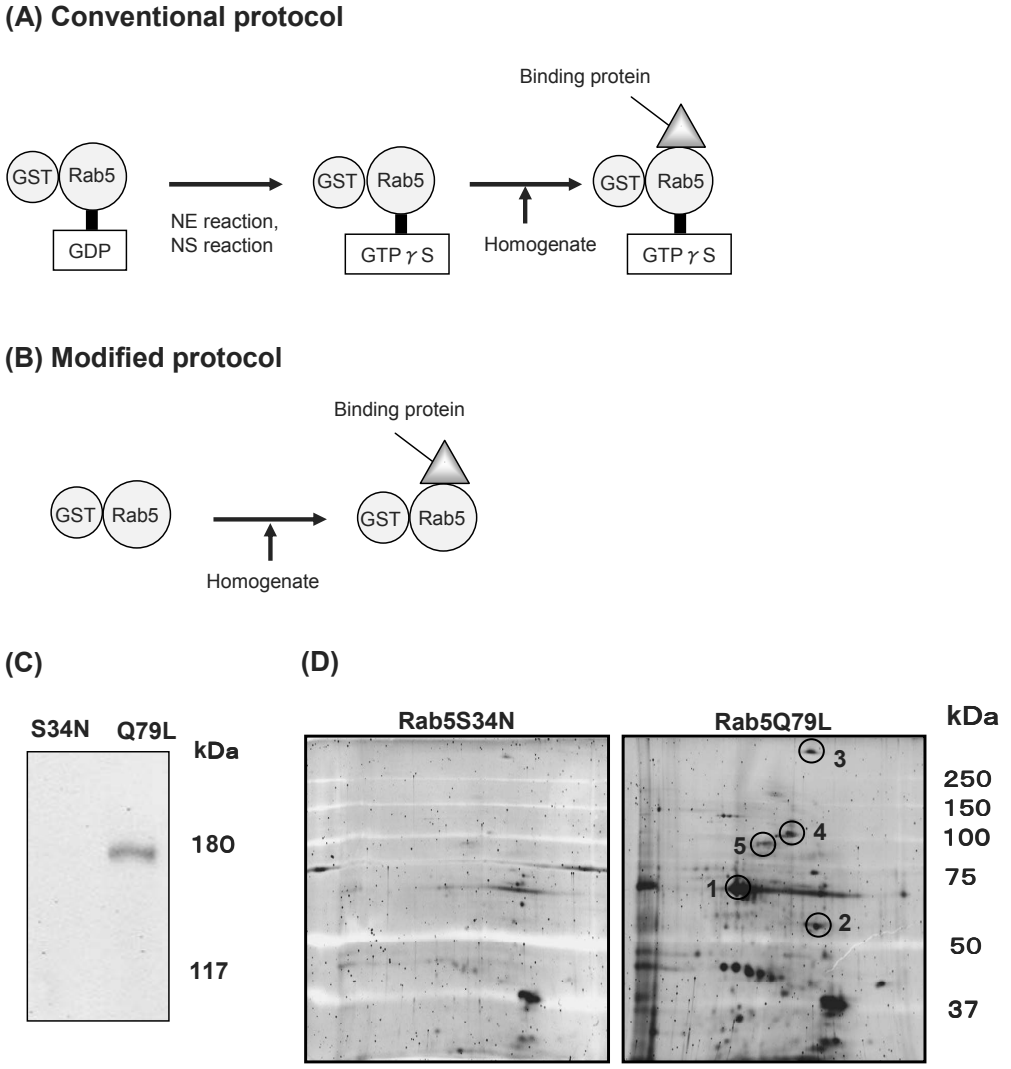

Fig. 1. The specific activated Rab5-binding protein EEA1 was detected by GST-Rab5Q79L affinity chromatography without nonhydrolyzable GTP analogue and the protocol reveals many Rab5-interacting proteins from bovine spleen cytosol. (A) Conventional Rab5 affinity chromatography was performed using the NE reaction and NS reaction with nonhydrolyzable GTP analogue and GST-Rab5 (wild-type) or GST-Rab5Q79L mutant. To test for the binding specificity of activated Rab5-interacting proteins, an identically treated GST-Rab5 (wild-type) or GST-Rab5S34N column was prepared for the NE reaction and NS reaction using GDP instead of nonhydrolyzable GTP analogue under the same conditions. (B) Our protocol using GST-Rab5Q79L does not require the NE reaction and NS reaction using nonhydrolyzable GTP analogue for Rab5 affinity chromatography. Inactive Rab5 analysis was performed according to the same procedure, except for using GST-Rab5S34N. (C) Rab5-binding proteins mixture was purified from bovine spleen cytosol by Rab5Q79L affinity chromatography without nonhydrolyzable GTP analogue. EEA1 was detected from the column by Western blotting analysis using specific anti-EEA1 antibody. On the other hand, EEA1 was not eluted from the Rab5S34N column. (D) The eluate was subjected to 2-dimensional electrophoresis and the gel was stained with Sypro Ruby. Many Rab5-binding proteins were detected from the GST-Rab5Q79L column.

Table I. Similar to protection of telomeres 1, L-plastin, ferritin heavy polypeptide 1, similar to vinculin isoform meta-VCL isoforms 1 and 2 were identified by LC-MS/MS as novel Rab5-binding proteins.

Spot no.	Name	Function	NCBI no.
1	Similar to protection of telomeres 1	Essential for chromosome end protection and telomere length homeostasis	XP 588599
2	L-Plastin	Actin-budding protein	AAI03002
3	Ferritin heavy polypeptide 1	Stores iron in a soluble, non toxic, readily available form; important for iron homeostasis	NP 776487
4	Similar to vinculin isoform meta-VCL isoform 1	Involved in cell adhesion	XP 001252007
5	Similar to vinculin isoform meta-VCL isoform 2	Involved in cell adhesion	XP 001252035

(Christoforidis and Zerial, 2001). By Western blotting analysis using anti-EEA1 specific antibody, we identified EEA1 eluted only from the GST-Rab5Q79L affinity chromatogram, thus confirming that this isolation method works in the absence of nonhydrolyzable GTP analogues such as GTPγS or GppNHp (Fig. 1C). We detected numerous Rab5-binding proteins from the Rab5Q79L column, in contrast with the Rab5S34N column (Fig. 1D). Christoforidis *et al.* (1999) and Christoforidis and Zerial, 2000, 2001) have shown that at least 22 cytosolic proteins from brain can bind to Rab5 by Rab5 affinity chromatography. However, there is no report claiming Rab5-binding proteins from spleen. The spots indicated in Fig. 1D as 1–5 were subjected to LC-MS/MS analysis (Gene World Co., Inc, Tokyo, Japan) and were identified as shown in Table I. None of them have been presented as Rab5-binding proteins and further analysis may provide the physiological importance of their interaction. In previous studies, using a magnetic bead-based method combined with immunoprecipitation, we showed that Rab5 interacts with tuberin, a tumour suppressor protein (Yamamoto *et al.*, 2002). Therefore, it may be interesting to study whether these candidates have any physiological interaction with tuberin.

Our result shows for the first time that, even in the absence of nonhydrolyzable GTP analogues, it is possible to identify binding proteins by using the Rab5Q79L mutant as a ligand. Our protocol will reduce experimental time and the cost of nonhydrolyzable GTP analogue during the NE and NS reaction. This modified protocol should serve as a screening method to identify novel Rab5-binding proteins, which should provide new insights into the understanding of the molecular mechanisms of intracellular vesicle transportation during endocytosis involving the small GTP-binding protein Rab5. Finally, by using a GTP hydrolysis defective mutant and/or a GTP-binding defective mutant as a ligand for affinity chromatography, it should be possible to identify novel binding proteins for small GTP-binding proteins.

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