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Faster and More Convenient Far-western Blotting for Identifying Rab5-binding Proteins

By

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Summary : The small GTP binding-protein Rab5 is known to be involved both in the vesicle-mediated transport from the plasma membrane to the early endosomes, and in the homotypic early endosome fusion. Previous reports showed that Rab5-binding proteins can be detected by using Far-western blotting in the presence of non-hydrolyzable GTP analogs such as GTPγS. However, this method requires significant quantities of non-hydrolyzable GTP analog and is thus time-consuming and expensive for the nucleotide exchange (NE) and nucleotide stabilization (NS) reactions. In this report we describe a faster and more cost-effective method that does not use non-hydrolyzable GTP analogs but rather, a constitutively active Rab5 mutant Q79L as a Far-western blotting probe. To validate this method, the binding of the previously characterized Rab5-binding protein EEA-1 was confirmed and actin beta was identified as a Rab5-binding protein by LC MS/MS. Our protocol can reduce the experimental time (by 3–4 h) and the cost (by 90–95%) for the experiment. We expect this method to provide fundamental insights into the molecular mechanism of intracellular transport by Rab5.

Key words : Rab5, endocytosis, GTPγS, Far-western blotting, overlay assay, Detergent insoluble fraction

Introduction

Rab5 is a small GTPase localized on early endosomes[1] and controls early endosome fusion during clathrin-dependent endocytosis[2], caveolae-dependent endocytosis[3,4], macropinocytosis[5,6], and phagocytosis[7]. Many Rab5-binding proteins have been identified, providing important clues as to how intracellular vesicle transportation during endocytosis by Rab5 is regulated. Far-western blotting (overlay assay) has been employed in the search for Rab5-binding proteins and has successfully led to identification of early endosome antigen (EEA1) through nucleotide exchange (NE) and stabilization (NS) reactions using wild-type bacterially expressed Rab5 with a non-hydrolyzable GTP analog such as guanosine 5'-3-O-(thio) triphosphate (GTPγS)[8]. The NE and NS reactions were used to obtain the activated form of Rab5 for Far-western blotting. The NE reaction uses EDTA to chelate magnesium ions (Mg^{2+}) and to exchange GDP for the non-hydrolyzable GTP analog. Following the NE reaction, the NS reaction stabilizes the activated form of Rab5 in the presence of excess non-hydrolyzable GTP analog and Mg^{2+} in the absence of EDTA. A serious disadvantage of the conventional protocol is that it takes a significant amount of the experimental time and, that it is expensive due to the cost of the non-hydrolyzable GTP analog.

We investigated whether or not a constitutively active, GTP hydrolysis-defective mutant Rab5 Q79L and an inactive, GTP binding defective-mutant Rab5 S34N could be used as alternative Far-western probes that do not require non-hydrolyzable GTP analogs. Using this modified method, we confirmed EEA1 as a binding partner of active Rab5 and further detected several putative protein candidates which may bind to GST-Rab5 Q79L and identified one of them by LC MS/MS. Thus, in this report we demonstrate that non-hydrolyzable GTP analogs are not necessary for Far-western blotting when using Rab5 Q79L as a probe.

Material and Methods

(1) Purification of Rab5

Rab5 Q79L and Rab5 S34N mutant cDNAs were subcloned into pGEX-2T vector (Amersham) to obtain GST-

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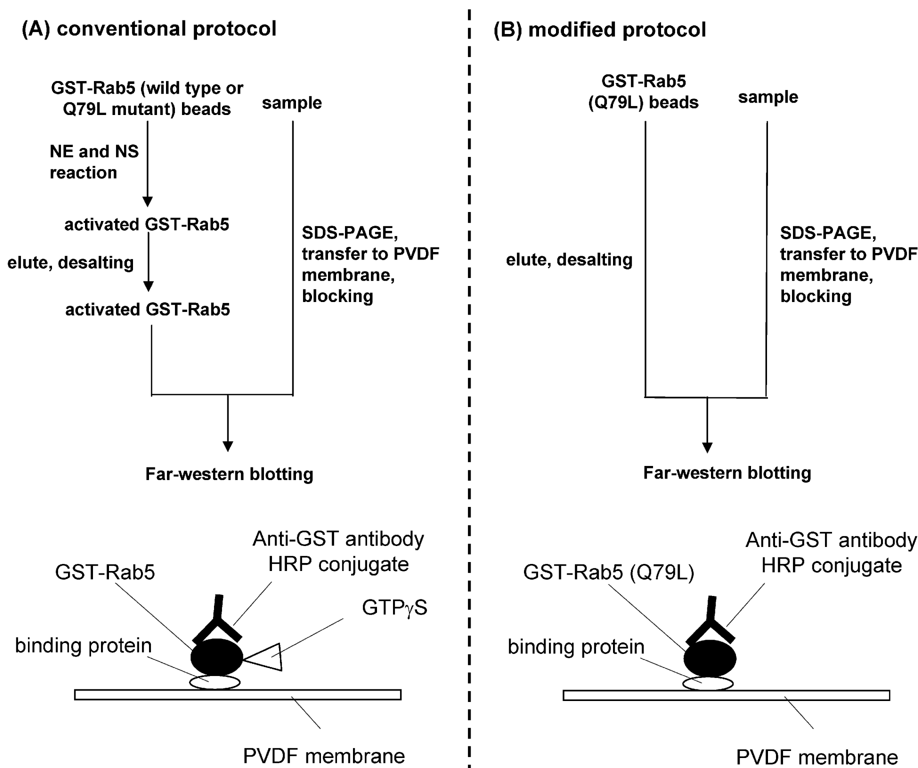

Fig. 1 Overview of the conventional Far-western blotting and our modified Far-western blotting without non-hydrolyzable GTP analog. (A) Conventional Far-western blotting using GST-Rab5 (wild type) or GST-Rab5 Q79L as a probe was performed with NE and NS reactions using a non-hydrolyzable GTP analog such as GTPγS. (B) Our protocol using GST-Rab5 Q79L does not require non-hydrolyzable GTP analog for Far-western blotting. Inactive Rab5 analysis was performed according to the same procedure, except that GST-Rab5 S34N was used.

Rab5 Q79L and GST-Rab5 S34N. These two plasmids were transformed into Escherichia coli BL21-CodonPlus (DE3)-RIL (STRATAGENE). GST-Rab5 Q79L and GST-Rab5 S34N fusion proteins were expressed and purified from *E. coli* with glutathione-Sepharose 4 Fast Flow (Pharmacia) through the standard procedures.

(2) Partial purification of EEA1

Bovine spleen was obtained from Tokyo Shibaura Zoki Co., Inc. (Tokyo). Bovine spleen homogenate was prepared in homogenization buffer (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5) which contains protease inhibitors (6 μg/m*l* chymostatin, 0.5 μg/m*l* leupeptin, 10 μg/m*l* antipain hydrochloride, 2 μg/m*l* aprotinin, 0.7 μg/m*l* pepstatin A, 10 μg/m*l* PMSF). The homogenate was centrifuged at 4,200×g for 20 min at 4℃ and then at 105,000×g for 60 min at 4℃. The supernatant was dialyzed against the homogenization buffer.

Furthermore, potential aggregates resulting from dialysis were removed by centrifuging the cytosol at 105,000×g for 60 min at 4℃. Subsequently, gel filtration chromatography using Sephacryl S-300 (Amersham) resulted in partial purification of EEA1. This fraction was enriched with Amicon Ultra (MILLIPORE). Aliquots were stored at −80℃ until usage. The partial purification of EEA1 fraction was confirmed by western blotting with a specific anti-EEA1 antibody (BD Bioscience).

(3) Preparation of 1% Triton X-100 insoluble fraction

Bovine spleen homogenate was prepared in the homogenization buffer as described above. The homogenate was centrifuged at 4,200×g for 20 min at 4℃ and then at 105,000×g for 60 min at 4℃. The precipitation was suspended in PBS with 1% Triton X-100 and incubated for 60 min on ice. 1% Triton X-100 insoluble fraction was obtained by centrifugation at 105,000×g for 60 min at 4℃ and resuspended in PBS. Aliquots were stored at −80℃ until usage.

(4) Far-western blotting

Proteins were subjected to SDS-PAGE, transferred to PVDF membrane. Transferred proteins were renatured on PVDF membrane in an 8 to 0 M urea gradient diluted in Tris-buffered saline and Tween 20 (TBST). The membrane was incubated overnight with TBST containing 5% blocking reagent (an accessory for ECL Ad-

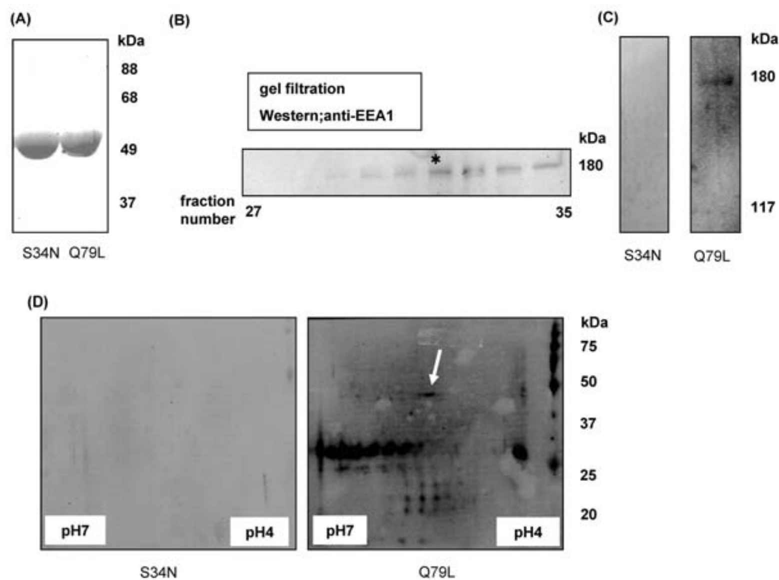

Fig. 2 Far-western blotting using GST-Rab5 Q79L mutant without non-hydrolyzable GTP analog. (A) GST-Rab5 Q79L and GST-Rab5 S34N were recombinantly expressed in *E. coli* and purified by glutathione Sepharose-4B affinity column chromatography. The gel was stained with Sypro Ruby. (B) Partially purified EEA1 used as a positive control for Rab5 binding was prepared from bovine spleen by gel filtration chromatography and detected in fraction number 32 (marked with an asterisk). The EEA1 signal was detected at approximately 180 kDa with specific anti-EEA1 antibody. (C) 800 ng of partially purified EEA1 protein was subjected to SDS-PAGE, transferred to PVDF membrane, and Far-western blotting was carried out using GST-Rab5 Q79L or GST-Rab5 S34N as a probe without the NE and NS reactions. Although the NE and NS reactions were not performed, GST-Rab5 Q79L bound to the 180 kDa protein EEA1. On the other hand, the GST-Rab5 S34N signal was not detected. Membranes incubated with GST-Rab5 Q79L and GST-Rab5 S34N were exposed for the same period of time. (D) Rab5-binding partners in the 1% Triton X-100 insoluble fraction from bovine spleen (100 μg) were detected by 2-dimensional Far-western blotting. Membranes incubated with GST-Rab5 Q79L and GST-Rab5 S34N were exposed for the same period of time.

vance product by Amersham) at 4℃. Subsequently, the membrane was washed with TBST containing 5 mM $MgCl_2$ and incubated for 1 h with TBST containing 5 mM $MgCl_2$ and 2.0×10^{-2} M active GST-Rab5 Q79L or inactive GST-Rab5 S34N. Next, it was washed with TBST containing 5 mM $MgCl_2$ and incubated for 1 h with HRP conjugated anti-GST antibody (Amersham) at a dilution of 1 : 50,000 in TBST containing 5 mM $MgCl_2$. Finally, the membrane was washed with TBST containing 5 mM $MgCl_2$, treated with ECL Advance (Amersham).

(5) LC MS/MS

A Rab5-binding protein was analyzed by LC MS/MS (Gene World Co., Inc, Tokyo).

Results and Discussion

A previous report[9] demonstrated a method to detect Rab5-binding proteins by Far-western blotting with the NE and NS reactions using wild Rab5 or Rab5 Q79L mutant with a non-hydrolyzable GTP analog such as GTPγS or GppNHp (Fig. 1A). In this report we demonstrate that non-hydrolyzable GTP analogs are not necessary for Far-western blotting when using Rab5 Q79L as a probe (Fig. 1B). First, we purified recombinant GST-Rab5 Q79L and GST-Rab5S34N proteins using affinity chromatography through *E. coli* expression. As a result, highly-purified proteins were obtained (Fig. 2A). EEA1 is the Rab5-binding partner that mediates tethering/docking of early endosomes[9]. We partially purified EEA1 from bovine spleen cytosol as a positive control for Far-western blotting following gel filtration chromatography. Partial purification of EEA1 fraction was confirmed by western blotting with a specific anti-EEA1 antibody (Fig. 2B). The EEA1 fraction was subjected to SDS-PAGE, transferred to PVDF membrane and Far-western blotting was performed by GST-Rab5 Q79L or GST-Rab5 S34N as a probe. Although GST-Rab5 Q79L did not go through the NE and NS reactions with a non-hydrolyzable GTP analog such as GTPγS, it bound to EEA1 (Fig. 2C). Rab5 Q79L has been reported

Bos taurus actin beta NP_776404

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  1 mdddiaalvv dngsgmckag faqddaprav fpsivgrprh qgvmvgmgqk dsyvgdeaqs
 61 krgiltlkyp iehgivtnwd dmekiwhhtf ynelrvapee hpvllteapl npkanrekmt
121 qimfetfntp amyvaiqavl slyasgrttg ivmdsgdgvt htvpiyegya lphailrldl
181 agrdltdylm kiltergysf tttaereivr dikeklcyva ldfeqemata assssleksy
241 elpdgqviti gnerfrcpea lfqpsflgme scgihettfn simkcdvdir kdlyantvls
301 ggttmypgia drmqkeital apstmkikii apperkysvw iggsilasls tfqqmwiskq
361 eydesgpsiv hrkcf
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Fig. 3 LC MS/MS sequence coverage of trypsin-digested Bos taurus actin beta. Matched peptides are underlined (sequence coverage : 22%).

to have the same function as wild-type Rab5-GTP[2-4]. Thus, this method can successfully identify Rab5.

Previous reports have shown that caveolae-coated protein, caveolin-1, was enriched in the 1% Triton X-100 insoluble fraction[10]. Rab5 is in a cytosolic protein and it is also localized to caveolin-1 positive organelles[3,4,11].

Christoforidis *et al.* have shown that at least 22 cytosolic proteins from bovine brain cytosol can bind to Rab5 by Rab5 affinity column chromatography[8]. However, this method is not suitable for identifying Rab5-binding partners from the insoluble fraction due to column clogging. Thus, we attempted to identify Rab 5-binding partners in the 1% Triton X-100 insoluble fraction from bovine spleen by Far-western blotting without non-hydrolyzable GTP analog. The sample was subjected to 2-dimensional electrophoresis and subsequent Far-western blotting as described above. Rab5-binding partners in 1% Triton X-100 insoluble fraction were detected by 2-dimensional Far-western blotting (Fig. 2D). The pattern of appearance of spots on the PVDF membrane were checked for reproducibility and one of them indicated by the arrow in Fig. 2D was selected as the constantly appearing spot. This spot was further subjected to LC MS/MS analysis and was identified as actin beta (Fig. 3). Actin has been implicated in early events in endocytosis by Rab5 in mammalian cells[12]. However, whether Rab5 can interact directly with actin has been unclear. Thus, our result leads us to a hypothesis that Rab5 binds directly to actin in early stages of endocytosis.

We have developed a novel protocol to identify Rab 5-binding proteins by Far-western blotting without using no-nhydrolyzable GTP analogs. Accordingly, this experimental procedure is simplified by omitting the NE and NS reactions (Fig. 1B). Our protocol can reduce the experimental time (by 3–4 h) and the cost (by 90–95%) for the experiment. We expect this strategy to provide fundamental insights into the molecular mechanism of intracellular transportation by the small GTP-binding protein Rab5. Furthermore, using a GTP hydrolysis-defective mutant and/or a GTP binding-defective mutant as ligands for Far-western blotting may prove a general strategy for identifying binding proteins for small GTP-binding proteins.

Acknowledgements

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Rab5 結合因子同定のための迅速且つ簡便な Far-western blotting

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要約：低分子量 G タンパク質 Rab5 は細胞膜から初期エンドソームへの小胞輸送や初期エンドソーム同士の融合に関与する因子であることが知られている。過去の報告では，GTPγS などの加水分解耐性 GTP アナログを使用し Far-western blotting を行うことによって Rab5 結合因子が検出されている。しかしながら，この方法では NE および NS 反応の際に多量の加水分解耐性 GTP アナログが必要であり，実験時間やコストが非常にかかる。本論文では Far-western blotting のプローブとして常時活性型変異体 Rab5Q79L を用いることによって，NE 反応と NS 反応が必要なく加水分解耐性 GTP アナログを使用しない簡便且つ実験コストが低い方法について記述する。この方法を評価するため，既知の Rab5 結合因子である EEA1 と Rab5 が結合するか否か検証したところ結合が確認された。さらに LC MS/MS によって Rab5 結合因子の同定を試みたところ actin beta が同定された。我々の方法では実験時間が 3–4 時間程短縮され，実験コストは 90–95% 削減された。我々はこの方法が Rab5 による細胞内小胞輸送における重要な知見をもたらすものと考える。

キーワード：Rab5，エンドサイトーシス，GTPγS，ファーウェスタンブロッティング，オーバーレイアッセイ，界面活性剤難溶性画分

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