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# Purification and Identification of Novel Rab Effectors Using Affinity Chromatography

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**Rab GTPases are central regulatory elements of the intracellular transport machinery of eukaryotic cells. To regulate vesicle docking and fusion as well as organelle dynamics Rab proteins interact with effector molecules in the GTP-bound active state. The identification of Rab effectors is, therefore, of primary importance for the mechanistic understanding of intracellular transport. Here we describe the experimental system we have developed to biochemically purify and identify effectors of the small GTPase Rab5. The method, which is based on an affinity chromatography procedure, results in the large-scale purification of Rab effectors in amounts sufficient for both their identification by microsequencing techniques and their functional characterization. In the case of Rab5, the procedure allows a comprehensive analysis of the downstream effectors and regulators of this GTPase. We expect this strategy to provide fundamental insights into the molecular mechanism of membrane transport but also to be applicable to several other GTPase-dependent biological functions.**


Intracellular trafficking is governed by Rab proteins, a subfamily of GTPases of the Ras superfamily. A number of studies in mammalian cells (1–5) and in yeast (6–8) have established that these proteins are key regulators of the docking and fusion process. The switch between the GDP-inactive and GTP-active forms ensures the kinetic control of membrane transport. The GDP/GTP nucleotide cycle is tightly controlled by a number of cytosolic and membrane proteins that maintain Rab proteins in a dynamic equilibrium between the cytosol and the organelle membrane. In cytosol Rab proteins are kept in the GDP-bound inactive form by GDP dissociation inhibitor (GDI) or the Rab escort protein (REP) (9–11). Given that Rab proteins are post-translationally modified by the addition of a hydrophobic $C_{20}$ geranylgeranyl group at the C terminus of the molecule, these two factors ensure the solubility of the protein in the cytosol. Rab GDI and REP are essential for delivery of the prenylated proteins to the membrane where a GDI displacement activity uncouples the Rab protein from its cytosolic cofactor. Following membrane association, a specific guanine nucleotide exchange factor (GEF) induces the exchange of GDP for GTP (5). The activated GTP form recruits distinct cytosolic effector proteins to the membrane which, together with other membrane components (SNAREs), are responsible for membrane docking and fusion. On effector binding, these factors also stabilize the Rab protein in the GTP-bound form. The regulation of the nucleotide cycle is completed by GTPase-activating proteins (GAPs) which stimulate the Rab-GTPase activity that hydrolyzes Rab-GTP to the inactive GDP form (12). Therefore, it becomes apparent that Rab proteins interact with multiple factors that both control the nucleotide cycle (GDI, REP, GEF, GAP) and mediate downstream functions (effectors). Recent studies provide evidence that, besides docking and fusion, Rab proteins can exert other functions such as ves-

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icle formation and organelle motility (13–15, 32). Given that membrane transport requires membrane–cytoskeleton interactions, Rab proteins may coordinate all these activities. This would imply that distinct effectors are required for a multiplicity of functions directed by each Rab GTPase.

Clearly, understanding the molecular mechanisms whereby Rab proteins function in membrane transport implies elucidating the activity of their specific effectors. Several methods have proven successful for the identification of effectors of small GTPases of the Ras superfamily and of Rab proteins in particular. For example, the first Rab effector, Rabphilin, was identified using chemical crosslinking of active Rab3 and crude membranes (16). Two-hybrid screenings led to identification of the Rab5 effectors Rabaptin-5 (17) and Rabaptin-5$\beta$ (18) and the Rho effectors Ser/Thr protein kinase and rhophilin (19). Proteins interacting with the activated forms of Rab6 (14), Rab8 (20), and Rab9 (21) have also been identified through yeast two-hybrid screens. The main disadvantage of this method, however, is the high incidence of false-positive results and the subsequent requirement for biochemical evidence to support the interaction.

The ligand overlay assay has also been employed in the search for effector proteins, and successfully led to identification of the Rab5 exchange factor Rabex-5 (22), as well as the Rho effector p160 Ser/Thr protein kinase (23). However, this method is limited by the ability of the proteins to refold after denaturation on gel electrophoresis. In addition, it requires purification of the candidate protein by conventional biochemical methods. Coimmunoprecipitation using anti-Rab protein-specific antibodies is limited by the use of detergents, which often interfere with protein–protein interactions and require the use of crosslinkers (24).

None of these methods have succeeded in revealing the complexity of the effector molecules for a given GTPase. To address this problem, we sought to use a biochemical approach to identify new Rab effectors and also obtain sufficient quantities of native proteins suitable for immediate functional analysis. For this we developed a Rab5-affinity chromatography method that could mimic the membrane recruitment of Rab effectors on activation of the GTPase. Affinity chromatography has been used in the past and led to the identification of three Rho effectors (25, 26). Here we have improved this method by optimizing a number of parameters such as increasing the density of the Rab protein on the matrix, the yield of nucleotide exchange, the stringency of the washes, and the specificity of the elution step. These conditions resulted in identification of a surprisingly large number of new Rab5-interacting proteins and effectors from bovine brain cytosol, thus underscoring the advantage of such a comprehensive strategy. We further describe the procedure we have designed to obtain these molecules in a form suitable for functional experiments either directly after the affinity chromatography procedure or after further biochemical fractionation.

## DESCRIPTION OF METHOD

### DNA Constructs

Human Rab5 cDNA was subcloned in the pMAL-C2 vector (New England Biolabs) encoding Rab5 fused to the C terminus of MBP. The glutathione *S*-transferase (GST)–Rab5 fusion protein was expressed using a pGEX vector (Pharmacia) generously provided by Harald Stenmark.

### Expression of GST–Rab5 and Preparation of the Affinity Chromatography Column

DH5$\alpha$ cells were transformed with cDNA expressing GST–Rab5. Expression was performed according to the instructions of the manufacturer. Briefly, 120 liters of LB–ampicillin medium divided in 80 $\times$ 5-liter flasks were grown at 37°C to a cell density of 0.8 (37°C). The culture was induced with 1 mM isopropylthiogalactoside (IPTG) and the bacteria were grown for an additional 3 h. The cells were harvested at 3000*g* at 4°C for 20 min and the cell pellet was resuspended in 2.5 liters cold phosphate-buffered-saline (PBS) buffer containing 200 $\mu$M GDP, 5 mM $MgCl_2$, 5 $\mu$g/ml DNase, 5 $\mu$g/ml RNase, 5 mM 2-mercaptoethanol (2-ME), and protease inhibitors (chymotrypsin, leupeptin, aprotinin, antipain, and pepstatin A, each concentration as recommended by supplier). The bacterial cell suspension was quick frozen in liquid $N_2$ and, after thawing, was lysed using a French press at 800–1000 psi. The lysate was centrifuged at 100,000*g* at 4°C for 60 min and the supernatant was incubated with 20 ml packed glutathione–Sepharose 4B beads (Pharmacia), pre-equilibrated with PBS, for 2 h at 4°C under rotation. Unbound proteins were washed with 200 ml PBS buffer containing 100 $\mu$M GDP, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. To determine the yield of the procedure, 10 $\mu$l of packed beads was

treated with elution buffer (100 mM Tris, 15 mM glutathione, 5 mM 2-ME, pH 8.5) for 10 min at 20°C and the eluted protein was measured using the Bio-Rad method. The estimated amount of GST–Rab5 on the column was 1 g per 20 ml of glutathione–Sepharose 4B beads.

### Preparation of the Active (GTP) and Inactive (GDP) Forms of GST–Rab5 on the Affinity Column

The procedure described above results in the production of GST–Rab5 mainly in the GDP (inactive) form due to the high intrinsic rate of GTP hydrolysis (4). Conversion to the active GTP conformation was performed by a nucleotide exchange method based on the use of EDTA which consists of the following steps: Beads containing immobilized GST–Rab5:GDP were washed with 40 ml nucleotide exchange buffer (NE buffer) containing 20 mM Hepes, 100 mM NaCl, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothroitol (DTT), 10 $\mu$M GTP$\gamma$S, pH 7.5, and incubated for 30 min at room temperature with 40 ml NE buffer containing 1 mM GTP$\gamma$S under rotation. Subsequently, buffer was drained out of the column, and the wash and incubation procedure described above was repeated twice. The above treatment results in the removal of $Mg^{2+}$-GDP from Rab5 due to the EDTA effect. Then, to stabilize Rab5 in the GTP conformation and remove EDTA, beads were washed with 40 ml nucleotide stabilization (NS) buffer containing 20 mM Hepes 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10 $\mu$M GTP$\gamma$S, pH 7.5, and further incubated with 40 ml NS buffer in the presence of 1 mM GTP$\gamma$S for 20 min at room temperature under rotation. The use of excess of $Mg^{2+}$ at this step stabilizes Rab5 in the GTP-active form. Since small GTPases exert intrinsic GTPase activity, the use of GTP$\gamma$S as a nonhydrolyzable analog of GTP is necessary. For consistency, the GST–Rab5:GDP column was prepared exactly as above with the difference that the NE and NS buffers contained GDP instead of GTP$\gamma$S.

### Bovine Brain Cytosol Preparation

Fourteen bovine brains were peeled and homogenized with 2.05 liters of buffer containing 20 mM Hepes, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5, using a blender homogenizer. The homogenate was centrifuged at 4200*g* at 4°C for 50 min. The resulting postnuclear supernatant was further centrifuged at 100,000*g* for 60 min at 4°C. The high-speed supernatant (1.5 liters) was dialyzed against NS buffer in the absence of nucleotide. Dialysis is necessary to remove endogenous nucleotides that interfere with the desired nucleotide state of Rab5. Dialyzed cytosol was cleared at 100,000*g* for 60 min at 4°C to remove any aggregated material resulting from the dialysis step.

### Purification of Rab5-Interacting Proteins

GST–Rab5:GTP$\gamma$S or GST–Rab5:GDP attached to glutathione–Sepharose 4B beads (prepared as described above) were incubated with bovine brain cytosol for 120 min at 4°C in the presence of 100 $\mu$M GTP$\gamma$S. Then beads were washed on a column with 200 ml NS buffer containing 10 $\mu$M GTP$\gamma$S, 200 ml NS buffer containing 250 mM NaCl final concentration and 10 $\mu$M GTP$\gamma$S, and 20 ml 20 mM Hepes, 250 mM NaCl, 1 mM DTT, pH 7.5. Bound proteins were eluted with 30 ml buffer containing 20 mM Hepes, 1.5 M NaCl, 20 mM EDTA, 1 mM DTT, 5 mM GDP, pH 7.5, and incubated with the beads for 20 min at room temperature under rotation. EDTA was used at this step to remove $Mg^{2+}$-nucleotide from Rab5, thereby releasing the effectors from the column.

The GST–Rab5:GDP column eluate was prepared as for the corresponding GTP$\gamma$S column with the following change: All buffers contained GDP instead of GTP$\gamma$S except for the elution buffer which contained GTP$\gamma$S (1 mM) instead of GDP. In the case of the GST column there was no nucleotide in the buffers.

Rab5-interacting proteins were analyzed by standard SDS–PAGE and Western blotting analysis.

### Preparation of Sample for Functional Assays

The eluate of the Rab5 affinity column contains GST–Rab5 (which leaks from the matrix) and high concentrations of salt, GDP, and EDTA which may interfere with functional tests. To remove these reagents, the eluate was reincubated with glutathione–Sepharose beads, followed by desalting columns. Prior to reincubation with the glutathione–Sepharose beads, the matrix was equilibrated with 10 ml PBS followed by a 1-h incubation with 10 mg/ml bovine serum albumin (BSA) in PBS. A final wash with 15 ml PBS removed unbound BSA. The eluate was incubated at 4°C for 45 min with 1.5 ml of the pretreated glutathione–Sepharose beads. This procedure is repeated twice to ensure complete removal of Rab5–GST. During removal of GST–Rab5

from the protein mixture, the presence of EDTA and GDP is necessary to prevent the reassociation of the proteins with GST–Rab5. Salt, EDTA, and GDP were subsequently removed using PD10 desalting columns (Pharmacia) at 4°C, preequilibrated in a buffer containing 20 mM Hepes, 150 mM NaCl, 1 mM DTT, pH 7.5. The desalted sample containing the Rab5-interacting proteins can now be used in functional tests.

### Fractionation of Rab5 Effectors

Since the eluate of the affinity column contained many Rab5 effectors, we added a further fractionation step. Further fractionation allowed the separation of proteins migrating at the same molecular mass that were impossible to resolve using standard gel electrophoresis. We found this separation also useful for the functional characterization of the effector(s) present in the eluate. All subsequent steps were performed at 4°C. The desalted eluate was diluted three times with 20 mM Hepes, 1 mM DTT, pH 7.5, resulting in a final volume of 135 ml. The diluted sample was loaded on a 1-ml Mono Q FPLC column (Pharmacia) at a flow rate of 0.5 ml/min. Bound proteins were eluted with 20 mM Hepes, 1 M NaCl, 1 mM DTT, pH 7.5, at a flow rate of 0.1 ml/min. Fractions of 0.15 ml were collected and those containing high protein concentration were pooled (maximum total volume, 1 ml). This step concentrates the sample for subsequent separation on a gel filtration column. Fractionation of the effectors was performed on a 24-ml Superose 6 FPLC gel filtration column (Pharmacia) at a flow rate of 0.12 ml/min. Fractions of 0.4 ml were collected, aliquoted, and frozen at −80°C.

## RESULTS

### Establishment of the Affinity Chromatography Procedure

The basic strategy consists of loading the fusion protein with either GDP or the nonhydrolyzable analog GTPγS, binding cytosolic factors to the immobilized protein, and specifically eluting the bound molecules by removing the nucleotide. This implies that only proteins binding to Rab5 in a nucleotide-dependent manner should be recovered in the eluate. This approach was developed by testing two different epitope-tagged Rab5 proteins for interaction with cytosolic proteins. We first used a fusion protein between Rab5 and MBP expressed in bacterial cells. The recombinant protein was purified and coupled by crosslinking to affi-gel 15 beads (Bio-Rad) using the manufacturer's protocol. After nucleotide exchange (to GDP or GTPγS), beads were incubated in the presence of cytosol and bound proteins were eluted with SDS sample buffer and analyzed by SDS–PAGE (Fig. 1A). A comparison between the eluate from the GDP-bound Rab5 column and that from the GTPγS-bound Rab5 column indicated a large number of nonspecific binding for both forms of Rab5 over a wide range of molecular masses. How-

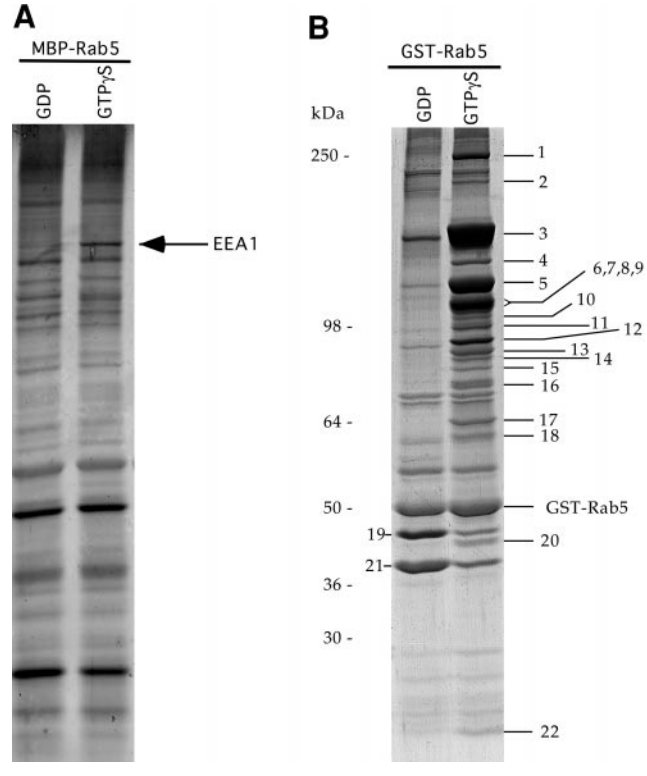


**FIG. 1.** Rab5 affinity chromatography reveals 22 Rab5-interacting proteins. (A) MBP–Rab5 was bacterially expressed and coupled to Affi-Gel 15 beads. On nucleotide exchange, two populations of beads (GDP and GTPγS form of Rab5) were incubated with bovine brain cytosol. Bound proteins were eluted with SDS sample buffer and loaded on SDS–PAGE and proteins were revealed by silver staining. (B) GST–Rab5 was expressed in bacteria and bound to glutathione–Sepharose beads. Following purification, beads were washed, nucleotide was exchanged, and the two populations of beads (GDP and GTPγS forms) were incubated with bovine brain cytosol. Bound proteins were eluted, loaded on SDS–PAGE, and silver stained. Molecular mass standards (kDa) are shown on the left. Numbers on the right- and left-hand sides of the gel represent distinct proteins bound to the Rab5 affinity column.

ever, the presence of a 170-kDa protein specifically interacting with the GTPγS form of Rab5 was evident. We identified this protein by Western blotting and mass spectroscopy as early endosome autoantigen 1 (EEA1) and demonstrated it to be a Rab5 effector necessary for endosome fusion (28). Independent work has also shown that EEA1 is required for fusion between early endosomes (27).

MBP–Rab5-based affinity chromatography has two major disadvantages. First, the efficiency of purification of the Rab5 effectors EEA1 and Rabaptin-5 is low (Fig. 2A). Second, analysis of the elution profile reveals a high background of nonspecific binding proteins as defined by the large number of bands interacting with the column matrix in a nucleotide-independent manner. In an attempt to improve this system, we used the GST–Rab5 fusion protein as a ligand for the affinity chromatography. This fusion protein has one main advantage compared with MBP–Rab5. Instead of crosslinking the protein to the matrix (which produces random coupling) the fusion protein is bound to glutathione beads (25, 26). Given that the tag specifies the orientation of the hybrid molecule on the bead, Rab5 may be better exposed and interact more efficiently with its cytosolic partners. Moreover, the GST tag may function as an arm that maintains Rab5 at distance from the bead, thereby avoiding steric hindrance problems. Finally, the elution of bound proteins was performed by switching the nucleotide from GTPγS to GDP rather than by using SDS sample buffer, thereby allowing the specific elution of proteins interacting with the GTP-bound form of Rab5. These changes significantly improved the results (Fig. 1B). First, nonspecific interactions (proteins that show no specificity for either the GDP or the GTPγS column) were reduced significantly compared with the MBP-Rab5 column (Fig. 1B). Second, more than 20 proteins were efficiently purified by the GTPγS form of Rab5 and two proteins were eluted from the GST–Rab5:GDP column.

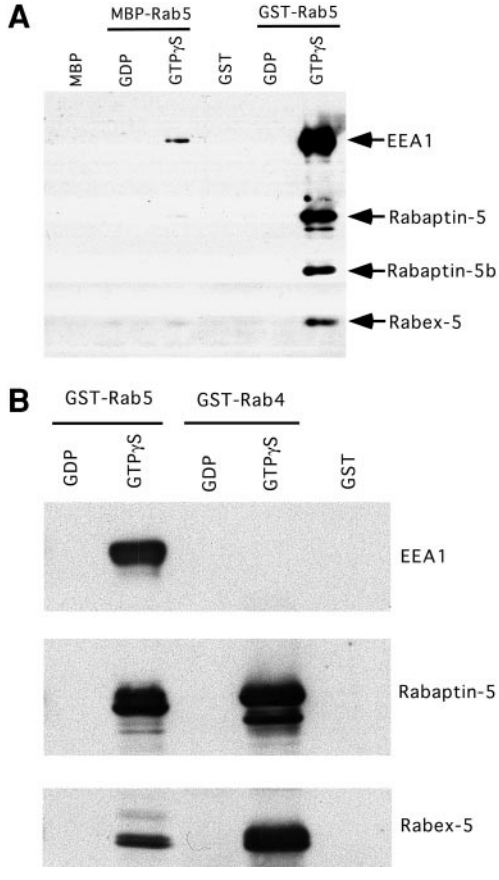


**FIG. 2.** Rab5 affinity chromatography binds the known Rab5 effectors. (A) MBP, MBP–Rab5 (GDP and GTPγS), GST, and GST–Rab5 (GDP and GTPγS) columns prepared as above were incubated with bovine brain cytosol. Eluted proteins were loaded on SDS–PAGE, transferred to nitrocellulose, and probed with anti-EEA1, -Rabaptin-5, -Rabaptin-5β, and -Rabex-5 polyclonal antibodies. Arrows demonstrate the positions of these proteins on the filter. (B) Eluates from GST–Rab5 (GDP and GTPγS), GST–Rab4 (GDP and GTPγS), and GST columns were treated as above. Western blotting was performed with anti-EEA1, -Rabaptin-5, and -Rabex-5 polyclonal antibodies. The positions of these proteins are indicated on the right-hand side of the gel.

### Monitoring Specificity

A number of observations suggest that the purified proteins interact specifically with Rab5. First, the GST–Rab5:GTPγS-interacting proteins do not bind the GDP form of Rab5 and vice versa, indicating that the interaction with cytosolic proteins is conformation-specific. Second, we performed similar binding experiments using GST–Rab4 and GST as controls and obtained a protein pattern different from that of GST–Rab5 (data not shown). These observations indicate that Rab proteins interact with a specific set of effectors probably to perform diverse functions. Third, the previously established Rab5 effectors are efficiently recruited on the affinity column. In fact, EEA1, Rabaptin-5/Rabex5, and Rabaptin-5β/Rabex-5 complex were detected in the GST–Rab5:GTPγS column eluate and not in the GDP form of Rab5 or GST control column (Fig. 2A). In comparison, the MBP–Rab5:GTPγS column showed much lower efficiency and only minor amounts of the Rabaptin-5

complexes could be detected by Western blot analysis. The specificity of these interactions is underscored by the observation that although the Rabaptin-5/Rabex-5 complex binds to both Rab5 and Rab4:GTP (Fig. 2B) (29), EEA1 binds only Rab5:GTP and not Rab4:GTP (Fig. 2B) as expected (28). Fourth, abundant cytosolic proteins do not interact unspecifically with the GTP-bound form of Rab5. We analyzed three cytosolic proteins ($\alpha$-actin, tuberin, XMAP215) which could be efficiently detected in cytosol by immunoblot labeling (Fig. 3). None of these proteins were recruited on the Rab5 column except for small amounts of $\alpha$-actin that bound in a nucleotide-independent manner. Fifth, compelling evidence for specificity was provided by functional studies that showed that the mixture of the Rab5 effectors could substitute for cytosol in an assay which measures homotypic early endosome fusion *in vitro* (30).

Given the complexity of the column eluate it is likely that the purified proteins do not simultaneously interact with the same Rab5 molecule. The multiplicity of effectors suggests that the interaction between these molecules and Rab5 must be subjected to spatial and temporal regulation *in vivo.* However, not all the proteins detected are likely to interact directly with Rab5. For example, Rabex-5 does not stably interact directly with Rab5:GTP, since it functions as an exchange factor for Rab5 (22). However, since this protein is in a stable complex with Rabaptin-5 (22), depletion of Rabaptin-5 from cytosol also abolished the binding of Rabex-5 to the column (Fig. 4). In contrast, depletion of Rabaptin-5 did not affect binding of EEA1 and vice versa.

### Fractionation of the Rab5 Column Eluate

To understand the specific role of each of the Rab5-interacting proteins, we performed additional fractionation of the eluate. This procedure consisted of the following steps. First, GST–Rab5 that leaked from the column was removed by incubation with fresh beads, followed by desalting and concentration on a Mono Q column. These steps did not significantly affect the overall protein composition of the eluate (Fig. 5A). Subsequently, the concentrated sample was fractionated on a Superose 6 gel filtration column (Figs. 5B, 5C). This separation revealed a very distinct property of EEA1. This protein eluted immediately after the void volume of the column. Such an elu-

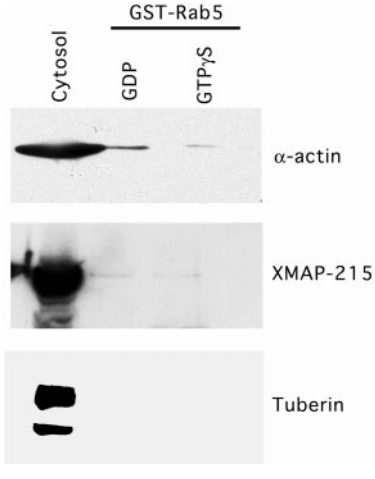


**FIG. 3.** Abundant cytosolic proteins show no preference for binding the GDP or GTP$\gamma$S form of Rab5. Cytosol and eluates from the Rab5–GST–GDP or -GTP$\gamma$S columns were loaded on SDS–PAGE, transferred to a nitrocellulose filter, and Western blotted using antibodies against $\alpha$-actin, XMAP-215, and tuberin. The positions of these proteins are marked on the right-hand side of the blots.

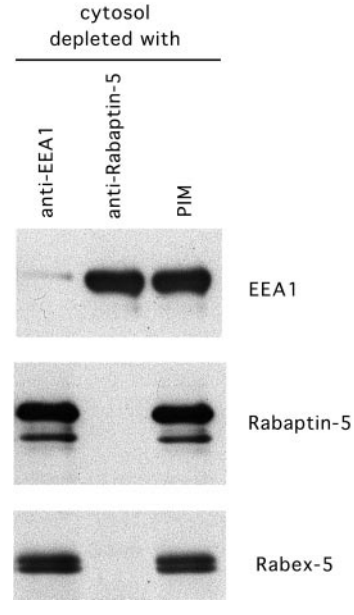


**FIG. 4.** Rabex5 binds Rab5–GTP$\gamma$S indirectly. Bovine brain cytosol was depleted with anti-EEA1, anti-Rabaptin-5, or control antibodies, and the treated cytosol was incubated with beads pre-loaded with a GST–Rab5:GTP$\gamma$S. Bound proteins were eluted and analyzed by SDS–PAGE followed by Western blotting using anti-EEA1, -Rabaptin-5, and -Rabex-5 antibodies. The positions of these proteins are shown on the right-hand sides of the blots.

tion profile is in agreement with a recent study showing that EEA1 in its native form exists as a dimer in a putative extended rodlike structure (31). Such a conformation would explain the chromatographic behavior of EEA1 which allowed the single-step isolation of this protein. Most importantly, this method resulted in a biologically active preparation of EEA1 as judged by its ability to support early endosome docking and fusion (30). We are in the process of functionally analyzing the effect of the different fractions in endosome membrane docking, fusion, and interactions with the cytoskeleton. This approach combined with the systematic identification of the polypeptides by

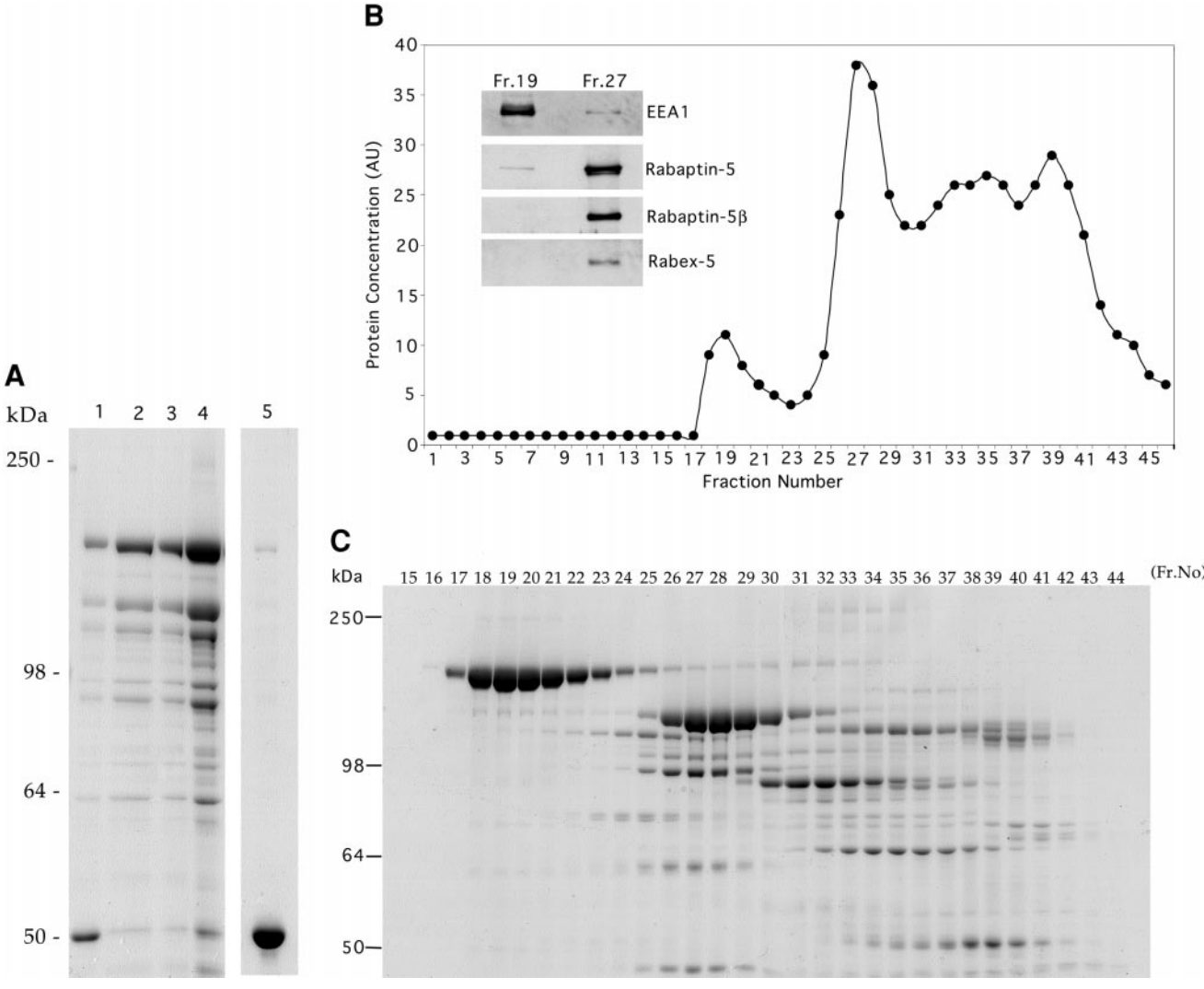


**FIG. 5.** Further purification of the Rab5 affinity column eluate. (A) SDS–PAGE analysis followed by Coomassie blue staining of samples taken from all steps of purification. GST–Rab5:GTPγS affinity column eluate (lane 1) was incubated twice with glutathione–Sepharose beads and the unbound material (lane 2) was desalted on PD10 columns. Desalted sample (lane 3) was loaded on a Mono Q FPLC column and step eluted with 1 M NaCl. Eluted sample (lane 4) was used for further purification on the Superose 6 column. GST–Rab5 depleted from the eluate is shown in lane 5. (B) Protein elution profile of Rab5-interacting proteins on a Superose 6 gel filtration column. Protein concentration is expressed in arbitary units. *Inset:* Localization of the known Rab5 effectors on the gel filtration fractions was tested by Western blotting on fractions 19 and 27. Nitrocellulose filters were probed using anti-EEA1, -Rabaptin-5, -Rabaptin-5$\beta$, and -Rabex-5 polyclonal antibodies. (C) SDS–PAGE analysis and Coomassie blue staining of the Superose 6 fractions. Fraction number is shown at the top of the gel, and molecular mass standards are depicted on the left-hand side. (C) is reprinted by permission from *Nature* (30), copyright (1999), Macmillan Magazines Ltd.

mass spectrometry analysis should lead to a precise molecular definition of the endocytic membrane transport machinery directed by the small GTPase Rab5.

## CONCLUDING REMARKS

Here we have described the optimization of a method that allows the identification and purification of Rab effectors. For the first time, a single method resulted in the identification and purification of a surprisingly large number of Rab effectors. This method can be applied to other members of the Ras superfamily of small GTPases (i.e., Ras, Rho, Arf). Given the complexity of Rab proteins in mammalian cells, this method is likely to provide a large number of novel important factors of the eukaryotic membrane transport and organelle function machinery.

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