[14] Purification of EEA1 from Bovine Brain Cytosol Using Rab5 Affinity Chromatography and Activity Assays

By SAVVAS CHRISTOFORIDIS and MARINO ZERIAL

Introduction

Vesicle docking and fusion as well as organelle dynamics are processes that are regulated by Rab proteins[1–8] via interactions with effector molecules. Therefore, understanding the molecular mechanisms underlying intracellular transport requires the identification and characterization of Rab effectors.

Transport through the early endocytic pathway is regulated by the small GTPase Rab5.[6] Work over many years has established multiple functions for Rab5 within the early endocytic route, including distinct roles in vesicle budding from the plasma membrane, motility along microtubules, and vesicle docking and fusion.[2,6,9,10] Consistent with this idea, we have shown that at least 22 cytosolic proteins can bind to the active form of Rab5.[11] Among these proteins we found, the previously characterized Rabaptin-5/Rabex-5 complex (see related chapter in this issue[11a]) and identified EEA1 as a new Rab5 effector.[12] EEA1 was initially described as an autoantigen in

[1] A. Salminen and P. J. Novick, *Cell* **49,** 527 (1987).
[2] C. Bucci, R. G. Parton, I. H. Mather, H. Stunnenberg, K. Simons, B. Hoflack, and M. Zerial, *Cell* **70,** 715 (1992).
[3] S. Ferro-Novick and P. Novick, *Annu. Rev. Cell Biol.* **9,** 575 (1993).
[4] M. Sogaard, K. Tani, R. R. Ye, S. Geromanos, P. Tempst, T. Kirchhausen, J. E. Rothman, and T. Sollner, *Cell* **78,** 937 (1994).
[5] V. Rybin, O. Ullrich, M. Rubino, K. Alexandrov, I. Simon, M. C. Seabra, R. Goody, and M. Zerial, *Nature* **383,** 266 (1996).
[6] P. Novick and M. Zerial, *Curr. Opin. Cell Biol.* **9,** 496 (1997).
[7] A. Mayer and W. Wickner, *J. Cell Biol.* **136,** 307 (1997).
[8] X. Cao, N. Ballew, and C. Barlowe, *EMBO J.* **17,** 2156 (1998).
[9] H. McLauchlan, J. Newell, N. Morrice, A. Osborne, M. West, and E. Smythe, *Curr. Biol.* **8,** 34 (1998).
[10] E. Nielsen, F. Severin, J. M. Backer, A. A. Hyman, and M. Zerial, *Nat. Cell Biol.* **1,** 376 (1999).
[11] S. Christoforidis, H. M. McBride, R. D. Burgoyne, and M. Zerial, *Nature* **397,** 621 (1999).
[11a] R. Lippe, H. Horiuchi, A. Runge, and M. Zerial, *Methods Enzymol.* **329,** [15], (2001) (this volume).
[12] A. Simonsen, R. Lippé, S. Christoforidis, J.-M. Gaullier, A. Brech, J. Callaghan, B.-H. Toh, C. Murphy, M. Zerial, and H. Stenmark, *Nature* **394,** 494 (1998).

0076-6879/00 $35.00

lupus erythematosus associated with early endosomes.[13] This protein was found to be required for fusion between early endosomes,[12,14] and shown to be an essential component of the endosome membrane docking machinery.[11] Besides interacting with Rab5, EEA1 also binds to phosphatidylinositol 3-phosphate (PI3P) via a conserved FYVE finger domain.[15–19] This finding provided a molecular explanation for the previously observed requirement for phosphatidylinositol-3-OH kinase (PI3K) in endosome fusion.[20–22]

Recently, we identified two PI3 kinases among the Rab5 interacting proteins, p85α/p110β and hVPS34, as new Rab5 effectors.[23] This finding implies that Rab5 not only interacts directly with EEA1 but also regulates the local production of PI3P, thereby creating a second binding site for EEA1 on the endosomal membrane. Therefore, on activation of Rab5, the combinatorial interactions between EEA1, Rab5, and PI3P would ensure the specific recruitment of the effector to the early endosome.

Here we describe the experimental system we developed to biochemically purify and characterize native EEA1 from bovine brain cytosol. This method is based on a combination of conventional biochemical techniques, and primarily on the use of affinity chromatography. Affinity chromatography has been used in the past and led to the identification of three RhoA effectors.[24,25] We have improved this method by optimizing a number of parameters such as increasing the density of Rab5 on the beads, the yield of nucleotide exchange, and the specificity of the elution step. This approach

[13] F. Mu, J. M. Callaghan, O. Steele-Mortimer, H. Stenmark, R. G. Parton, P. L. Campbell, J. McCluskey, J. P. Yeo, E. P. C. Tock, and B. H. Toh, *J. Biol. Chem.* **270,** 13503 (1995).
[14] I. G. Mills, A. T. Jones, and M. J. Clague, *Curr. Biol.* **8,** 881 (1998).
[15] V. J. Patki, W. S. Lane, B. H. Toh, H. S. Shpetner, and S. Corvera, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 7326 (1997).
[16] J.-M. Gaullier, A. Simonsen, A. D'Arrigo, B. Bremnes, H. Stenmark, and R. Aasland, *Nature* **394,** 432 (1998).
[17] V. Patki, D. C. Lawe, S. Corvera, J. V. Virbasius, and A. Chawla, *Nature* **394,** 433 (1998).
[18] C. G. Burd and S. D. Emr, *Mol. Cell* **2,** 157 (1998).
[19] C. Wiedemann and S. Cockcroft, *Nature* **394,** 426 (1998).
[20] G. Li, C. D'Souza-Schorey, M. A. Barbieri, R. L. Roberts, A. Klippel, L. T. Williams, and P. D. Stahl, *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10207 (1995).
[21] A. T. Jones and M. Clague, *Biochem. J.* **311,** 31 (1995).
[22] D. J. Spiro, W. Boll, T. Kirchhausen, and M. Wessling-Resnick, *Mol. Biol. Cell* **7,** 355 (1996).
[23] S. Christoforidis, M. Miaczynska, K. Ashman, M. Wilm, L. Zhao, S. C. Yip, M. D. Waterfield, J. M. Backer, and M. Zerial, *Nat. Cell Biol.* **1,** 249 (1999).
[24] K. Amano, H. Mukai, Y. Ono, K. Chihara, T. Matsui, Y. Hamajima, K. Okawa, A. Iwamatsu, and K. Kaibuchi, *Science* **271,** 648 (1996).
[25] T. Matsui, M. Amano, T. Yamamoto, K. Chichara, M. Nakafuku, M. Ito, T. Nakano, K. Okawa, A. Iwamatsu, and K. Kaibuchi, *EMBO J.* **15,** 2208 (1996).

has the advantage of allowing a comprehensive analysis of the Rab5 effectors in addition to EEA1. Therefore, we have developed a strategy that will allow us to better understand the complexity and dynamics of Rab5-regulated transport within the endocytic pathway.

Purification of Native EEA1 from Bovine Brain

EEA1 was purified from cytosol derived from bovine brain, and the entire procedure involves four principal steps: (1) preparation of bovine brain cytosol, (2) preparation of a Rab5 affinity chromatography column, (3) isolation of Rab5 effectors via affinity chromatography, and (4) purification of EEA1 using ion-exchange and size-exclusion chromatography. A flowchart of the entire purification procedure is shown in Fig. 1.

Preparation of Bovine Brain Cytosol

Solutions

Wash buffer: Phosphate-buffered saline (PBS) consisting of 2.7 m*M* KCl, 1.8 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, 137 m*M* NaCl, pH 7.2

Homogenization buffer (HB): 20 m*M* HEPES, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), pH 7.5

Procedure. Fourteen fresh bovine brains are put on ice and processed immediately. All steps of the purification are performed in the cold room, unless otherwise stated. First, meninges and cerebellum are removed and discarded and the remaining tissue washed twice with ice-cold PBS. Protease inhibitors are added freshly to ice-cold homogenization buffer (2.05 liters) in the following concentrations: 6 μg/ml chymostatin, 0.5 μg/ml leupeptin, 10 μg/ml antipain hydrochloride, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin, 10 μg/ml 4-Amidinophenylmethane sulfonyl fluoride (APMSF) (Sigma, St. Louis, MO). 220 ml of homogenization buffer (containing the protease inhibitors) is added to each 1.5 brain and homogenized in a Waring blender (Bender + Hobein, Zurich, Switzerland) on the highest speed for 50 sec. The total homogenate is centrifuged in a GS3 rotor (Sorvall) at 4200*g* at 4° for 50 min. The resulting postnuclear supernatant (PNS) is further centrifuged in a Ti 45 rotor (Beckman ultracentrifuge) at 100,000*g*, 4°, for 1 hr. The 100,000*g* supernatant (cytosol, 1.5 liter) is dialyzed twice against 50 liters (each time) of homogenization buffer (without protease inhibitors) to remove endogenous nucleotides that would interfere with the desired nucleotide state of Rab5 at later steps. The dialyzed cytosol is aliquoted in 50-ml Falcon tubes and snap-frozen in liquid nitrogen for storage at −80°. Potential aggregates resulting from dialysis and snap-freezing are removed by preclearing the thawed cytosol at 100,000*g* for 60 min at 4°

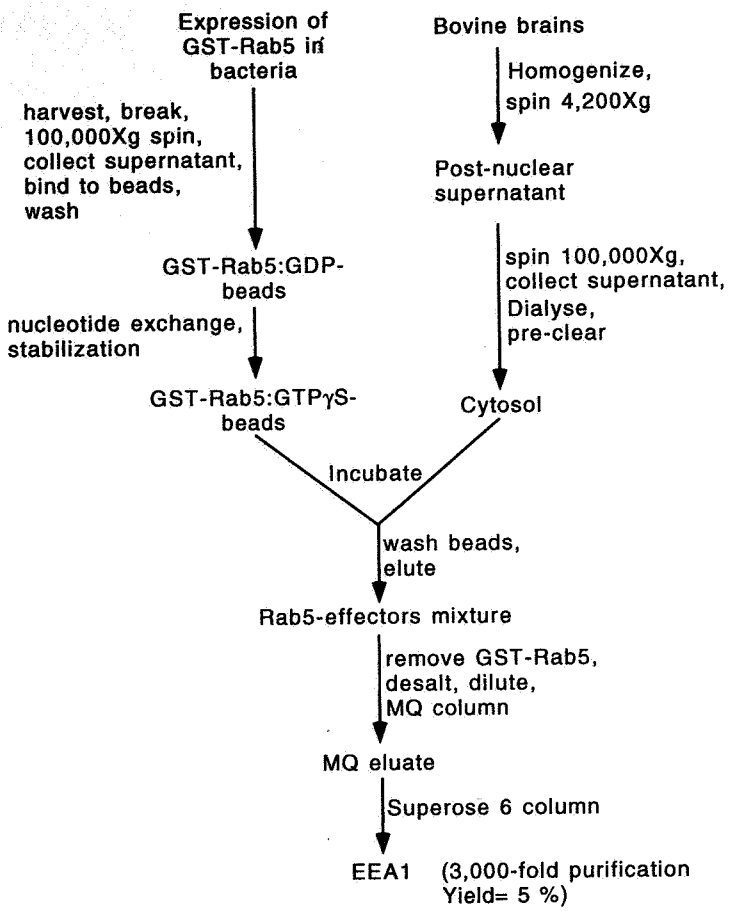

FIG. 1. Schematic representation of EEA1 isolation from bovine brain cytosol. Yield and purification fold are indicated in the final preparation of EEA1.

prior to use. This method typically results in a preparation of 1.5 liter cytosol at a protein concentration of 14 mg/ml.

Preparation of Rab5 Affinity Chromatography Column

Solutions

Lysis buffer: PBS, 5 m*M* 2-mercaptoethanol, 5 m*M* $MgCl_2$, 200 μM GDP, 5 μg/ml DNase, 5 μg/ml RNase, 6 μg/ml chymostatin, 0.5 μg/ml leupeptin, 10 μg/ml antipain hydrochloride, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin, 10 μg/ml APMSF

Nucleotide exchange buffer (NE): 20 m*M* HEPES, 100 m*M* NaCl, 10 m*M* EDTA, 5 m*M* $MgCl_2$, 1 m*M* DTT, pH 7.5
Nucleotide stabilization buffer (NS): 20 m*M* HEPES, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, pH 7.5

Procedure. Rab5 cDNA, tagged at the N terminus with GST in the pGEX-5X3 vector (Pharmacia, Piscataway, NJ), is transformed into DH5α bacteria cells. An overnight culture is grown in Luria–Bertani (LB) medium containing 100 μg/ml ampicillin. 150 flasks, each containing 0.8 liter LB + ampicillin (100 μg/ml), are inoculated with 20 ml of overnight culture and grown to an OD_{600} of 0.7–0.9 at 37°. The cultures are induced with 1 m*M* isopropylthiogalactoside (IPTG) (final) at 37° for 3 hr. Cells are harvested by centrifugation at 6000 rpm, for 15 min, 4°, in a Sorvall Avanti J-20 centrifuge, in a JLA 8100 rotor. The supernatant is discarded and the bacterial pellet resuspended in cold lysis buffer in a total volume of 2.5 liters, snap-frozen in liquid nitrogen, and kept at −20° until use.

Frozen bacteria are thawed and lysed using a French press at 800–1000 psi. The lysate is centrifuged at 33,000 rpm in a Ti 45 rotor, for 1 hr at 4°, and the supernatant incubated with 20 ml glutathione Sepharose beads (Pharmacia) (prewashed with PBS, 5 m*M* 2-mercaptoethanol) for 2 hr under rotation at 4°. Then the beads are pelleted at 500*g* for 15 min, loaded on an empty column, and washed with 200 ml PBS, 5 m*M* 2-mercaptoethanol, 5 m*M* $MgCl_2$, and 200 μ*M* GDP. This preparation yields 800 mg of glutathione *S*-transferase (GST)–Rab5 (Fig. 2C).

The procedure described above results in the production of GST–Rab5 : GDP form (inactive) due to the high intrinsic rate of GTP hydrolysis by Rab5.[5] To obtain the active (GTP) from of Rab5 on the beads, we developed a two-step reaction, first a nucleotide exchange (NE) reaction to release the bound nucleotide, followed by the stable loading of empty Rab5 with the desired nucleotide, such as GTPγS, a slowly hydrolyzble analog of GTP. The nucleotide exchange reaction is based on the use of EDTA to strip Mg^{2+} ions and exchange GDP for GTPγS on Rab5. Following the nucleotide exchange, Rab5 is stabilized in the active form in the presence of excess GTPγS and magnesium in the absence of EDTA. Nucleotide exchange is performed by first dividing the beads (20 ml) containing GST–Rab5 in 20 small empty columns (Bio-Rad, Hercules, CA). All volumes for incubations and washes described below refer to each column. The columns are washed with 5 ml NE buffer containing 10 μ*M* GTPγS and then closed and incubated with 2 ml NE buffer containing 1.5 m*M* GTPγS, for 30 min, at room temperature under very slow rotation. Then the columns are set up on racks, the solution drained out, and the beads

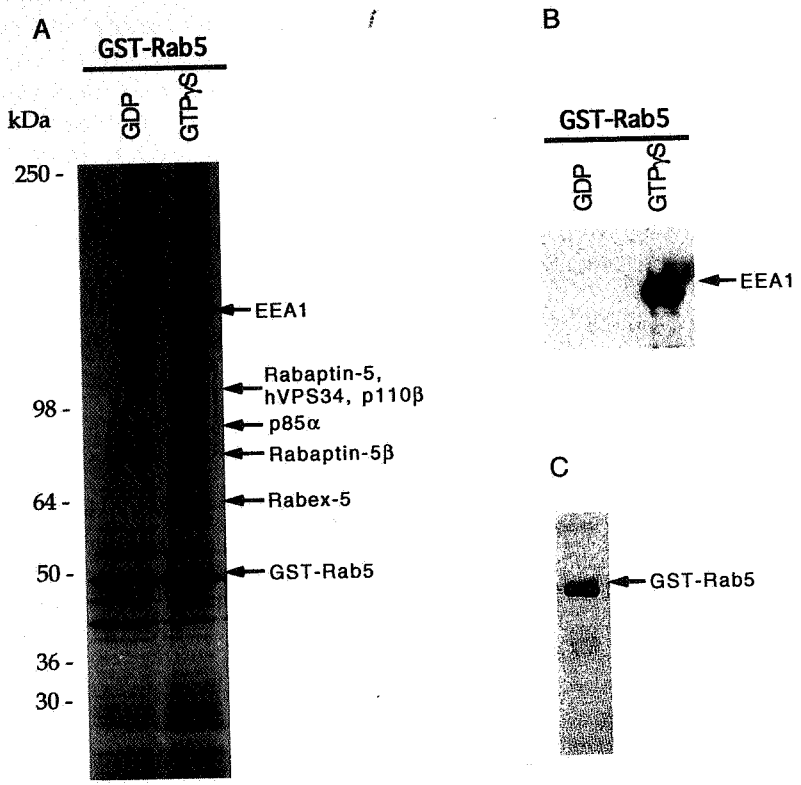

FIG. 2. Rab5 affinity chromatography reveals 22 Rab5 interacting proteins, and in this mixture of proteins EEA1 is the most abundant. (A) GST–Rab5 was expressed in bacteria, bound to glutathione Sepharose beads (see part C) and the corresponding nucleotide form was prepared. After incubation with cytosol, proteins bound to either form of GST–Rab5 were eluted. 50 μl of the eluate was separated on SDS–PAGE and the gel was silver stained. The positions of the known (so far) Rab5 effectors are shown on the right-hand side and molecular weight standards on the left-hand side of the gel. (B) Western blotting analysis of the eluate (10 μl) obtained from the Rab5 affinity chromatography using anti-EEA1 rabbit antibodies raised against a synthetic peptide corresponding to the N terminus of EEA1. (C) GST–Rab5 was produced in bacteria and bound to glutathione Sepharose beads. 10 μl of the beads were used to elute the protein in order to test its purity by SDS–PAGE analysis (loaded 5 μg) followed by staining with Coomassie blue.

washed with 5 ml NE buffer containing 10 μM GTPγS. Subsequently, the columns are closed again and incubated as above. The whole procedure is repeated a total of 3 times, which allows for complete removal of all the GDP released from Rab5. Then, the columns are washed with 5 ml NS buffer containing 100 μM GTPγS and incubated for 20 min

with 2 ml NS buffer, 2 m*M* GTPγS, at room temperature and slow rotation (excess of Mg^{2+} at this step serves to stabilize Rab5 in the GTPγS active form). To test for the binding specificity of Rab5:GTP interacting proteins, an identically treated GST–Rab5:GDP column is prepared except that the NE and NS buffers contained GDP instead of GTPγS at the same concentrations.

Isolation of Rab5 Effectors via Affinity Chromatography

Solutions

Wash buffer A: 20 m*M* HEPES, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, pH 7.5

Wash buffer B: 20 m*M* HEPES, 250 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, pH 7.5

Wash buffer C: 20 m*M* HEPES, 250 m*M* NaCl, 1 m*M* DTT, pH 7.5

Elution buffer: 20 m*M* HEPES, 1.5 *M* NaCl, 20 m*M* EDTA, 1 m*M* DTT, pH 7.5

Procedure. Beads containing GST–Rab5:GTPγS are incubated with cytosol prepared as described above (50 ml of cytosol per 0.7 ml of beads) for 2 hr at 4° under rotation in the presence of 100 μ*M* GTPγS. Then the beads are pelleted at 500*g*, for 15 min, at 4°, and loaded back on the empty plastic columns used before. The supernatant (unbound cytosol) is divided in the 20 columns and passed through again. The columns are washed first with 10 ml NS buffer containing 10 μ*M* GTPγS, followed by a wash with 10 ml buffer B containing 10 μ*M* GTPγS, and a third wash with 2 ml buffer C. Then columns are taken at room temperature and loaded with 0.3 ml elution buffer containing 5 m*M* GDP and allowed to drain (void volume). Subsequently, the beads are incubated in the columns with 1 ml elution buffer containing 5 m*M* GDP for 20 min with occasional mixing at room temperature. Then the eluate is collected and 0.7 ml new elution buffer containing 5 m*M* GDP passed through the column and pooled with the previous eluate, to give a final volume of 1.7 ml eluate per column. EDTA used at this step in the elution buffer serves to remove Mg^{2+} nucleotide from Rab5, thereby releasing the effectors from the column. The eluate (34 ml total volume) has a protein concentration of 0.3 mg/ml, and the pattern of molecules is shown in Fig. 2A. The presence of EEA1 in this preparation is shown by Western blotting using anti-EEA1 specific antibodies (Fig. 2B). EEA1 is the most abundant protein in the mixture of Rab5 effectors (Fig. 2A).

The GST–Rab5:GDP column is prepared like the corresponding GTPγS column with the following change: all buffers contain GDP instead

of GTPγS except for the elution buffer, which contains GTPγS (1 m*M*) instead of GDP. The inactive form of Rab5 results in a different protein pattern than that of the active form (Fig. 2A), indicating the specificity of the method.

Purification of EEA1 Using Ion-Exchange and Size-Exclusion Chromatography

Solutions

Buffer D: 20 m*M* HEPES, 150 m*M* NaCl, 1 m*M* DTT, pH 7.5
Buffer E: 20 m*M* HEPES, 1 m*M* DTT, pH 7.5
Buffer F: 20 m*M* HEPES, 1 *M* NaCl, 1 m*M* DTT, pH 7.5

Procedure. The eluate from the affinity column prepared as described above contains an excess of GDP, EDTA, and NaCl as well as GST–Rab5, which leaks from the beads. These components must be removed prior to further purification of EEA1. Glutathione Sepharose beads (1.5 ml) are washed with 15 ml PBS, and blocked with 10 mg/ml BSA in PBS for 1 hr under rotation. Then the beads are washed with 15 ml PBS to remove excess BSA, and incubated with the eluate at 4° for 45 min. Beads are removed and the procedure is repeated again with 1.5 ml fresh beads to obtain the complete removal of GST–Rab5. These incubations remove leaked GST–Rab5 without significant loss of effectors (Fig. 3A), due to the presence of EDTA and GDP, which prevent the interaction between Rab5 and effectors from occurring.

To remove NaCl, EDTA, and GDP, the eluate is passed through PD10 desalting columns (Pharmacia) previously equilibrated with buffer D, at 4°. The desalted eluate (50 ml) is diluted with buffer E to a final volume of 150 ml to reduce the salt concentration enough for subsequent ion-exchange chromatography. The diluted sample is loaded on a 1-ml Mono Q fast protein liquid chromatography column (FPLC) (Pharmacia) at a flow rate of 0.5 ml/min and the column washed with 10 ml buffer E. Bound proteins are step-eluted with buffer F at a flow rate of 0.1 ml/min, 0.15-ml fractions are collected, and the protein concentration is determined. Fractions with the highest protein concentrations are pooled (fractions 9–15, Fig. 3B) to give a final volume of up to 1 ml. This sample is then injected into a size-exclusion column (Superose 6, 25-ml, FPLC column from Pharmacia) preequilibrated with buffer D. The column is run at a flow rate of 0.12 ml/min (Fig. 4A), and 0.4-ml fractions are collected and analyzed by SDS–PAGE (Fig. 4B). Fractions containing the effectors are finally snap-frozen in liquid nitrogen and stored at −80°. EEA1 is obtained in the first peak [fractions 17–20 (Fig. 4A

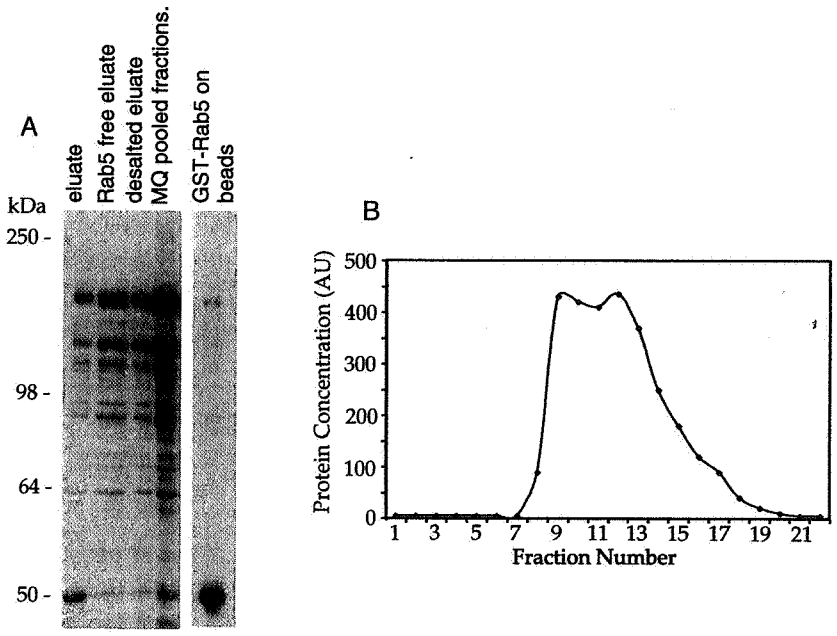

FIG. 3. Anion-exchange Mono Q (MQ) column chromatography. (A) Comparison of protein pattern by SDS–PAGE analysis and Coomassie blue staining of samples obtained from the purification procedure of EEA1 prior and after the MQ chromatography step. The following samples were used: eluate obtained from the affinity column (10 μl), eluate after the removal of GST–Rab5 (30 μl), desalted eluate (40 μl), and pooled fractions after the MQ column (10 μl). The last lane shows GST–Rab5 retained on glutathione beads after its removal from the eluate. (B) Elution profile of proteins from the MQ column chromatography. Fractions 9 to 15 contained the highest protein concentration (expressed here as arbitrary units) and were pooled.

and B)] and is well separated from the bulk of the other proteins. The peak fraction of EEA1 (fraction 18) shows a protein concentration of 0.3 mg/ml. Using Western blotting analysis as a quantitation method, and the purified EEA1 as a standard, we found that the endogenous concentration of EEA1 in cytosol is approximately 4 μg/ml. Therefore, we estimate that the above method results in approximately 3000-fold purification of EEA1.

Functional Assays of Purified EEA1

The activity of purified EEA1 was tested by two means. First we tested whether the whole purification procedure affects the ability of EEA1 to

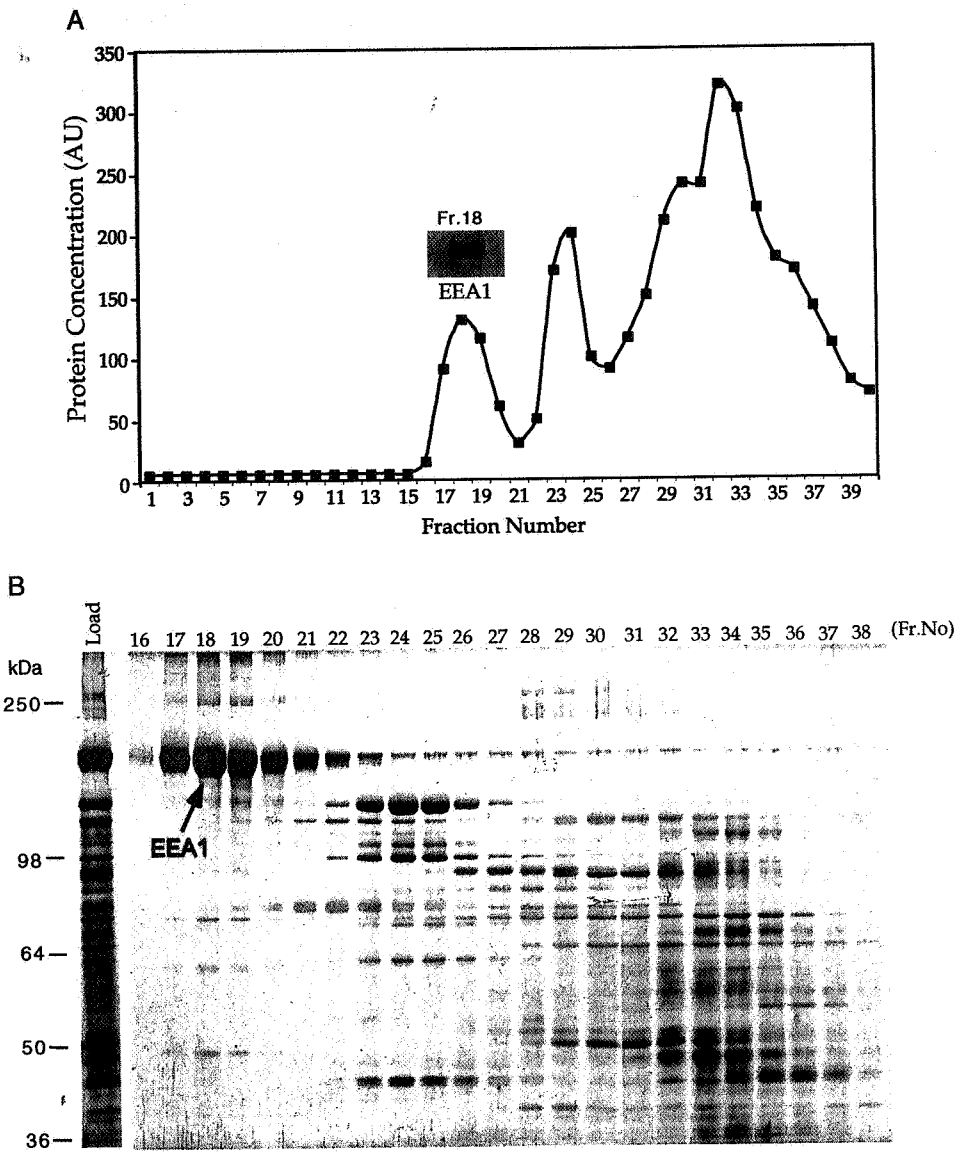

FIG. 4. Final purification step of EEA1 using size-exclusion Superose 6 column chromatography. (A) Elution profile of Rab5 effectors on a Superose 6 column. Protein concentration is expressed in arbitrary units. The peak of EEA1 (fraction 18) was identified by Western blotting (*Inset*). (B) SDS–PAGE analysis and Coomassie blue staining of the Superose 6 fractions. Fraction number is shown on the top of the gel, and molecular weight standards are depicted on the left-hand side. For comparison, 5 μl of the sample loaded on the column was run in parallel in the left-hand side of the gel. The position of EEA1 (peak in fraction 18) is shown.

interact with Rab5 using a ligand overlay assay, and second whether pure EEA1 is able to stimulate fusion between early endosomes in the absence of cytosol.

Solutions

Glutathione elution buffer: 100 m*M* Tris-HCl, 5 m*M* 2-mercaptoethanol, 15 m*M* glutathione, 5 m*M* $MgCl_2$

Blocking buffer: 50 m*M* HEPES, 5 m*M* magnesium acetate, 100 m*M* potassium acetate, 1 m*M* DTT, 1% (w/v) bovine serum albumin (BSA), 0.1% (w/v) Triton X-100, 0.1% (w/v) Tween 20, 1 μM $ZnCl_2$, pH 7.4

Binding buffer: 12.5 m*M* HEPES, 1.5 m*M* magnesium acetate, 75 m*M* potassium acetate, 1 m*M* DTT, 0.2% (w/v) BSA, 0.005% (w/v) Triton X-100, 4 m*M* *n*-octylglucoside, pH 7.4

Procedure. The ligand overlay assay is based on the renaturation of proteins immobilized on a nitrocellulose filter in order to test for potential protein–protein interactions with a soluble ligand, which can be easily detected. In this case, purified EEA1 is renatured on nitrocellulose and incubated with soluble GST–Rab5:GTPγS or GST–Rab5:GDP, followed by the detection of bound GST–Rab5 using anti-GST antibodies. Active (GTPγS) and inactive (GDP) forms of GST–Rab5 are made as described above for the preparation of the affinity column. The stable nucleotide-bound Rab5 is eluted from the beads with glutathione elution buffer with 1 m*M* of the corresponding nucleotide. Glutathione is removed by desalting on a PD-10 column preequilibrated with NS buffer containing 100 μM of the corresponding nucleotide. This procedure resulted typically in 3 mg/ml of GST–Rab5 of either form.

Purified EEA1 (Superose 6 column, fraction 18) is loaded on a SDS–PAGE gel (without prior boiling) and transferred to nitrocellulose membrane. The blot is then washed 2× for 10 min with PBS containing 0.1% Tween 20, and blocked overnight by incubation with blocking buffer (all incubations are done at 4° unless otherwise stated). Then the blots are incubated 2× for 1 hr with binding buffer, followed by incubation with 1 μg/ml GST–Rab5:GTPγS or GST–Rab5:GDP in binding buffer containing 100 μM GTPγS or GDP, and 1% BSA, for 1 hr. Subsequently, the blots are washed 5× for 5 min with binding buffer containing 10 μM of the appropriate nucleotide (all subsequent washes contained this concentration of nucleotide), and incubated with anti-GST antibody (Pharmacia) at a dilution of 1:2000 in binding buffer containing 1% BSA. The blots are washed 5× for 5 min with binding buffer and incubated with rabbit anti-goat IgG conjugated to HRP at a dilution of 1:1500 in binding buffer containing 1% BSA, followed by five 5-min washes with binding buffer,

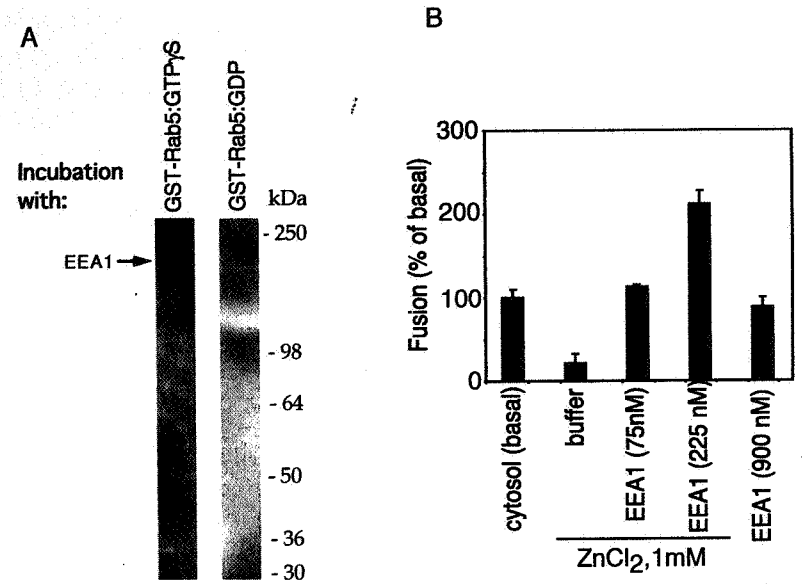

FIG. 5. Assays of purified EEA1. (A) Purified EEA1 (fraction 18, 400 ng) was loaded on a 7–17% gradient SDS–PAGE followed by transferring on a nitrocellulose filter. Then the blots were renatured, incubated with the indicated form of Rab5 (1 μg/ml), and the position of bound GST–Rab5 was identified using goat anti-GST antibodies, followed by incubation with rabbit anti-goat IgG coupled to HRP and detection by ECL. The expected molecular weight of EEA1 (on the left-hand side) and molecular weight standards (on the right-hand side) are shown in the figure. (B) Fusion between early endosomes in the presence of cytosol (basal), or buffer, or different concentrations of EEA1 in the presence or absence of $ZnCl_2$. The extent of fusion is expressed as percent of basal.

and 2 quick washes with PBS. Finally, the blots are treated with the ECL (enhanced chemiluminescence) detection system (Amersham) and exposed to autoradiography. This method shows that purified EEA1 is able to bind GST–Rab5:GTPγS but not GST–Rab5:GDP (Fig. 5A).

The activity of EEA1 is also tested in an *in vitro* early endosome fusion assay that has been described before.[26] In this case, isolated early endosomes are mixed with purified EEA1[11] and the percent of fusion is measured using an Origen analyzer (IGEN, Gaithersburg, MD). The fraction from the size-exclusion chromatography containing EEA1 (fraction 18) is able to support fusion between early endosomes in the absence of cytosol (Fig. 5B). When EEA1 is used in the absence of $ZnCl_2$, fusion is significantly reduced and requires much higher concentration of the fraction containing

[26] H. Horiuchi, R. Lippé, H. M. McBride, M. Rubino, P. Woodman, H. Stenmark, V. Rybin, M. Wilm, K. Ashman, M. Mann, and M. Zerial, *Cell* **90,** 1149 (1997).

EEA1 in order to compensate for the reduction of $ZnCl_2$. It is likely that the purification procedure (treatment with 20 m*M* EDTA during elution from the affinity column) has stripped the Zn^{2+} ions from the FYVE finger of EEA1, consistent with previous reports.[27,28] Our efforts to elute the effectors from the affinity column in the absence of EDTA using high salt concentrations and detergent proved unsuccessful.

The above two criteria used for testing the activity of EEA1 (binding to active Rab5 and stimulation of endosome fusion) show that the described purification method results in an active preparation of this Rab5 effector.

Acknowledgments

We are grateful to Harald Stenmark for the cDNA of GST–Rab5, and Heidi McBride and Marta Miaczynska for critical reading of the manuscript. S.C. is a recipient of a Max Planck fellowship. This work was supported by the Max Planck Gesellschaft, grants from the Human Frontier Science Program (RG-432/96), EU TMR (ERB-CT96-0020), and Biomed (BMH4-97-2410) (M.Z.).

[27] H. Stenmark, R. Aasland, B. H. Toh, and A. D'Arringo, *J. Biol. Chem.* **271,** 24048 (1996).
[28] S. Misra and J. H. Hurley, *Cell* **97,** 657 (1999).

[15] Expression, Purification, and Characterization of Rab5 Effector Complex, Rabaptin-5/Rabex-5

By Roger Lippé, Hisanori Horiuchi, Anja Runge, and Marino Zerial

Introduction

The small GTPase Rab5 is a molecule regulating the endocytic pathway and is an important component of the docking and fusion apparatus.[1–3]

[1] J.-P. Gorvel, P. Chavrier, M. Zerial, and J. Gruenberg, *Cell* **64,** 915 (1991).
[2] C. Bucci, R. G. Parton, I. H. Mather, H. Stunnenberg, K. Simons, B. Hoflack, and M. Zerial, *Cell* **70,** 715 (1992).
[3] G. Li, M. A. Barbieri, M. I. Colombo, and P. D. Stahl, *J. Biol. Chem.* **269,** 14631 (1994).

0076-6879/00 $35.00