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

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

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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]

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Hence, in concert with the SNARE machinery,[4–6] it regulates heterotypic fusion between clathrin-coated vesicles and early endosomes, as well as homotypic fusion between early endosomes. In addition, Rab5 has also been shown to regulate the motility of early endosomes along microtubules[7] and the formation of clathrin-coated vesicles at the plasma membrane.[8] So far, several Rab5 effectors have been documented, including EEA1,[9–11] the PI3 kinases hVPS34 and p85–p110,[12] Rabaptin-5,[13,14] Rabaptin-5β,[15] and recently a large number of other potential effectors.[11] Given the multiple roles of Rab5, the characterization of its effectors is essential.

Rabaptin-5 was the first identified Rab5 effector[13] and is essential for the Rab5 regulated docking and fusion machinery.[13,14] It reduces hydrolysis of the Rab5-bound GTP into GDP, possibly by preventing a GAP from binding to Rab5 and hence preserving Rab5 in its active conformation.[16] Interestingly, Rabaptin-5 does not support fusion of early endosomes by itself and must be bound to yet another Rab5 interacting molecule called Rabex-5 to be functional.[14] Rabex-5 is a guanine nucleotide exchange factor (GEF) that mediates the GDP-to-GTP nucleotide exchange on Rab5. Surprisingly, the effector Rabaptin-5 and the exchange factor Rabex-5 are stably associated in cytosol, which is the first identification of an effector–GEF complex for the Rab family.[14] Although the combined function of this complex is to drive the equilibrium of Rab5 toward its GTP active

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form, the exact role and mechanism of action of the complex await a more detailed characterization. It has been suggested, however, that the association of Rabaptin-5 and Rabex-5 may be functionally important to generate local clusters of active Rab5 at the docking and fusion site.[14] In addition, Rabaptin-5 specifically interacts not only with Rab5 through its carboxyl terminus, but also with Rab4 through a distinct binding domain located at the amino end of Rabaptin-5, suggesting the complex may also be important to link endocytosis to recycling from early endosomes to the plasma membrane.[17]

Following the discovery of Rabaptin-5, a related molecule, Rabaptin-5β, was subsequently identified. It shares significant sequence homology (40% homology and 60% similarity) with Rabaptin-5 and, as a result, also binds to Rabex-5.[15] Importantly, Rabaptin-5β, which is also recognized by the Rabaptin-5α antibody we use, can readily be distinguished from Rabaptin-5α on the basis of its different molecular mass (70 and 100 kDa, respectively). Thus, Rabaptin-5 and Rabaptin-5β are not found in the same complex because Rabex-5 associates exclusively with either Rabaptin protein.[14,15] Although the Rabaptin-5β complex is also involved in the homotypic fusion between early endosomes, it appears to work cooperatively with the functionally predominant Rabaptin-5 complex,[15] hence our focus on the latter complex.

To further understand the significance and function of the Rabaptin-5/Rabex-5 complex and to molecularly dissect the Rab5-mediated pathway, purified molecules are needed. Two main approaches exist, namely, the purification of native complex from cytosol and the expression and purification of complex as recombinant proteins. This chapter first details the purification of the native Rabaptin-5/Rabex-5 complex from bovine brain cytosol. It subsequently describes the recent purification of the recombinant complex from insect cells using baculovirus.

Description of Method

Purification of Native Complex

Preparation of Bovine Brain Cytosol. The purification of the native complex is performed from bovine brain cytosol (Fig. 1). For this purpose, obtain four fresh brains from the slaughterhouse and quickly put on ice to avoid protein degradation. All steps must be performed at 4°. Wash them in cold phosphate-buffered saline (PBS) to remove the blood and prepare

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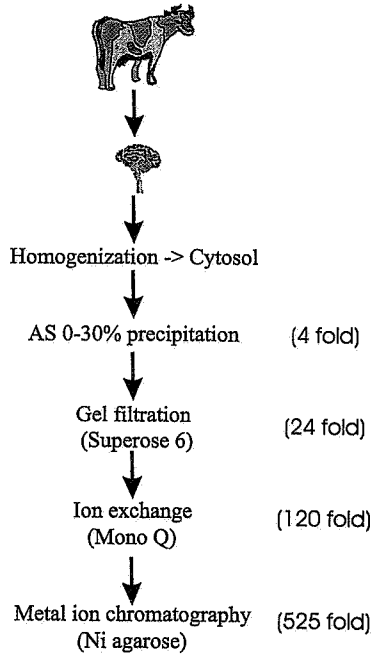

FIG. 1. Purification scheme of the native Rabaptin-5/Rabex-5 complex from bovine brain. Schematic representation of the purification protocol to obtain native Rabaptin-5 complex from bovine brain cytosol. The fold purification is indicated for each step. It is determined by Western analysis against Rabaptin-5, using the L1-46 polyclonal antibody, and by determination of the total protein content with the Bio-Rad protein assay.

the cytosol as follows. First remove the meninges, cerebellum, and remains of the spinal cord and homogenize the brains in a blender with 600 ml of ice-cold homogenization buffer [20 m*M* HEPES, pH 7.4, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), 100 m*M* NaCl, and a cocktail of protease inhibitors, including 6 μg/ml chymostatin, 0.5 μg/ml leupeptin, 10 μg/ml antipain, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin, and 10 μg/ml APMSF]. Spin the homogenate at 4200*g* for 50 min at 4°. Keep the postnuclear supernatant and centrifuge at 100,000*g* for 1 hr at 4°. Collect the cytosol and proceed immediately to purification, as this is important for the activity of the complex (see Comments).

Ammonium Sulfate Precipitation. The Rabaptin-5 complex mostly precipitates from cytosol at 30% ammonium sulfate but requires 40% ammonium sulfate for a quantitative recovery. To achieve this, place the cytosol in a large beaker at 4° atop a stirrer and slowly add 40% of ammonium sulfate (243 g per liter of cytosol). Make sure there is extensive stirring to

avoid high local concentrations of ammonium sulfate. Once all the ammonium sulfate is added, stir for another 30 min. Spin the suspension at 100,000*g* for 10 min and solubilize the pellet with a Dounce homogenizer in 50 ml of HMD (20 m*M* HEPES, pH 7.4, 5 m*M* $MgCl_2$, 1 m*M* DTT). Dialyze the sample against HMD/100 m*M* NaCl. At this point, samples can be snap-frozen in 20-ml aliquots. Two other Rab5 effectors, EEA1 and Rabaptin-5β, which are also precipitated during this step, are separated from the Rabaptin-5 complex at later stages.

Fractionation by Gel Filtration. The second purification step consists of the separation of the 0–40% ammonium sulfate fraction on a 125-ml (bed volume) preparative grade Superose 6 column (Pharmacia, Stockholm). Preequilibrate the column with filtered and degassed HMD/100 m*M* NaCl buffer. If the sample was frozen, spin one of the 20-ml aliquots at 150,000*g* for 30 min (4°) and filter the supernatant first through a 0.45 μm then a 0.22 μm filter to remove aggregates. This is enough material for two runs on the column. Load half of the sample, perform the fractionation at 0.5 ml/min in HMD/100 m*M* NaCl, and collect 2-ml fractions. Wash the column with HMD/100 m*M* NaCl until the UV reading has returned to its baseline. Repeat immediately with the second half of the sample. Meanwhile take a 15 μl aliquot of each fraction, run a 10% SDS–PAGE gel, and identify the Rabaptin-5 positive fractions by Western analysis using an anti-Rabaptin-5 antibody such as L1-46.[13] It is critical at this stage to work as fast as possible to preserve the activity of the complex. If possible, use minigels and short incubation times for the Western analysis. Alternatively, the complex can be monitored at this stage by an anti-Rabex-5 antibody, if available. As a reference point, the Rabaptin-5 complex should elute approximately 10–14 fractions before the hemoglobin peak, easily visible by eye during the fractionation. Pool the positive fractions (around six fractions of 2 ml per run). Note that this step efficiently purifies the Rabaptin-5 complex away from the Rab5 effector EEA1, but not yet from the related Rabaptin-5β.

Ion-Exchange Chromatography. In addition to further purifying the complex, the ion-exchange column serves the purpose of a concentration step as well. Pool the positive fractions from two gel filtration runs (usually 20–22 ml in total) and load all onto a 1-ml prepacked Mono Q column (Pharmacia, Stockholm) preequilibrated with HMD. Fractionate at 0.5 ml/min by first washing the column with 10 column volumes of HMD, eluting with 15–20 column volumes of a 0–500 m*M* NaCl gradient (starting buffer, HMD; final buffer, HMD/500 m*M* NaCl), and a final wash step of five column volumes of HMD/1 *M* NaCl. Collect 2-ml fractions during the initial wash step, and 0.5-ml fractions during the subsequent elution step. Identify the positive fractions by Western analysis as above. The complex elutes at 200–300 m*M* salt in a peak of about five fractions (2.5 ml).

Final Purification Step on Metal Ion Column. Purification on nickel columns is typically used for recombinant proteins tagged with a His_{6-9} tail, tags which are obviously absent in native proteins. Although Rabaptin-5 contains 16 histidine residues (1.9% of total amino acids), it has no single contiguous stretch of histidine residues. The same is similarly true for its partner protein Rabex-5 (six histidine residues scattered throughout the whole protein). Despite this, Rabaptin-5 binds to nickel-coated beads (Horiuchi and Zerial, unpublished observation, 1999). Therefore, it is possible to use such an unconventional approach to purify the native Rabaptin-5 complex.

To purify the complex, proceed as follows. Wash 300–400 μl (packed volume) of nickel NTA agarose beads (Qiagen, Hilden) twice with HMD. Incubate in batch the Mono Q Rabaptin-5 containing fractions (2–2.5 ml) with the nickel beads for 1–2 hr in two 2-ml Eppendorf tubes. Transfer the beads into a single empty 10-ml chromatographic column (Bio-Rad, Munich) and drain the excess liquid by gravity flow. Wash the beads with 10–15 ml of wash solution (HMD/10 m*M* imidazole). Do not use a higher concentration of imidazole because the native protein is eluted starting at 25 m*M* imidazole (Lippé, Runge, Horiuchi, and Zerial, unpublished observation, 1999). Note that the Rabaptin-5β is quantitatively eluted by the 10 m*M* wash. Elute the Rabaptin-5 complex with 1 ml of elution buffer (HMD/100 m*M* imidazole), omitting the first two drops to get more concentrated material. Aliquot the nickel agarose eluate in small volumes and snap-freeze in liquid nitrogen before storing at −80°.

Comments. Rabex-5 will elute in both the 10 m*M* imidazole wash and the 100 m*M* imidazole elution because it forms distinct complexes with both Rabaptin-5 and Rabaptin-5β. Therefore, it is not advisable to monitor the purification of the Rabaptin-5 complex with an antibody directed against Rabex-5. The expected yield from four bovine brains is low (1 ml of roughly 200 n*M* complex), but the material is active and in sufficient amounts for functional assays. The association of Rabaptin-5 and Rabex-5 is maintained during all of these steps, indicating that they interact with high affinity. It is therefore not necessary to follow Rabex-5 during the purification. By this procedure, the complex is not purified to homogeneity, but it is of reasonable purity (Fig. 2). In our hands, further purification of the complex is deleterious for its activity and is not recommended. The complex is highly sensitive to freeze-thawing and care should be taken to avoid such processing. In addition, the successful purification of the complex is dependent on avoiding freezing during the purification and working as quickly as possible.

Analysis of Activity of Complex. The Rabaptin-5/Rabex-5 complex is an essential component of the Rab5-mediated endocytic pathway. It has

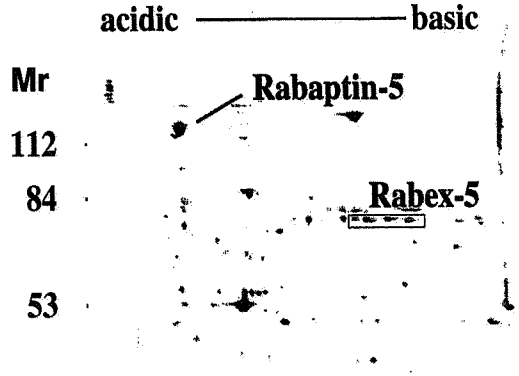

FIG. 2. Silver staining of the purified native complex. Analysis of the purified native complex by isoelectrofocusing electrophoresis (first dimension) and SDS–PAGE (second dimension). The gel was then silver stained. The positions of Rabaptin-5 and Rabex-5 are indicated, as confirmed by Western blot analysis. The molecular mass markers in the second dimension are indicated in kilodaltons.

been shown to be necessary in the *in vitro* early endosome homotypic fusion assay and to stimulate fusion when added exogenously to the fusion reaction.[14] In addition, depletion of the complex from the cytosol used in the assay significantly impairs the fusion of the endosomes. Rescue of such depleted cytosol can be achieved by the addition of exogenous Rabaptin-5 complex.[14] These observations therefore provide means to assay for the functionality of the purified complex. Finally, the nucleotide exchange activity of Rabex-5 provides an additional and complementary method for evaluating the quality of the preparation.[14]

In Vitro Fusion Assay. To evaluate the purified Rabaptin-5 complex, one can use an *in vitro* homotypic endosome fusion assay. Although the detailed description of such assay is beyond the scope of this chapter and has also been reported elsewhere,[14] the general guidelines are here provided. Briefly, the assay consists of the incubation of biotinylated transferrin-labeled early endosomes with anti-transferrin antibody-labeled early endosomes in the presence of HeLa cytosol, an ATP-regenerating system, an unlabeled holotransferrin quencher, buffer, and water in a total volume of 20 μl. Measurements of fusion scores are taken for the content mixing between the endosomes containing antibody and its antigen, using streptavidin-coated magnetic beads and an Origen analyzer. To test the Rabaptin-5 complex, simply replace 10 μl of water by 10 μl of complex (Fig. 3A).

Alternatively, the complex can be used in the fusion assay to rescue cytosol depleted of the Rabaptin-5 complex. To deplete the complex from

cytosol, wash 10 μl of protein A agarose beads twice with phosphate-buffered saline (PBS) and incubate with 10 μl of crude anti-Rabaptin-5 antibody for 2 hr on a rotator at 4° in a total volume of 1 ml in PBS. Spin the beads at low *g* force for 30–60 sec and wash them twice with cytosol buffer (50 m*M* HEPES, pH 7.4, 50 m*M* KCl, 10 m*M* EGTA, 2 m*M* $MgCl_2$). In this case, a wash means a 20-min incubation of the beads with cytosol buffer at 4° on the rotator to allow desorption of the nonspecific material from the beads. Add the beads to 100 μl of the cytosol used in the fusion assay and incubate 30 min on the rotator (4°). Sediment the beads and collect the depleted cytosol. Verify the depletion by SDS–PAGE and Western blot analysis using a Rabaptin-5 antibody. Use this cytosol in the fusion assay and rescue the inhibitory effect of the depletion with 10 μl of purified native complex (Fig. 3A).

Guanine Nucleotide Exchange Assay. Dilute recombinant GTPase (e.g., Rab5) produced in *Escherichia coli* or purified from the membrane fraction of overexpressing Sf9 insect cells[18,19] to 1 mg/ml in exchange buffer (20 m*M* Hepes/NaOH, pH 7.2, 5 m*M* $MgCl_2$, 1 m*M* DTT). Incubate 10 p*M* of the GTPase with different amounts of Rabaptin-5 complex or Rabex-5 (0–50 n*M*), together with 2 μM [^{35}S]GTPγS (20,000 cpm/pmol) for a final reaction volume of 20–25 μl in exchange buffer. Incubate the samples at 30° for different times (0–25 min). Stop the exchange reaction by adding 2 ml of ice-cold buffer (20 m*M* Tris, pH 7.4, 20 m*M* $MgCl_2$, 100 m*M* NaCl) to each sample, filter through nitrocellulose filters (2-cm-diameter BA85, Schleicher & Schuell, Keene, NH), wash the filters twice with 4 ml of the above ice-cold buffer, and dry them. Count the filter-bound radioactivity with a beta scintillation counter. Express the results as folds of the counts obtained with complex or Rabex-5 compared with those obtained without complex or Rabex-5. See Fig. 3B for an example.

Purification of Recombinant Complex

Although the purification of the Rabaptin-5 complex from bovine cytosol has been instrumental in identifing Rabex-5, further characterization of the role and significance of the complex requires the use of recombinant proteins. Furthermore, this is needed to improve the complex yields and to have it free of the contaminating proteins present in the native material. Finally, a recombinant complex would be essential to ultimately reconstitute the entire Rab5 docking and fusion apparatus *in vitro*. Although its expres-

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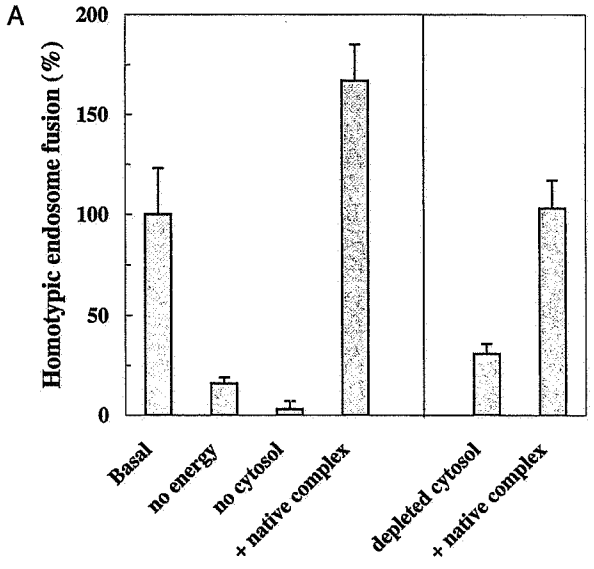

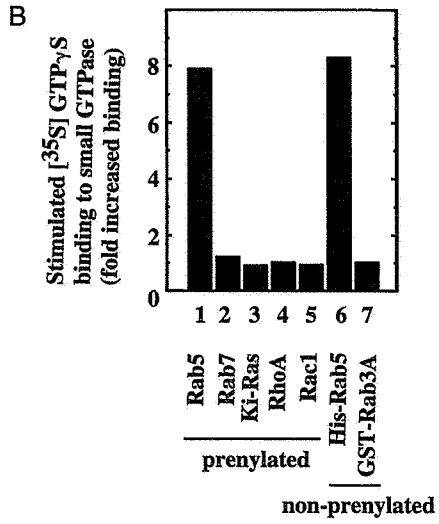

sion in bacteria is possible, expression in an insect cell system is preferable due to potential posttranslational modifications of the complex and the ease of expression of multiple subunits in insect cells. For these reasons, we have chosen to express the Rabaptin-5 complex in insect cells using baculoviruses.

Construction of Vectors. The Bac-to-Bac baculovirus expression system (Gibco, Karlsruhe) is used for these experiments. This system is particularly useful, because it is versatile and relies solely on bacteria to perform all the cloning steps and the clonal expansion of recombinant viral genomes, thereby avoiding tedious and time-consuming plaque purification steps. In addition, the blue/white selection procedure of the Bac-to-Bac system allows a quick and easy distinction between wild-type and recombinant viral genomes. The recombination efficiency, in fact a transposition event, is also much higher than in insect cells (10–25%).[20] Finally, the presence of extensive multiple cloning sites (MCS) and the strong *Autographa californica* polyhedrin promoter are an asset. Cloning of Rabaptin-5 and Rabex-5 is designed to permit their coexpression to allow the formation of complexes *in vivo* and their subsequent copurification. Although a complex of Rabaptin-5 and Rabex-5 can be formed *in vitro* from individually produced recombinant proteins, complex formation *in vivo* is a key aspect since it is significantly more efficient (Lippé, Horiuchi, and Zerial, unpublished observation, 1999). Subclone the full-length Rabaptin-5 cDNA into the MCS of the proper pFAST BAC HT donor vector (a, b, or c depending on the frame), yielding a histidine-tagged recombinant protein. In addition, subclone Rabex-5 into the MCS of the pFAST BAC1 donor vector, yielding an untagged recombinant protein.

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FIG. 3. Functional analysis of the purified native complex. (A) Analysis of the purified complex with the use of the *in vitro* homotypic early endosome assay. Basal fusion represents the fusion in presence of donor and acceptor early endosomes, cytosol, and energy regenerating system. The depleted cytosol lane consists of the above but using cytosol that has been depleted of the Rabaptin-5 complex using the L1-46 anti Rabaptin-5 antibody. For the native complex lanes, 100 n*M* of exogenous purified bovine Rabaptin-5/Rabex-5 was added to the reaction. (B) Analysis of the complex in the nucleotide exchange assay. The ability of the complex to load [^{35}S]GTPγS nucleotide onto various recombinant small GTPases, including Rab5, is shown. Prenylated GTPases (Rab5, Rab7, Ki-Ras, RhoA, and Rac1) were purified from overexpressing insect cells.[18] Nonprenylated GTPases (Rab5 and Rab3A) were produced in and purified from *Escherichia coli.* In this experiment, the data indicate the fold of stimulation of [^{35}S]GTPγS binding in 20 min at 30° in the presence of 20 n*M* Rabex-5 over the [^{35}S]GTPγS binding in the absence of Rabex-5. Although thus far specific for Rab5, a nucleotide exchange activity of Rabex-5 on GTPases other than the ones tested here cannot formally be excluded.

Generation of Recombinant Viruses and Viral Stocks. Generate the recombinant viruses separately for each construct by transforming 1 ng of the recombinant donor plasmids into the *A. californica* baculoviral genome present in DH10Bac *E. coli* cells (100 μl of bacteria) according to the manufacturer's instructions. It is critical to incubate the transformed cells 4 hr at 37° prior to plating them on selection plates [50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracycline, 100 μg/ml Bluo-Gal (Sigma, Taufkirchen), and 40 μg/ml isopropylthiogalactoside (IPTG)]. Three hundred micrograms of X-Gal per ml of culture can alternatively be use with equivalent results instead of the Bluo-Gal. Develop the blue color for 2 days, pick the resulting white recombinant colonies (inactivation of the *lacZ* gene), and replate for a second round of blue/white selection to avoid false positives. Collect the white colonies and grow them overnight in 2 ml of liquid selection media (50 μg/ml kanamycin, 7 μg/ml gentamycin, and 10 μg/ml tetracycline). Extract the viral DNA manually without the use of column plasmid kits, for example from Qiagen, because the large viral DNA will be sheared. Follow instead the instructions in the Gibco Bac-to-Bac user manual. Confirm by polymerase chain reaction (PCR) the presence of the recombinant gene using the BacA and BacB amplification primers

oligo(A) 5′ GTTTTCCCAGTCACGACGTTGTAAAACGAC3′
oligo(B) 5′ AGCGGATAACAATTTCACACAGGAAACAGC 3′

and the following PCR conditions (94° for 5 min, followed by 35 cycles of 94° for 1 min/55° for 1 min/72° for 6 min, and finally 1 cycle at 72° for 7 min).

Transfect separately by the $CaCl_2$ technique[21] 5 μl of the viral DNAs (Rabaptin-5 and Rabex-5) into one 25-cm^2 dish each of subconfluent *Spodoptera frugiperda* (Sf9) insect cells. The Sf9 cells are cultured in TNM-FH medium (Sigma, Deisenhofen), 10% fetal calf serum (FCS), and penicillin/streptomycin. No selection is necessary because only the released virus is of interest. Collect the P1 viruses 4 days later. Amplify the viruses twice by infecting Sf9 cells with a small amount of virus (for example, 100 μl of P1, then P2 into a 175-cm^2 flask of cells) to obtain large stocks of virus (P2, then P3). The precise time to harvest depends on the titer of the viruses and the culture conditions.

Protein Expression. The use of Sf9 cells is particularly suited for the expansion of baculoviruses. However, protein expression tends to be significantly better in general in High Five insect cells.[20] These latter cells are also easier to manipulate. Although it is recommended to titrate the viruses by plaque assay (plaque forming units/ml), it is also possible by protein

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expression, an approach that we generally employ. It is important to optimize the infection conditions for each stock of baculovirus. Therefore, for an optimal protein expression, add different amounts of recombinant virus to High Five cells in 175-cm^2 flasks and titrate by SDS–PAGE and Coomassie blue staining, the amounts of virus and optimal time required to obtain maximal protein expression. This will also be a good starting point to evaluate the amounts of viruses to be used for coinfections. Follow the guidelines provided in the Gibco Bac-to-Bac user manual. Verify the degradation of the complex by Western analysis with a Rabaptin-5 antibody and if available one against Rabex-5. Once identified, perform a coinfection with the appropriate amounts of both Rabaptin-5 and Rabex-5 recombinant viruses and harvest the cells by scraping them in PBS at the predetermined optimal time (usually 40–50 hr postinfection). Wash the pellet twice with PBS to remove all traces of serum albumin and freeze the pellet at −80° until ready to purify the Rabaptin-5 complex. Typically, 10–20 245 × 245 mm^2 plates of cells yield roughly 5–10 ml of pellet.

Purification of Complex by Affinity Chromatography. The key feature for the purification scheme is the presence of a histidine tag on Rabaptin-5 and the lack of tag on Rabex-5. This implies that the complex can be purified in a single purification step on a nickel agarose column. For this reason, a stringent wash regime is used to minimize contamination by insect proteins. To purify the complex, thaw the above pellet on ice and resuspend in 5 ml of lysis buffer per gram of pellet [lysis buffer: 50 m*M* Tris, pH 8.5, 4 m*M* 2-mercaptoethanol (2-ME), 5 μg/ml DNase I, and a cocktail of protease inhibitors (see above)]. Break the cells once in a French press (pressure of 800–1100 psi) and spin the lysate at 125,000*g* for 45 min at 4°. Meanwhile, preequilibrate 0.25–1 ml of nickel-NTA agarose beads (Qiagen, Karlsruhe)/20 plates of cells with lysis buffer. Filter the supernatant through a 0.22 μm filter and incubate in batch with the nickel beads for 1 hr at 4° on a rotator. Sediment the beads at low *g* force for 2–5 min and wash with 10 column volumes of buffer A [20 m*M* Tris, pH 8.0, 500 m*M* NaCl, 20 m*M* imidazole, 4 m*M* 2-ME, and 10% (v/v) glycerol] followed by 10 column volumes of buffer B [20 m*M* Tris, pH 8.5, 1 *M* NaCl, 4 m*M* 2-ME, and 10% (v/v) glycerol]. Resuspend the beads in buffer C (20 m*M* HEPES, pH 7.5, 100 m*M* NaCl, 20 m*M* imidazole, 4 m*M* 2-ME, and 10% glycerol) and load them in an empty 10-ml chromatographic column (Bio-Rad, Munich). Wash the beads with 10 column volumes of buffer C and elute the Rabaptin-5 complex with 10 column volumes of buffer D (20 m*M* HEPES, pH 7.4, 100 m*M* NaCl, 4 m*M* 2-ME, 200 m*M* imidazole, and 10% glycerol), collecting 10 fractions of 1 column volume each. Take aliquots to analyze by SDS–PAGE and Coomassie blue staining. Dilute the fractions to 20 m*M* HEPES, pH 7.4/100 m*M* NaCl/4 m*M* 2-ME/100 m*M* imidazole/10% glycerol to

avoid precipitation and freeze aliquots of the fractions. It is best not to pool the fractions, because one of the fractions will be of much higher concentration than the other ones.

Analysis of the Purified Complex. Analyze the fractions by SDS–PAGE on a 10% polyacrylamide gel and by Coomassie blue staining. Figure 4 shows a typical purification preparation. The activity of the recombinant complex can be accessed as described above. It is as active as the native complex (data not shown).

Comments. The amount of nickel-NTA agarose beads influences both the concentration of the sample and the total yield. For instance, 0.25 ml of beads/20 plates of cells gives the highest concentration of complex, but also the lowest yield as some complex ends up in the flowthrough and washes. On average, the yield of complex is low overall (≤70 μg/20 plates of cells), but nevertheless up to 20-fold better than for the native complex. In addition, the peak fraction of the recombinant complex is 5–15 times more concentrated than the native sample depending on the volume of nickel-NTA agarose beads used (≤2700 NM). Furthermore, the purified complex is relatively pure (≥70–80%) and devoid of the contaminating

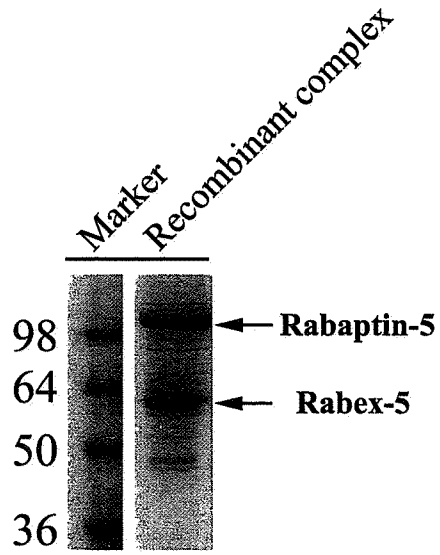

FIG. 4. Coomassie staining of the purified recombinant complex. On purification, 10 μl of the sample was loaded onto a 10% SDS–PAGE gel and stained with Coomassie blue. The molecular mass markers are indicated in kilodaltons. The identity of the Rabaptin-5 and Rabex-5 bands was confirmed by Western blot analysis.

mammalian proteins that are present in the native preparation and perhaps able to modulate the fusion of the early endosomes. Finally, this recombinant material is sufficient to perform many functional assays.

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[16] Measurement of Rab5 Protein Kinase B/akt and Regulation of Ras-Activated Endocytosis

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Introduction

Endocytosis is a carefully orchestrated process required by all cells for nutrition and defense. Whereas an increasing number of Rab GTPases localize to the endocytic pathway, including Rab5, Rab4, Rab11, and Rab7,[1] the endocytic rate appears to be regulated by Rab5.[2] Rab5 is also rate limiting for endosome fusion reconstituted *in vitro*.[2–4] Earlier work has shown that Rab5, in turn, is regulated by phosphatidylinositol 3-kinase (PI3K) and Ras.[5,6] Ras is the prototype of a large family of 20- to 35-kDa monomeric GTPases that serve as molecular switches in regulating diverse

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