[13] Expression and Properties of Rab4 and Its Effector Rabaptin-4 in Endocytic Recycling

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Introduction

Rab4 is associated with early endocytic compartments and regulates receptor recycling from early endosomes back to the plasma membrane.[1–5] To understand how Rab4 mediates its function, we searched for effector proteins interacting with the GTP-bound form of Rab4 using the two-hybrid system. One of the clones we identified in this screen encoded a Rabaptin-5 homolog,[6] which we named Rabaptin-4.[7] Rabaptin-4 interacts preferentially with Rab4 and Rab5 in the GTP-bound form, a property shared by Rabaptin-5.[8] Rab4 binds to an N-terminal domain of Rabaptin-4, whereas two binding sites are present for Rab5, one in the N-terminal and one in the C-terminal half of Rabaptin-4. Rabaptin-4 is recruited by Rab4 to the perinuclear region where it colocalizes with endocytosed transferrin (Tf) and cellubrevin. Because Rabaptin-4 binds Rab4–GTP and reduces the intrinsic GTP hydrolysis rate of Rab4, we think that Rab4 and Rabaptin-4 coordinately regulate transport from early endosomes. We describe here some of the assays that we used to study the function of Rabaptin-4.

Methods

Expression of His$_6$-Tagged Rab4 and His$_6$-Tagged Rabaptin-4ΔC in Escherichia coli

Expression Constructs. To investigate binding of Rab4 to the N terminus of Rabaptin-4 *in vitro,* we first generated the expression plasmid pRSET/

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C–Rabaptin-4ΔC. This construct encodes amino acids 11–388 of Rabaptin-4 and is prepared by releasing an *Eco*RI–*Hin*dIII fragment from pGADGH-#16B[7] and ligating it in the same sites of pRSET/C (Invitrogen, Leek, The Netherlands).

Buffers

Buffer A: 300 m*M* NaCl, 50 m*M* Tris-HCl, pH 8.0, 20 m*M* imidazole, 2 mg/ml lysozyme, 3 μg/ml aprotinin, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 1 μ*M* pepstatin

Buffer B: 300 m*M* NaCl, 50 m*M* KOH–MES, pH 6.5, 10% (v/v) glycerol, 20 m*M* imidazole, 3 μg/ml aprotinin, 1 m*M* PMSF, 10 μg/ml leupeptin, 1 μ*M* pepstatin

Buffer C: 50 m*M* KOH–PIPES, pH 8.0, 150 m*M* KCl, 1 m*M* EGTA, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT) 0.1% CHAPS

Expression and Purification Protocol. Hexahistidine-tagged (His_6) Rab4 and His_6-tagged Rabaptin-4ΔC are produced as fusion proteins in *E. coli.* Constructs are transformed into competent *E. coli* BL21(DE3)pLysS. Transformants are grown at 37° and selected on Luria–Bertani (LB) agar plates containing 100 μg/ml ampicillin + 50 μg/ml chloramphenicol. The next day a colony is transferred to 2 ml LB + 100 μg/ml ampicillin + 50 μg/ml chloramphenicol. Precultures are grown for 6 hr at 37°, diluted into 50 ml, and grown overnight. Next morning the culture is diluted 20 times with LB + 100 μg/ml ampicillin + 50 μg/ml chloramphenicol. After the culture reaches $OD_{600} \sim 0.6$, isopropylthiogalactoside (IPTG) (Boehringer Mannheim, Almere, The Netherlands) is added to a final concentration of 0.1 m*M* and the cells are grown for 3 hr at 37°. Bacteria are harvested after centrifugation of the culture for 10 min at 5000 rpm in a Sorvall GSA rotor. Pellets are resuspended with cold phosphate-buffered saline (PBS), combined, and centrifuged for 10 min at 5000 rpm in a Sorvall SS34 rotor. At this stage, pellets are snap-frozen in liquid nitrogen and stored at −80°.

The cell pellet is resuspended in 50 ml cold buffer A and sonicated 3 times for 45 sec on ice. Cell debris is pelleted by centrifugation at 4° for 15 min at 16,000 rpm in a Sorvall SS34 rotor. The supernatant containing soluble protein is retrieved and loaded on a 5-ml HiTrap affinity column (Pharmacia Biotech, Roosendaal, The Netherlands) and washed with buffer B until OD_{280} is ~0. Proteins are eluted at a flow rate of 0.4 ml/min using a continuous 0.02–0.5 *M* imidazole gradient in buffer B and collected in fractions of 0.25 ml. Column fractions (5 μl) are analyzed by 12.5% SDS–PAGE and stained with Coomassie Brilliant Blue. Peak fractions are pooled and desalted on two PD10 columns (Pharmacia), equilibrated with buffer C. Fractions of 0.5 ml are collected, and protein content is measured with

the BCA protein assay (Pierce, Rockford, IL). Peak fractions are pooled, snap-frozen in liquid nitrogen, and stored at -80°.

Comments. For the expression of His_6-tagged Rab4 and His_6-tagged Rabaptin-4ΔC, we recommend starting with fresh transformants because they provide the most reproducible results in terms of protein yield. We have tried to increase the yield of His_6-tagged Rabaptin-4ΔC by growing cultures at room temperature; however, this provided little improvement. For purification of His_6-tagged Rab4, all buffers are supplemented with 5 μM GDP to stabilize the small GTPase. Typical yields are 1.5 mg of His_6-tagged Rab4 and 0.3 mg of His_6-tagged Rabaptin-4ΔC per liter of culture. The purity of recombinant proteins is ~90% as assessed by silver staining.

In Vitro Binding Assay

A simple and rapid procedure to assay guanine nucleotide-dependent binding of Rab4 to Rabaptin was developed using an enzyme-linked immunosorbent assay (ELISA) format in which His_6-tagged Rabaptin-4 is coated in microtiter plates. Subsequently wells are incubated with increasing amounts of His_6-tagged Rab4 in the presence of GDP or GTP and bound Rab4 is determined with Rab4 antibody.

Reagents

Binding buffer: 100 mM $NaHCO_3/Na_2CO_3$, pH 9.5
TBST: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20
Blotto: 5% skimmed milk powder in TBST
Stock solutions of GDP (0.25 M) and GTP (0.25 M)

Procedure. Wells are coated for 1 hr at room temperature with 300 ng His_6-tagged Rabaptin-4ΔC in binding buffer and blocked for 1 hr with Blotto. Increasing amounts of His_6-tagged Rab4 are added in the presence of 5 mM $MgCl_2$, and 1 mM GDP or GTP (final concentrations) in TBST and incubated for 1 hr in a cold room. Wells are washed 5 times with 200 μl Blotto and incubated for 45 min with a rabbit antibody against Rab4 (1 : 2000). Wells are again washed 5 times with Blotto, and incubated 45 min with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Westgrove, PA). After 5 washes, HRP enzyme activity is detected colorimetrically with 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine (Sigma, Zwijndrecht, The Netherlands) in 0.03‰ H_2O_2, 0.111 M sodium acetate, pH 5.5. Reactions are stopped after 5–10 min by addition of 100 μl 2 M H_2SO_4 and OD_{450} is assayed in a microtiter plate reader. Results of a representative binding assay are shown in Fig. 1.

Comments. As controls we use Blotto, or His_6-tagged Rabaptin-4ΔN (amino acids 469–830). When wells are coated with these proteins, a con-

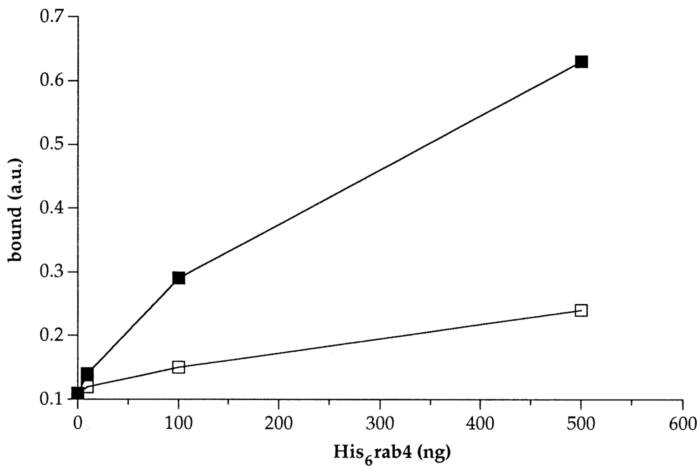

FIG. 1. *In vitro* binding of His_6-tagged Rab4 to His_6-tagged Rabaptin-4ΔC. His_6-tagged Rabaptin-4ΔC is immobilized in a microtiter plate and incubated with His_6-tagged Rab4 in the presence of GDP (open squares) or GTP (closed squares). Binding is detected using anti-Rab4 antibodies and a HRP-conjugated secondary antibody. An approximately fourfold increase in binding is observed in the presence of GTP as compared to GDP, showing that the interaction prefers the GTP-bound conformation of His_6-tagged Rab4. Reprinted with permission from *Biochemical Journal* **346,** 593 (2000).

stant background signal of ~0.3 OD_{450} units is obtained, irrespective of the amount of His_6-tagged Rab4 that is added. This background is subtracted from experimental data points. Three to four times more Rab4 is bound to rabaptin in the presence of GTP as compared to GDP. Replacement of GTP by the nonhydrolyzable analog GTPγS should further increase this ratio.

Intrinsic GTPase Assay for His_6-Tagged Rab4 in Presence of His_6-Tagged Rabaptin-4ΔC

To investigate the effect of the bound effector molecule on the intrinsic GTPase activity of Rab4 we use a slightly modified charcoal precipitation assay[9] that measures release of hydrolyzed [^{32}P]P from [γ-^{32}P]GTP pre-bound to His_6-tagged Rab4.

Procedure. Reactions are done at 37° in a volume of 350 μl and contain 200 n*M* active His_6-tagged Rab4 (determined as described in Ref. 10), 500

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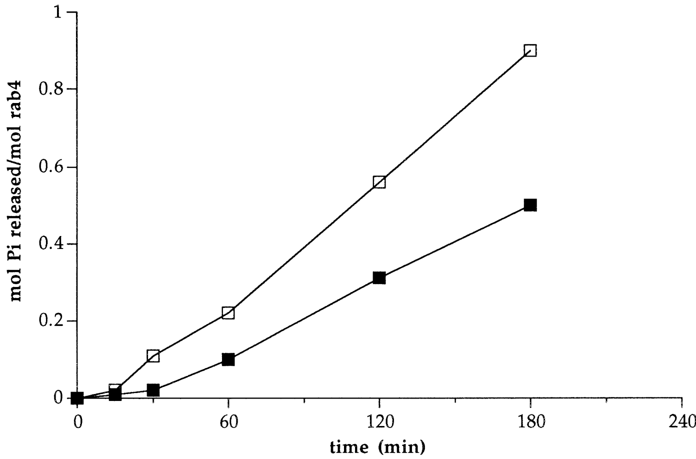

FIG. 2. Effect of His_6-tagged Rabaptin-4ΔC on intrinisic GTP hydrolysis of His_6-tagged Rab4. [γ-^{32}P]GTP loaded His_6-tagged Rab4 is incubated with His_6-tagged Rabaptin-4ΔC (closed square) or bovine serum albumin (open squares) for different periods of time. In the presence of His_6-tagged Rabaptin-4ΔC the intrinsic rate of GTP hydrolysis is reduced 45% compared to the incubations with BSA. Reprinted with permission from *Biochemical Journal* **346,** 593 (2000).

n*M* [γ-^{32}P]GTP (7.4×10^{11} Bq/mmol), and 2 μ*M* His_6-tagged Rabaptin-4ΔC or BSA (control) in 50 m*M* KOH–PIPES, pH 8.0, 150 m*M* KCl, 1 m*M* EDTA, 10 m*M* $MgCl_2$, 1 m*M* DTT, 0.1% CHAPS. After different periods of time, 50-μl aliquots are retrieved and added to 750 μl 5% (w/v) activated charcoal (Serva, Heidelberg, Germany) in 50 m*M* NaH_2PO_4 on ice. The mixture is vortexed and centrifuged in a microcentrifuge at 3000*g* for 10 min at 4°. The supernatant is saved and 500 μl is mixed with 5 ml Ultima Gold scintillation fluid (Packard Biosystems, Groningen, The Netherlands) to quantitate hydrolyzed [^{32}P]P_i. Background signals are determined in parallel reactions lacking His_6-tagged Rab4 and subtracted from experimental values.

Comments. In the presence of a 10-fold molar excess of His_6-tagged Rabaptin-4ΔC, the intrinsic GTP hydrolysis rate of His_6-tagged Rab4 is reduced from 0.046 to 0.025 min^{-1}, as shown in Fig. 2. An equimolar amount of His_6-tagged Rabaptin-4ΔC does not effect GTP hydrolysis. To formally show that Rabaptin-4 is a true effector of Rab4 requires knowledge of whether it can effect the exchange of Rab4-bound guanine nucleotide. We have tested this in an *in vitro* guanine nucleotide exchange assay and found

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that a 10-fold molar excess of His_6-tagged Rabaptin-4ΔC does not effect guanine nucleotide exchange on His_6-tagged Rab4.

Expression in Mammalian Cells

Expression Constructs. For the analysis of Rab4 binding to Rabaptin-4, and recruitment assays of Rabaptin-4 to early endosomes, we generated constructs from which Rabaptin-4, the N-terminal region (aa 11–388), and C-terminal region (aa 469–830) are expressed as hexahistidine fusion proteins in mammalian cells. An *Eco*RI–*Xba*I Rabaptin-4 fragment encoding aa 11–830 is excised from pGADGH-#16B to generate pcDNA3.1His/B-Rabaptin (Invitrogen). The N-terminal Rabaptin-4 construct pcDNA3.1His/B-Rabaptin-4ΔC is made by inserting a *Bam*HI–*Dra*I fragment between the *Bam*HI–*Eco*RV sites of pcDNA3.1His/B. The C-terminal construct pcDNA3.1His/A-Rabaptin-4ΔN is made by inserting a Rabaptin-4 *Hin*dII–*Xba*I fragment between the *Eco*RV–*Xba*I sites of pcDNA3.1His/A. For NHRab5 expression (canine Rab5a with an N-terminal X31 influenza hemagglutinin epitope tag, we use the previously described Rab5 expression plasmid pcDNA3-NHRab5.[11]

Transfection Protocol. 20 μg sterile DNA is mixed with 0.5 ml 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 6 m*M* dextrose, 21 m*M* HEPES, pH 7.1 (HBS), in a polycarbonate tube. A calcium phosphate precipitate is prepared by adding 31 μl 2 *M* $CaCl_2$ while flicking the tube 2–3 times. After 45 min, the cloudy suspension is added to an aspirated 10-cm plate of Chinese hamster ovary (CHO) cells that is 25% confluent. Cells are incubated 30 min at room temperature on a rocking platform. At the end of this period, cells receive 10 ml of minimal essential medium-α (αMEM), 10% fetal calf serum (FCS), and are grown in a 37° incubator. After 4 hr, medium is aspirated and 2.5 ml 15% glycerol in HBS is added for precisely 3.5 min at 37°. The cells are washed twice with culture medium and grown in αMEM, 10% FCS. After 24 hr, cells are trypsinized and grown for a week in αMEM, 10% FCS, 0.6 mg/ml G418 (Gibco-BRL, Breda, The Netherlands). For immunofluorescence microscopy, cells are seeded on round (11-mm diameter) #1 coverslips. Transfectants receive 5 m*M* sodium butyrate (Fluka, Buchs, Germany) 17 hr prior to the experiments to enhance expression of cytomegalovirus promotor (CMV)-driven expression plasmids.

Comments. The glycerol shock is used to enhance transfection efficiency and is optional. When this step is omitted, the medium containing calcium phosphate and DNA should be replenished with fresh culture medium the next morning. Sodium butyrate affects the ultrastructure of secretory and endocytic compartments at concentrations above 5 m*M*. We grow trans-

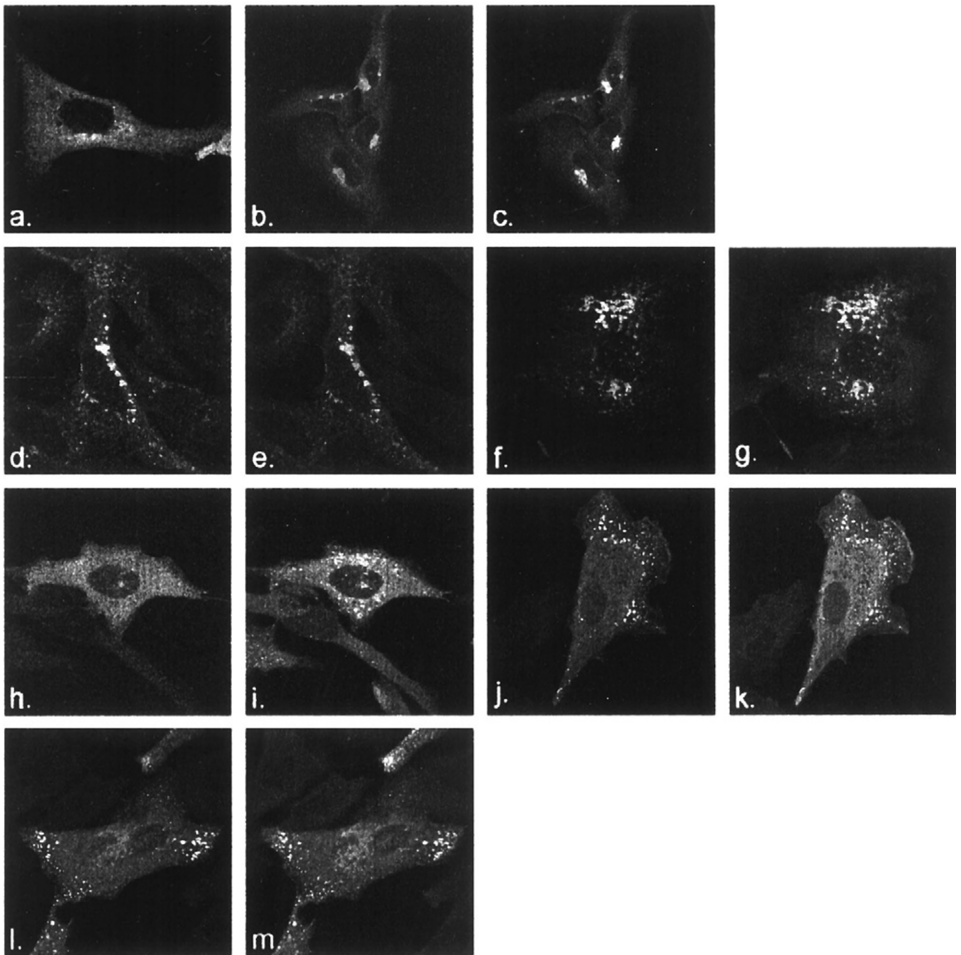

FIG. 3. Recruitment of His_6-tagged Rabaptin-4 to early endosomes. In CHO cells transfected with His_6-tagged Rabaptin-4, His_6-tagged Rabaptin-4 predominantly shows a diffuse cytosolic labeling (a). In His_6-tagged Rabaptin-4–Rab4 transfectants, His_6-tagged Rabaptin-4 (b) and Rab4 (c) colocalize on aciniform organelles in the perinuclear region. This is an endocytic organelle since it contains endocytosed Cy3-labeled transferrin (d) that also is decorated with His_6-tagged Rabaptin-4 (e). The region required for targeting His_6-tagged Rabaptin-4 to Rab4 containing endosomes is localized in the N terminus of Rabaptin-4, as the His_6-tagged Rabaptin-4ΔC (f) colocalizes with Rab4 (g) in a His_6-tagged Rabaptin-4ΔC–Rab4 transfectant, while His_6-tagged Rabaptin-4ΔN (h) does not colocalize with Rab4 (i) in His_6-tagged Rabaptin-4ΔN–Rab4 CHO cells. Both His_6-tagged Rabaptin-4ΔN (j) and His_6-tagged Rabaptin-4ΔC (l) are are recruited to Rab5 containing endosomes (k, m) in His_6-tagged Rabaptin-4ΔN–NHRab5 and His_6-tagged Rabaptin-4ΔC–NHRab5 CHO cells, showing that Rabaptin-4 contains two domains that bind Rab5. Reprinted with permission from *Biochemical Journal* **346,** 593 (2000).

fected cells for a week on selection to increase the number of transfectants and because it improves morphology of the cells.

Immunofluorescence Microscopy

To detect compartments of the transferrin pathway, Cy3-labeled transferrin is internalized in cells grown on coverslips as described.[5] Coverslips are fixed in 1 ml 3% paraformaldehyde for 1 hr and subsequently washed with PBS. Excess fixative is quenched in 1 ml 50 m*M* NH_4Cl in PBS for 5 min. The cells are permeabilized and blocked for 30 min in 1 ml 0.1% saponin, 0.5% bovine serum albumin in PBS (blocking buffer) and incubated with primary antibodies in a volume of 25 μl. Rab4 and NHRab5 are detected with affinity purified antibodies raised against glutathione *S*-transferase(GST)–Rab4 and the NH epitope tag.[4,11] The Rabaptin constructs are detected with the XPRESS monoclonal mouse antibody (Invitrogen). The coverslips are washed for three periods of 15 min with 1 ml blocking buffer and then incubated in 25 μl for 30

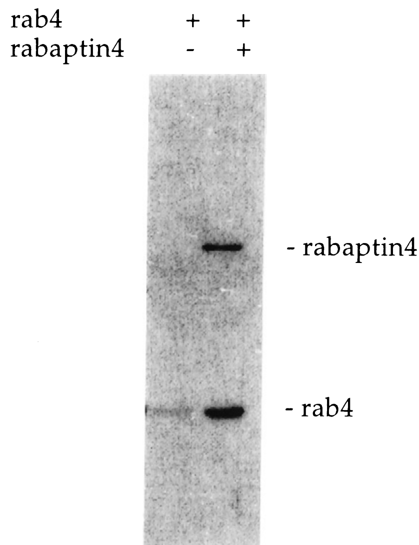

FIG. 4. *In vivo* binding assay of Rab4 to rabaptin4. A Rab4 CHO cell line was either mock-transfected or transfected with pcDNA3.1His/B-Rabaptin. Postnuclear supernatants are incubated with Ni^{2+}-NTA resin to bind His_6-tagged Rabaptin-4. Resin fractions are washed with RIPA buffer and resolved by SDS–PAGE on 12.5% gels under reducing conditions followed by Western blotting using antibodies against Rab4 and His_6-tagged Rabaptin-4. In the cells expressing His_6-tagged Rabaptin-4, ~6 times more Rab4 is isolated on the beads than in mock transfected Rab4 CHO cells.

min with indocarbocyanine Cy3-conjugated goat anti-mouse IgG and 5-[(4,6-dichlorotriazin-2-yl)amino] (DTAF) fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories), diluted 200 times and 50 times, respectively, in blocking buffer. The coverslips are washed 3 times with blocking buffer, once with PBS, and finally mounted in Moviol 4-88 (Calbiochem, La Jolla, CA) containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma, Zwijndrecht, The Netherlands) and examined with a 63× planapo objective on a Leitz DMIRB fluorescence microscope (Fig. 3) (Leica, Voorburg, The Netherlands).

Coprecipitation of Rab4 with His_6-Tagged Rabaptin-4 on Ni^{2+}-NTA Resin

To demonstrate the association of Rab4 and Rabaptin-4 *in vivo,* we use Rab4 CHO transfected with pcDNA3.1His/B-Rabaptin and control (mock transfected) Rab4 CHO cells.

Procedure. Three 10-cm plate subconfluent cells are homogenized in 1 ml 100 m*M* KOH–HEPES, pH 7.4, 0.1 m*M* GTP, 1 m*M* $MgCl_2$, 50 m*M* NaF, and 1 m*M* PMSF by passing them 10 times through a 25-gauge needle. Homogenates are spun 10 min at 1500 rpm to prepare a postnuclear supernatant. The postnuclear supernatant is next incubated with 50 μl Ni^{2+}-NTA beads (Qiagen-Westburg, Leusden, The Netherlands) by end-over-end rotation for 1 hr at 4° to collect His_6-tagged Rabaptin-4–Rab4 complexes. Resin fractions are washed four times with 1 ml 150 m*M* NaCl, 100 m*M* Tris-HCl, pH 8.3, 0.1% SDS, 0.5% deoxycholate (DOC), 0.5% Nonidet P-40. After the third wash, the resuspended beads are transferred to a clean tube, pelleted, and boiled for 5 min in reducing Laemmli sample buffer. Samples are resolved by SDS–PAGE on 12.5% minigels, transferred to PVDF membrane, and analyzed by Western blotting using the anti-Xpress antibody (at 1 : 4000 dilution) to detect His_6-tagged Rabaptin-4 and a rabbit antibody against Rab4 (at 1 : 2000 dilution) (Fig. 4).

Acknowledgment

This work was supported by grants from NWO-MW, NWO-ALW and the Dutch Cancer Society (to PvdS).