[46] Expression and Properties of the Rab4, Rabaptin-5α, AP-1 Complex in Endosomal Recycling

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Abstract

We previously showed that the small GTPase Rab4 regulates formation of recycling vesicles from early endosomes. To understand how Rab4 accomplishes this task, we started to identify the Rab4 effector protein network. In this chapter, we describe experiments leading to the characterization of a complex consisting of Rab4GTP, its effector Rabaptin-5α, and the adaptor protein complex AP-1, which regulates recycling from endosomes.

Introduction

Early endosomes are an important sorting station where proteins and lipids that are destined for late endocytic compartments are sorted away from cargo molecules that are recycled back to the plasma membrane. Early endosomes receive material from the plasma membrane by endocytosis as well as from the *trans*-Golgi network (TGN) via exocytic routes. Exit routes from early endosomes lead to the cell surface and intracellular compartments such as late endosomes and the TGN. The massive flow of membrane through early endosomes needs to be tightly controlled to maintain the structural and architectural integrity of this compartment (see Gruenberg, 2001). Indeed the small GTPases Rab5 and Rab4 have been shown to be important regulators that act sequentially in controlling entry into and recycling from early endosomes, respectively (see Deneka *et al*., 2003b). They accomplish this feat through the spatial and temporal recruitment of a structurally diverse group of cytoplasmic effector proteins that bind to their active GTP forms. Effector proteins generally have been found to cooperate with their Rab protein partners at multiple stages in a transport pathway that include vesicle formation, transport along the cytoskeleton, and tethering and docking on a target membrane (see Zerial and McBride, 2001). Interestingly, a subgroup of the Rab5 effectors, including rabaptin-5, rabaptin-5α, rabaptin-5β as well as some of the FYVE domain proteins such as rabenosyn-5 and rabip4′ also interact with Rab4 in a separate binding site (de Renzis *et al*., 2002; Fouraux *et al*., 2003; Nagelkerken *et al*., 2000; Vitale *et al*., 1998). The bivalent nature of these effectors is thought to coordinate the activities of Rab5 and Rab4 in distinct but overlapping

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microdomains on early endosomes (de Renzis *et al.*, 2002; Sönnichsen *et al.*, 2000). We found before that Rab4 controls recycling vesicle formation from endosomes and that Rab4 and brefeldin A act in the same recycling pathway (de Wit *et al.*, 2001; Mohrmann *et al.*, 2002). Because the hetero-tetrameric adaptor complex AP-1 localizes to early endosomes (Stoorvogel *et al.*, 1996) and is involved in transport vesicle formation (Odorizzi and Trowbridge, 1997), it is important to investigate the functional relationships of the Rab4, rabaptin-5α, and AP-1 adaptor in endosomal recycling.

Methods

Yeast Two-Hybrid Interaction Assay of Rabaptin-5 Constructs and AP Complex Subunits

To assess interactions between rabaptins and AP-1 subunits we used initially Gal4-based two-hybrid assays since this format is well suited for rapid testing of direct interactions (Fig. 1A and B).

Expression Constructs. pGBT9-rabaptin-5α, pGBT9-rabaptin-5, pGBT9-rabaptin-5α(1–390), pGBT9-rabaptin-5α(509–830), pGBT9-rabaptin-5α (509–863), pGBT9-rabaptin-5α(1–592), pGBT9-rabaptin-5α(301–830), pGBT9-rabaptin-5α(301–863), pGBT9-rabaptin-5α(301–592), pGBT9-rabaptin-5α(301–449), +pGBT9-rabaptin-5α(449–592), pACT2-α-adaptin, pACT2-β2-adaptin, pACT2-μ1-adaptin, pACT2-μ2-adaptin, pGADH-γ1-adaptin, pACT2-δ-adaptin, and pACT2-σ1-adaptin have been described (Deneka *et al.*, 2003). The two-hybrid plasmids pGBT9, pACT2, and pGADGH were obtained from Clontech.

Reagent. SC medium: 6.7 g yeast nitrogen base lacking amino acids (Difco), 0.75 g Complete Supplement Medium without leucine, tryptophan, and histidine (Bio 101), 20 g glucose (Sigma), 0.1 g adenine hemisulfate (Sigma), brought to 1 liter with ddH_2O. The pH of the medium is 5.8.

Protocol. YGH1 (*MATa trp1 leu2 his3 LYS2::GAL1-HIS3 URA3::GAL1-LacZ*) cells (Spaargaren and Bischoff, 1994) were transformed with 3–6 μg DNA and grown at 28° on SC medium lacking tryptophan and leucine. Colonies were patched on SC plates lacking leucine, tryptophan, and histidine (growth selection) and SC plates without leucine and tryptophan for β-galactosidase assay. For the latter, plates were overlaid with Hydrobond-N nylon membrane (Amersham Pharmacia Biotech) under light pressure. Filters are quickly lifted and immersed for 20 s in liquid nitrogen. After a brief warming, filters are overlaid (cells on top) with Whatman 3 paper soaked in 2 ml 60 m*M* NaH_2PO_4, pH 7, 10 m*M* KCl, 1 m*M* $MgSO_4$, containing 50 m*M* α-mercaptoethanol and 0.02% X-gal (both freshly added).

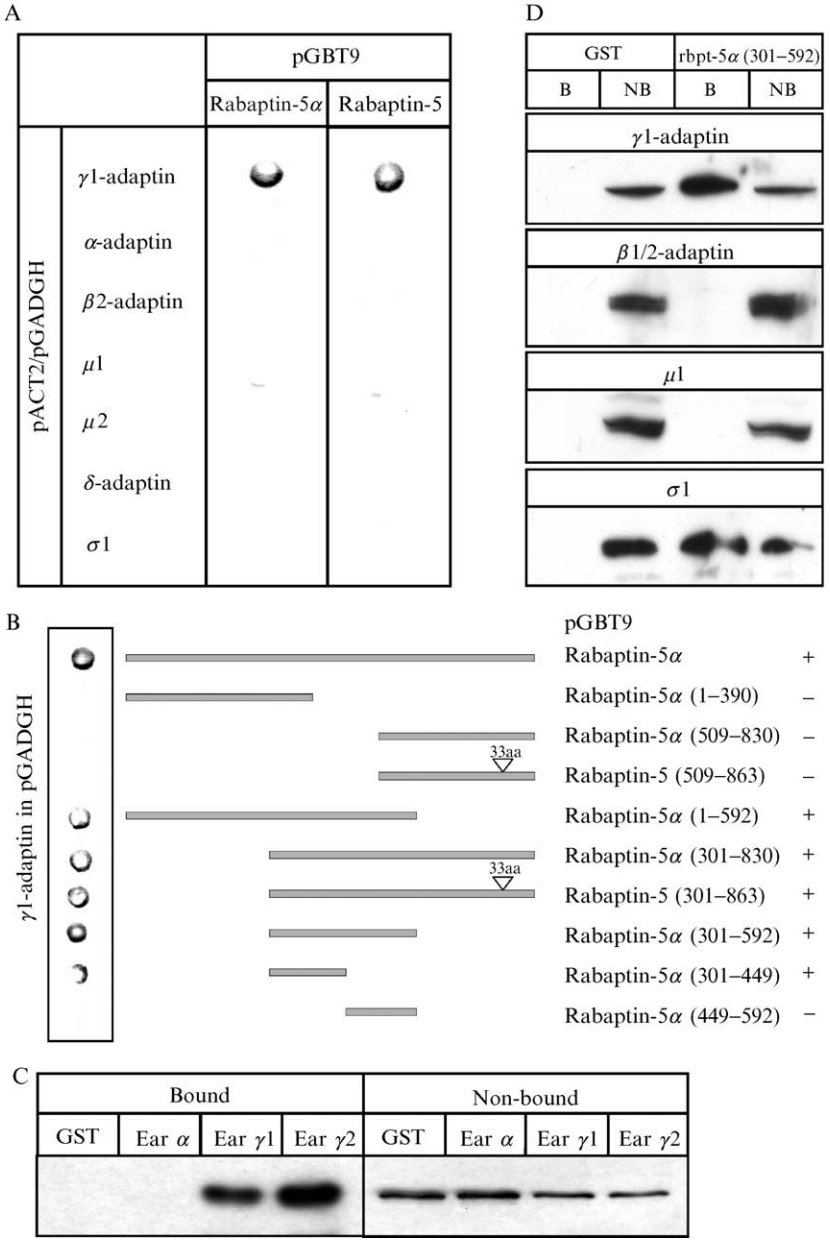

FIG. 1. The hinge region of rabaptin-5/5α binds to the ear domain of γ1-adaptin (A) β-galactosidase reporter activation of YGH1 cells cotransformed with pGBT9-rabaptin-5α or pGBT9-rabaptin-5 and pGADH-γ1-adaptin. None of the other tested subunits of AP-1

Comments. Over the past several years a number of rabaptin-5 variants have been identified that with the exception of rabaptin-5β are thought to arise by alternative splicing (Gournier *et al.*, 1998; Korobko *et al.*, 2002; Nagelkerken *et al.*, 2000). Rabaptin-5α contains a C-terminal 33-amino acid internal deletion compared to rabaptin-5, immediately adjacent to the Rab5-binding site. So far we have not found functional differences between rabaptin-5 and rabaptin-5α, and indeed binding of γ1-adaptin to rabaptin-5 and rabaptin-5α is identical and occurs in a completely conserved region. Korobko *et al.* (2005), however, observed that rabaptin-5δ, which contains an in-frame deletion in the Rab4-binding site, lost the ability to interact with Rab4 while Rab5 binding is not affected.

Binding Assay of Rabaptin-5α to Glutathione S*-transferase (GST) Fusions of Adaptor Ears*

An independent biochemical method was developed to confirm the results of the yeast two-hybrid interaction trap. The method is based on the immobilization of recombinant adaptin ear domains as GST fusions on GSH beads, followed by the retrieval of rabaptin-5α from detergent lysates and detection by Western blot (Fig. 1C).

Reagents

Buffer A: 25 m*M* Tris–HCl, pH 7.5, 0.5 m*M* ethylenediaminetetrascetic acid (EDTA), 10% sucrose, 1 m*M* dithiothreitol (DTT)
Buffer B: 25 m*M* Tris–HCl, pH 7.5, 0.5 m*M* EDTA, 1 m*M* DTT
Buffer C: 25 m*M* Tris–HCl, pH 7.5, 0.5 m*M* EDTA, 1% Triton X-100, 1 m*M* DTT
Buffer D: 50 m*M* Tris–HCl, pH 8.0, 25 m*M* GSH
Protease inhibitors stocks: 5 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mg/ml pepstatin, 100 m*M* phenylmethylsulfonyl fluoride (PMSF) (all stored at -20°)
1 *M* DTT stock (stored at -20°)
250 m*M* reduced glutathione (GSH, stored at -80°)

(μ1- and σ1-adaptin), AP-2 (α-, β2-, and μ2-adaptin), or AP-3 (δ-adaptin) bound to rabaptins. (B) Binding of rabaptin-5α from a brain extract to GST fusions containing the ear domain of γ1-adaptin and γ2-adaptin. (C) Summary of the two-hybrid assays with rabaptin-5α truncations showing that the domain required for γ1-adaptin binding is between amino acids 301 and 449; binding is indicated by +. (D) Pull-down assay showing binding of AP-1 from a HeLa cell extract to GST-rabaptin-5α. The Western blot of bound (B) and nonbound (NB) fractions was probed with antibodies against γ1-, β1,2-, σ1-, and μ1-adaptin. Since rabaptin-5 and rabaptin-5α behave identically in the assays and have complete sequence identity in the hinge region, we performed the other experiments with rabaptin-5α (Adapted with permission from Deneka *et al.*, 2003a).

Expression and Isolation of GST Adaptin Ear Fusion Proteins. pGEX4T3, pGEX1λT ear α-adaptin, pGEX4T3 ear γ1-adaptin, and pGEX4T3 ear γ2-adaptin are transformed in *Escherichia coli* BL21(DE3) and bacteria are grown at 37° on Luria–Bertani (LB) agar plates containing 50 μg/ml ampicillin. The next day a colony is transferred to 2.5 ml LB containing 50 μg/ml ampicillin (medium) and grown for 5 h. The cultures are subsequently diluted to 125 ml and grown overnight at 30°. The next morning suspension cultures are diluted four times with medium and maintained at 30° until OD_{600} reached 0.6. Freshly dissolved isopropylthiogalactoside (IPTG) is then added to 1 m*M* (final concentration) and the cells are grown for 3 h at 30°. Bacteria are harvested by centrifugation for 10 min at 5000 rpm in a Sorvall SLA-3000 rotor. Pellets are washed in cold phosphate-buffered saline (PBS), combined, and recentrifuged for 10 min at 4500 rpm in a Beckman table centrifuge. Bacterial pellets are snap-frozen in liquid nitrogen and stored at −80°. All steps of this protocol are performed at 4°. Glutathione (GSH) Sepharose beads are washed twice with Buffer B and twice with Buffer C before use in protein isolation. Bacterial pellets corresponding to 2 liter culture volume are resuspended in 20 ml Buffer A containing 2 mg/ml lysozyme and kept for 15 min on ice. The suspension is then diluted with 80 ml Buffer A containing protease inhibitors (5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 1 m*M* PMSF), sonicated two times 1 min on ice, and centrifuged for 15 min at 15,000 rpm in a Sorvall SS-34 rotor. Fresh protease inhibitors and 1% Triton X-100 (final concentration) are added to the supernatant, which is subsequently incubated with 1.5 ml washed GSH beads by end-over-end rotation for 2 h. Beads are washed with Buffer C and four times with Buffer B in batch mode. Fusion protein is then eluted by end-over-end rotation with 1.5 ml Buffer D. After 30 min, beads are centrifuged and supernatant containing fusion protein is saved. The last incubation is repeated once with the same volume of Buffer D, and the eluates are combined, aliquotted, snap-frozen in liquid nitrogen, and stored at −80°.

Preparation of Pig Brain Extract and Binding Assay with GST-Ear Adaptins. All steps of this protocol are performed at 4°. Fresh pig brain is obtained from a local slaughterhouse and 100 g of brain is homogenized with a Waring blender at maximal speed in two volumes phosphate-buffered saline (PBS) containing 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 m*M* PMSF (protease inhibitors). NP-40 is added to 0.1% (final concentration) and the mixture is stirred slowly on ice. After 30 min, the detergent extract is centrifuged at 10,000 rpm in a Sorvall SLA1500 rotor for 40 min. The supernatant is subsequently retrieved and recentrifuged for 60 min at 33,000 rpm in a Beckman Ti45 rotor. The pellet is discarded and the supernatant is snap-frozen in liquid nitrogen and stored at −80°.

For binding experiments, brain extract is thawed on ice and centrifuged for 15 min at 50,000 rpm in a Beckman TLA100.2 rotor. Aliquots (7.5 mg protein) of extract are precleared by end-over-end incubation with 100 μg GST and 200 μl GSH beads. In the meantime GST adaptin ear fusion proteins are defrosted and centrifuged as described earlier. In a typical binding assay 200 μl precleared brain extract (1.5 mg protein) is incubated with 20 μg clarified fusion protein and 60 μl GSH beads by end-over-end rotation. After 2 h, the beads are washed five times with PBS containing 0.1% NP-40. Bound material is boiled off the beads in 20 μl reducing Laemmli buffer. The proteins are resolved on a 10% sodium dodecyl sulfate–polyacrylic acid (SDS-PAA) gel and transferred to a PVDF membrane for 1.5 h at 225 mA. Bound rabaptin-5α is then assayed by Western blot using a rabbit antibody as previously described (van der Sluijs *et al.*, 2000).

Comment. We found that rabaptin-5α binds equally well to the ear domains of both γ1-adaptin and γ2-adaptin. The γ2-adaptin variant, however, appears to have a more distinct function than γ1-adaptin or is expressed in different tissues because a γ1-adaptin knockout cannot be compensated for by γ2-adaptin (Meyer *et al.*, 2000).

Binding Assay of AP-1 to Recombinant Rabaptin-5α Hinge Fragment

To investigate whether other subunits of AP-1 bind to rabaptin-5α, we slightly modified the biochemical binding assay. This alternative binding assay capitalizes on the observation made in the two-hybrid assay that the hinge region (aa 301–592) of rabaptin-5α contains the information required to interact with γ1-adaptin. In contrast to full-length rabaptin-5α, which expresses poorly in *E. coli*, the hinge fragment can be obtained readily as a GST fusion protein (Fig. 1D).

Preparation of HeLa Cell Lysate and Binding Assay. pRP269 and pRP269-rabaptin-5α(301–592) are transformed in *E. coli* BL21(DE3) and proteins are isolated and stored as described previously for the adaptin ear fusion proteins. Five confluent 10-cm dishes of HeLa cells are washed twice with 10 ml ice-cold PBS and lysed in 1 ml PBS containing 0.5% NP-40 for 30 min on a rocking platform. Dishes are scraped and lysates are shaken for 20 min in a cold room and centrifuged for 15 min at 14,000 rpm in a microcentrifuge. The HeLa cell lysate (5 ml) is precleared for 2 h by incubation with 200 μl GSH beads and 100 μg GST. Precleared lysate is divided in two equal aliquots and incubated with 20 μg fusion protein and 60 μl GSH beads for 2 h under rotation. The beads are washed three times with 1 ml PBS containing 0.1% NP-40. Bound proteins are eluted in 70 μl reducing Laemmli buffer, resolved on 7.5% SDS-PAA gels, and transferred to a PVDF membrane for 50 min at 400 mA. Bound AP-1 subunits are

analyzed by Western blot using antibodies against γ1-adaptin (Sigma) and σ1-adaptin (generously provided by Linton Traub). Dilutions of antibodies are made in PBS containing 5% skimmed milk and 0.2% Tween-20.

Comments. To detect the small σ1-subunit and medium μ1-subunit, we use 12% (instead of 7.5%) SDS-PAA gels. Initially we could detect only γ1-adaptin and the σ1-subunit in the bound protein fraction (Deneka *et al.*, 2003a). More recently we found the entire set of AP-1 subunits in the eluted protein pool using a freshly prepared extract. Possibly this reflects differences between freshly prepared and stored detergent extracts.

Analysis of the Rabaptin-5α/AP-1 Complex by Fluorescence Microscopy

Transfection and Immunolabeling Protocol. The pcDNA3-rabaptin-5α, pcDNA3.1His-rabaptin-5α, pEYFP-Rab4, and pEGFP-Rab5 constructs used in the following experiments are generated as described before (Deneka *et al.*, 2003a). None of the available Rab4 and rabaptin-5α antibodies allows for the detection of the endogenous protein by immunofluorescence microscopy, thus requiring ectopic expression for this type of analysis. We generally employ HeLa cells as recipient since they can be easily transfected and contain a convenient number of transferrin (Tf) receptors that serve as markers for early endosomes. Typically, HeLa cells are grown to 40% confluency on 10-mm #1 coverslips in 6-cm dishes. Six microliters Fugene 6 (Roche) is mixed with 94 μl serum-free minimal essential medium (SFMEM). After 5 min at room temperature, 3 μg expression construct is added, and after 15 min the mixture is added to the cells. After 24 h transfected cells are processed for further analysis. Cells are washed once with PBS, fixed with 3% paraformaldehyde for 30 min, washed once with PBS, and incubated for 5 min with PBS containing 50 m*M* NH_4Cl. Fixed cells are washed once with PBS and incubated for 1 h in PBS containing 0.5% bovine serum albumin (BSA) and 0.1% saponin (block buffer). Incubation with primary antibodies is done for 1 h and is followed by three 5-min washes with block buffer. Staining with appropriately labeled secondary antibodies is done for 1 h and is followed by three 5-min washes with block buffer and two washes with PBS. Coverslips are mounted on objective glasses with 3 μl Moviol (Hoechst).

Assay for Rabaptin-5α and γ1-Adaptin Colocalization on Rab4-Containing Endosomes

HeLa cells transfected with pcDNA3.1His-rabaptin-5α are grown for 24 h on coverslips and incubated for 30 min in SFMEM, washed once with uptake medium (SFMEM, 20 m*M* HEPES, pH 7.4, 0.1% BSA), and incubated with 15 μg/ml human Alexa594-Tf (Molecular Probes) for 60 min

at 37° in uptake medium. Cells are fixed for 15 min with 2.5% PFA in PBS at 37°, followed by a fixation for 30 min at room temperature. Cells are subsequently labeled with a rabbit antibody against rabaptin-5α followed by Alexa488-conjugated IgG. So far the antibodies against rabaptin-5α and γ1-adaptin do not allow for a triple label experiment. We therefore perform double label immunofluorescence in a parallel dish with cells that are not exposed to Alexa594-Tf, with a rabbit antibody against rabaptin-5α and a monoclonal antibody against γ1-adaptin. Staining is done with Alexa488 goat anti-rabbit IgG and Cy3 goat anti-mouse IgG (Fig. 2A and B). γ1-Adaptin localizes to both the *trans*-Golgi network (TGN) and endosomes, although we find γ1-adaptin and rabaptin-5α complex only on endosomes. This suggests that an endosome-specific factor is required for the unique localization of the complex, which could be either Rab5 or Rab4, of which rabaptin-5α is an effector. This can be tested in coexpression studies of either pEYFP-Rab4 or pEGFP-Rab5 in combination with pcDNA3-rabaptin-5α (Fig. 2C). Cells are then labeled separately with a monoclonal antibody against γ1-adaptin or a rabbit antibody against rabaptin-5α followed by secondary Cy3 goat anti-rabbit IgG or Cy3 goat anti-mouse IgG.

Comment. The enlarged endosomes that are seen by overexpression of rabaptin-5α look similar to those observed after overexpression of a GTP-hydrolysis-deficient Rab5 mutant (Stenmark *et al.*, 1995). Experiments with rabaptin-5α truncation mutants lacking the Rab5 binding site rule out an involvement of Rab5 (Deneka *et al.*, 2003a).

Assay for the Interaction of Rab4 with Endogenous Rabaptin-5α–AP-1 Complex

To investigate whether Rab4 can interact with a rabaptin-5α–AP-1 complex we use pulldown assays with recombinant GST-Rab4 and brain cytosol (Fig. 2D).

Buffers

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

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

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

Elution buffer: 20 m*M* HEPES, pH 7.5, 1.5 *M* NaCl, 20 m*M* EDTA, 1 m*M* DTT, 5 m*M* GDP

Isolation of GST-Rab Fusion Protein and Guanine Nucleotide Loading. The expression constructs Rab4-pGEX2T, Rab5-pGEX1λT, and

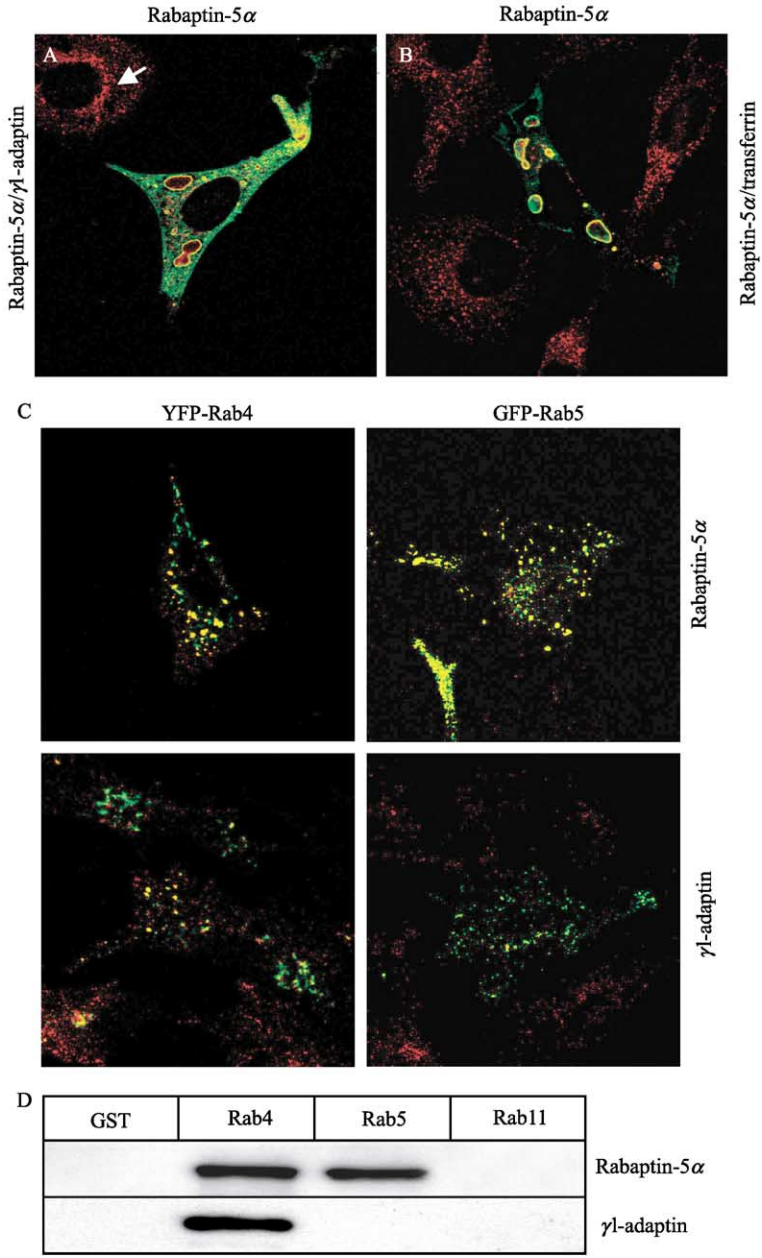

FIG. 2. Rab4-dependent localization of the rabaptin-5α/AP-1 complex to endosomes. (A) HeLa cells were transfected with pcDNA3.1His-rabaptin-5α. The cells are labeled for rabaptin-5α (green) and endogenous γ1-adaptin (red). Note the colocalization of rabaptin-5α

Rab11-pGEX5X3 have been described (Deneka *et al*., 2003a), and expression of GST, GST-Rab4, GST-Rab5, and GST-Rab11 in *E. coli* is done as for the GST ear adaptin constructs. Bacterial pellets of 1-liter cultures are resuspended in 20 ml PBS containing 5 m*M* $MgCl_2$, 5 m*M* α-mercaptoethanol, 2 mg/ml lysozyme, 10 μg/ml RNase, 10 μg/ml DNase, protease inhibitors (cf. above), and 0.2 m*M* GDP. Lysates are sonicated two times 1 min on ice and centrifuged for 1 h at 33,000 rpm in a Beckman Ti45 rotor. The supernatants are incubated with 0.6 ml GSH beads (washed three times with PBS, 5 m*M* $MgCl_2$, and 5 m*M* α-mercaptoethanol prior to use) for 2 h by end-over-end rotation. Beads are washed four times with PBS, 5 m*M* $MgCl_2$, 5 m*M* α-mercaptoethanol, and 0.1 m*M* GDP, and stored overnight at 4° in PBS, 5 m*M* $MgCl_2$, 5 m*M* α-mercaptoethanol, 0.1 m*M* GDP, and 0.02% sodium azide. To establish the fusion protein concentration on the beads, 10-μl beads are boiled in 50 μl reducing Laemmli buffer and analyzed on a 12.5% SDS-PAA gel that is also loaded with a concentration range of BSA. This also allows for normalization of the amount of beads to be used in binding experiments. Empty GSH beads are added to have the same amount of beads in the assay. Beads are washed with NE buffer containing 10 μ*M* GTPγS, and incubated for 30 min under rotation with NE buffer containing 1 m*M* GTPγS at room temperature. The wash and incubation steps are repeated twice to ensure optimal nucleotide loading. Beads were washed once with NS buffer containing 10 μ*M* GTPγS and further incubated with NS buffer containing 1 m*M* GTPγS for 20 min at room temperature and immediately used in binding assays.

Pig Brain Cytosol Preparation and Binding Assay

Pig brain (200 g) is homogenized on ice (as described earlier for the preparation of brain extract) in 350 ml NS buffer containing 1 m*M* DTT and protease inhibitors. The homogenate is centrifuged for 40 min at

and γ1-adaptin in transfected cells and the distinct localization of γ1-adaptin in nontransfected cells (arrow). (B) HeLa cells were incubated with Alexa594-Tf for 60 min at 37° and subsequently labeled with anti-rabaptin-5α antibody and Alexa488-IgG, showing that the rabaptin-5α/AP-1 complex localizes to early endosomes. (C) Localization of γ1-adaptin to endosomes depends on Rab4. HeLa cells expressing His_6-rabaptin-5α in combination with YFP-Rab4 or GFP-Rab5 and labeled with anti-γ1-adaptin antibody and anti-rabaptin-5α antibody, detected with Cy3-labeled secondary antibodies. Note that GFP-Rab5 colocalizes with rabaptin-5α, but not with γ1-adaptin. (D) Rab4 recruits rabaptin-5α/AP-1 complex. GST-Rab4, GST-Rab5, and GST-Rab11 were isolated on GSH beads, loaded with GTPγS and incubated with pig brain cytosol. Bound fractions were immunoblotted with antibodies against rabaptin-5α and γ1-adaptin. Note that GST-Rab4 and GST-Rab5 both retrieve rabaptin-5α, but that only GST-Rab4 bound material includes γ1-adaptin (Adapted with permission from Denaka *et al*., 2003d).

10,000 rpm in a Sorvall SLA1500 rotor, and the supernatant is recentrifuged for 1 h at 33,000 rpm in a Beckman Ti45 rotor. The cytosol is dialyzed overnight against NS buffer to remove free nucleotides, and used immediately in binding experiments. Beads (250 μl) (corresponding to 2 mg protein) are rotated with 8 ml cytosol for 2.5 h at 4° in the presence of 100 μM GTPγS. Beads are next washed twice with 2.5 ml NS buffer in the presence of 10 μM GTPγS, twice with 2.5 ml NS containing 250 mM NaCl, 10 μM GTPγS, and finally washed once with 1.5 ml wash buffer. Bound proteins are eluted with 150 μl elution buffer for 30 min at room temperature. Eluates are either resolved immediately on 10% and 12% SDS-PAA gels and analyzed by Western blot with antibodies against rabaptin-5α and γ1-adaptin or snap-frozen and stored at −80° until further analysis.

Comments. Instead of the nonhydrolyzable GTP analog GTPγS, we now routinely use guanosine-5′-(β,γ-imido)triphosphate (GMP-PNP) and obtain the same results with the considerably cheaper alternative. The beads with bound GST-Rab proteins can be stored several days at 4°. In our hands, best results are obtained when storage time is kept to a minimum.

Analysis of Rabaptin-5α Function in Recycling from Endosomes

To determine the role of rabaptin-5α, we analyze transferrin recycling using a pulse–chase protocol in cells transfected with rabaptin-5α. In principle it is also possible to perform functional experiments in HeLa cells in which rabaptin-5α is knocked down with RNAi. Rab5-mediated endocytosis is, however, critically dependent on rabaptin-5α, and knock down of the effector precludes the analysis of recycling since insufficient amounts of transferrin are internalized (Deneka *et al.*, 2003a) (Fig. 3).

Pulse–Chase Protocol for Fluorescently Labeled Transferrin. HeLa cells on coverslips are transfected with pcDNA3.1His-rabaptin-5α alone or in combination with pcDNA3-γ1-adaptin (1.5 μg DNA of each/6-cm dish). After 24 h, cells are depleted of endogenous transferrin during a 30-min incubation in bicarbonate, free SFMEM, 20 mM HEPES, pH 7.4, 0.1% BSA (uptake medium). The cells are then incubated with uptake medium containing 15 μg/ml Alexa488-transferrin (single transfectants) or Alexa594-transferrin (double transfectants) at 16°, which causes accumulation of the endocytic tracer in early endosomes (de Wit *et al.*, 2001). Medium is aspirated after 30 min, and cells are briefly washed with 16° uptake medium. Recycling is initiated by adding 37° uptake medium containing 100 μM desferal. The iron chelater serves to inhibit reinternalization of recycled transferrin. After different periods of time, the cells are fixed and processed for double label fluorescence microscopy with anti-Xpress (against His_6 rabaptin) followed by Cy3-labeled anti-mouse IgG (single transfectants) or

Alexa488-labeled anti-mouse IgG (double transfectants). A semiquantitative estimate of the effect of rabaptin-5 on Tf kinetics can be made by selecting five rabaptin-5α-positive endosomes (per cell and per chase time) and count how many of those contain Tf. This analysis is performed on 10 cells per time point and in duplicate transfections.

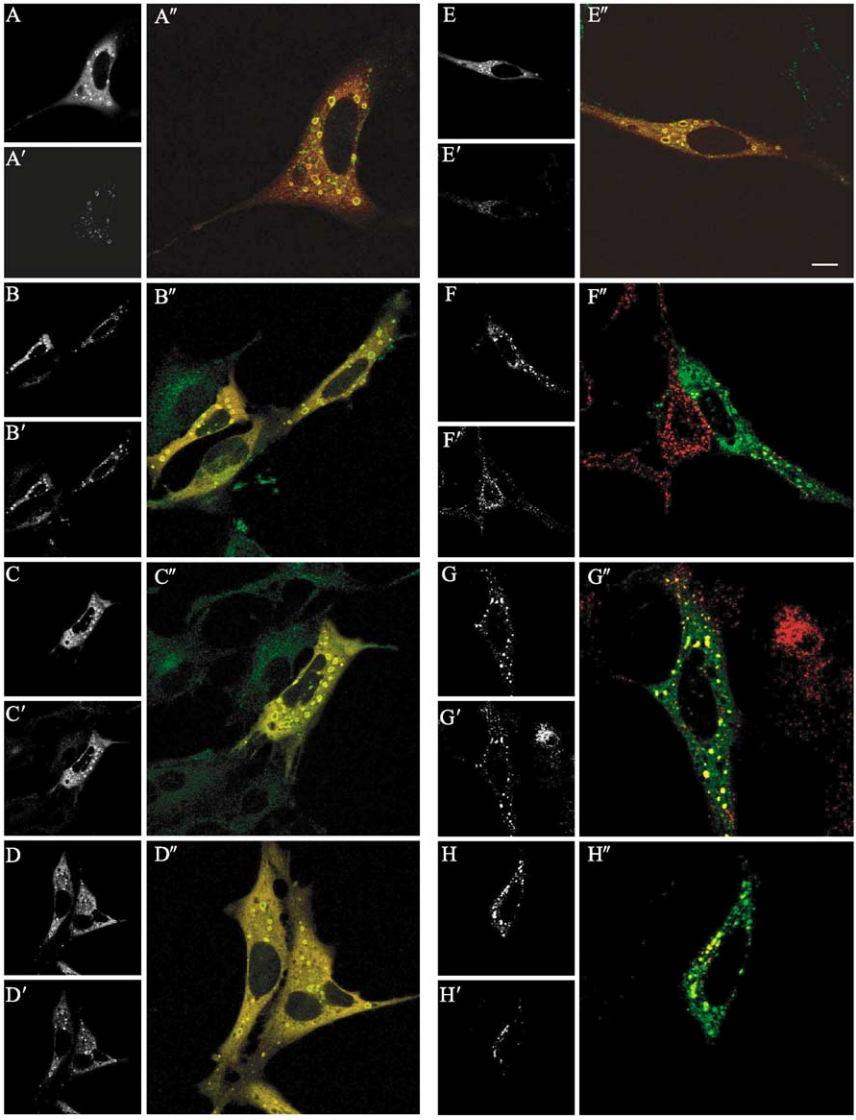

FIG. 3. (*continued*)

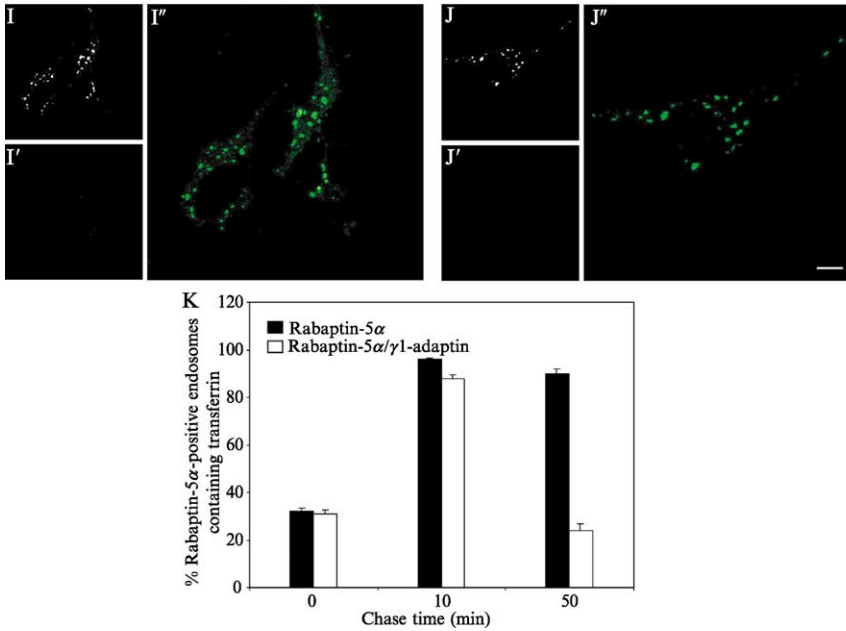

FIG. 3. Transfected rabaptin-5α delays transferrin recycling. HeLa cells were transfected with pcDNA3.1 His-rabaptin-5α (A–E) alone or in combination with pcDNA3-γ1-adaptin (F–J). The cells were incubated with 15 μg/ml Alexa488-Tf (A–E) or Alexa594-Tf (F–J) at 16° for 30 min, subsequently chased at 37° for 0 min (A′, F′), 10 min (B′,G′), 20 min (C′, H′), 40 min (D′, I′), and 50 min (E′, G′) and labeled with anti-Xpress followed by Cy3-labeled anti-mouse IgG (A–E) or Alexa488-labeled anti-mouse IgG (F–J). Merged images are shown in A″–J″. Bar = 10 μm. (K) Quantitation of the fraction of rabaptin-5α-positive endosomes containing Alexa-Tf at 0, 10, and 50 min of chase. Error bars denote the standard deviation ($n = 10$). Note that entry of Alexa-Tf into early endosomes is the same in single rabaptin-5α transfectants as in the double transfectants, showing that Rab5-dependent internalization is not affected. Recycling of Alexa488-Tf in the pcDNA3.1 His-rabaptin-5α transfectants, however, is strongly diminished compared to pcDNA3.1 His-rabaptin-5α/pcDNA3-γ1-adaptin transfected (or control) cells. Others reported a similar negative regulatory function of rabaptin-5 in an *in vitro* endosome recycling assay (Pagano *et al.*, 2004) (Adapted with permission from Denaka *et al.*, 2003a).

Comments. We perform the pulse on a 16° waterbath and the chase on a 37° waterbath. This allows for better temperature control and allows precise chase times to be obtained, which is especially important for short periods of chase.

Concluding Remarks

Rabaptin-5 and rabaptin-5α belong to a rapidly expanding group of accessory proteins that bind with low affinity to the heterotetrameric AP-1

and monomeric GGA adaptors (Hirst *et al*., 2001; Mattera *et al*., 2003; Mills *et al*., 2003; Neubrand *et al*., 2005; Ritter *et al*., 2004; Shiba *et al*., 2002). It is reasonable to assume that they assist in AP-1 and GGA-dependent formation of clathrin-coated vesicles from intracellular membranes. To understand, the precise mechanism of their activity, however, needs further investigations, especially since some of these acessory proteins also bind to the plasma membrane adaptor AP-2. The indirect link between the active form of the small GTPase Rab4 and AP-1 suggests an extra layer of control of AP-1 function and testifies to the complexity of the mechanisms controlling membrane dynamics between endosomes, the plasma membrane, and the TGN. The assays described in this chapter provide tools that might contribute to our understanding of regulatory factors in transport pathways from endosomes.

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