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In Vitro Formation of Recycling Vesicles from Endosomes Requires Adaptor Protein-1/Clathrin and Is Regulated by Rab4 and the Connector Rabaptin-5

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The involvement of clathrin and associated adaptor proteins in receptor recycling from endosomes back to the plasma membrane is controversial. We have used an in vitro assay to identify the molecular requirements for the formation of recycling vesicles. Cells expressing the asialoglycoprotein receptor H1, a typical recycling receptor, were surface biotinylated and then allowed to endocytose for 10 min. After stripping away surface-biotin, the cells were permeabilized and the cytosol washed away. In a temperature-, cytosol-, and nucleotide-dependent manner, the formation of sealed vesicles containing biotinylated H1 could be reconstituted. Vesicle formation was strongly inhibited upon immunodepletion of adaptor protein (AP)-1, but not of AP-2 or AP-3, from the cytosol, and was restored by readdition of purified AP-1. Vesicle formation was stimulated by supplemented clathrin, but inhibited by brefeldin A, consistent with the involvement of ARF1 and a brefeldin-sensitive guanine nucleotide exchange factor. The GTPase rab4, but not rab5, was required to generate endosome-derived vesicles. Depletion of rabaptin-5/rabex-5, a known interactor of both rab4 and γ-adaptin, stimulated and addition of the purified protein strongly inhibited vesicle production. The results indicate that recycling is mediated by AP-1/clathrin-coated vesicles and regulated by rab4 and rabaptin-5/rabex-5.

INTRODUCTION

Early endosomes are a major sorting station for proteins and membranes in eukaryotic cells (Gruenberg, 2001; Maxfield and McGraw, 2004). They receive material from the cell surface by endocytosis and from the exocytic pathway via the *trans*-Golgi network (TGN), and they distribute it further to late endosomes and back to the TGN and the plasma membrane. Transport receptors like the transferrin receptor, the low-density lipoprotein (LDL) receptor, and the asialoglycoprotein (ASGP) receptor cycle continuously between the plasma membrane and early endosomes (Spiess, 1990; Trowbridge *et al*., 1993). Receptor-positive early endosomes can be subdivided into sorting endosomes and recycling endosomes (or endocytic recycling compartment, ERC). Primary endocytic vesicles fuse to sorting endosomes where ligands dissociate from their receptors because of a reduced internal pH. The receptors exit into tubular membranes that form recycling endosomes, whereas the ligands with the main fluid volume mature to late endosomes ("geometry-based sorting"; Maxfield and McGraw, 2004). There seem to be two main recycling pathways from early endosomes to the plasma membrane: a fast one directly from sorting endosomes and a slower one via recycling endosomes (Sheff *et al*., 1999; Hao and Maxfield, 2000; van Dam *et al*., 2002).

The mechanisms to generate endosome-derived recycling vesicles are not clear, because there are seemingly contradictory findings. In general, formation of transport vesicles between organelles of the endocytic and secretory pathways requires cytosolic coat proteins to be recruited at the membrane of the donor compartment. Among the established coat complexes, clathrin is classically responsible for endocytosis from the plasma membrane in combination with the adaptor protein (AP) complex AP-2 and for TGN-to-endosome transport with AP-1 adaptors (Hirst and Robinson, 1998). However, clathrin coats were also detected on endosomal membranes positive for the transferrin receptor (Stoorvogel *et al*., 1996). Consistent with a role of clathrin-coated vesicles in receptor recycling, expression of a temperature-sensitive mutant of dynamin, a GTPase involved in clathrin-coated vesicle release (van der Bliek, 1999), inhibited recycling of transferrin receptor (van Dam and Stoorvogel, 2002). In addition, brefeldin A (BFA), an inhibitor of guanine nucleotide exchange factors of the GTPase ADP-ribosylation factor 1 (ARF1) involved in recruiting AP-1– and AP-3–containing coats, partially inhibited transferrin receptor recycling in nonpolarized cells (van Dam and Stoorvogel, 2002; van Dam *et al*., 2002). Overexpression of rabaptin-5, an interactor of the γ-subunit of AP-1 (Hirst *et al*., 2000; Shiba *et al*., 2002) as well as of rab4 and rab5 (Vitale *et al*., 1998), was shown to inhibit transferrin receptor recycling, unless γ-adaptin was overexpressed simultaneously (Deneka *et al*., 2003).

AP-1/clathrin coats on endosomes may, however, be involved in other functions than recycling to the plasma membrane (Hinners and Tooze, 2003): Shiga toxin B-chain, which

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Abbreviations used: AP, adaptor protein; ARF1, ADP-ribosylation factor 1; ASGP, asialoglycoprotein; BFA, brefeldin A; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; TGN, *trans*-Golgi network.

is transported via endosomes to the Golgi, was found to be colocalized in recycling endosomes with AP-1 and clathrin and accumulated in transferrin receptor-positive endosomes upon BFA treatment (Mallard *et al*., 1998). In cells lacking the μ1A subunit of AP-1, the mannose-6-phosphate receptor was found to accumulate in endosomes, rather than in the TGN, supporting an AP-1–dependent pathway from endosomes to the TGN (Meyer *et al*., 2000). Furthermore, retrograde transport of furin and other proteins containing acidic-cluster motifs from endosome-to-TGN is mediated by the protein PACS-1 and its interaction with AP-1 (Crump *et al*., 2001).

Some experiments directly argued against an involvement of clathrin-coated vesicles in receptor recycling. Perturbation of clathrin coat formation by regulated expression of a dominant-negative clathrin hub domain in HeLa cells reduced endocytosis by ~50% and caused perinuclear aggregation of early endosomes, but it reduced recycling by only ~20% (Bennett *et al*., 2001). Similarly, drug-induced cross-linking of an FK506-binding protein-clathrin light-chain fusion, which dominantly interferes with clathrin function, reduced LDL or transferrin uptake by half, but minimally affected receptor recycling in Chinese hamster ovary (CHO) cells (Moskowitz *et al*., 2003). Elimination of clathrin heavy-chain in DT40 lymphocytes substantially inhibited receptor-mediated and fluid phase internalization, but it reduced transferrin receptor recycling by only ~50% (Wettey *et al*., 2002).

Another consideration is that generally clathrin/adaptor coats mediate cargo concentration by interaction with specific sorting signals, whereas receptor recycling in nonpolarized cells seems to be signal independent. Mutation or deletion of the cytoplasmic domain of the transferrin receptor, for example, resulted in a reduction of endocytosis, but it did not significantly affect its recycling rate (Jing *et al*., 1990; Johnson *et al*., 1993). An isoform of the μ1 subunit of AP-1 complexes, μ1B, has recently been discovered in epithelial cells and shown to mediate basolateral sorting of the receptors for transferrin, LDL, and ASGPs in a signal-dependent manner (Ohno *et al*., 1999; Sugimoto *et al*., 2002; Fölsch *et al*., 2003). Indeed, AP-1B was found to mediate specific basolateral recycling from endosomes (Gan *et al*., 2002), supporting earlier proposals that implicated clathrin/AP-1 coats in polarized cells in recycling transferrin receptor back to the basolateral surface (Futter *et al*., 1998). Consistent with this, BFA treatment caused loss of polarity in recycling to the cell surface in Madin-Darby canine kidney (MDCK) cells (Futter *et al*., 1998; Mohrmann *et al*., 2002).

The apparent discrepancy between experimental results and their interpretations may be due to the complexity and plasticity of the endosomal system. Long-term experiments such as overexpression or elimination of proteins, which takes hours to days to take effect, may cause indirect effects or adaptation. In this study, we have reconstituted the formation of recycling vesicles in vitro by using permeabilized cells. Because the formation of a single cohort of vesicles is analyzed, indirect effects on the organization of endosomes are unlikely to affect the results. We found that formation of endosome-derived vesicles containing ASGP receptor depends on AP-1 and clathrin, requires rab4, and is inhibited by rabaptin-5, an interactor of both AP-1 and rab4.

MATERIALS AND METHODS

Reagents and Antibodies

Cell culture media and reagents were obtained from Invitrogen (Carlsbad, CA). Adenylyl imidodiphosphate (AMP-PNP), guanylyl imidodiphosphate (GMP-PNP), ATP, GTP, creatine kinase, and creatine phosphate were from

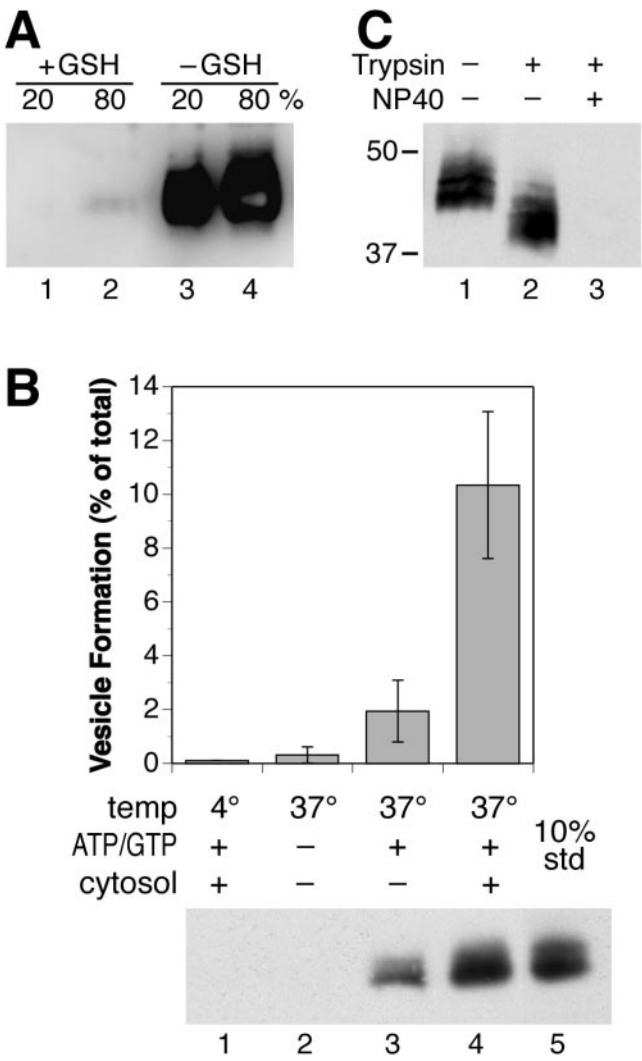


Figure 1. Biotinylated, internalized ASGP receptor H1 is released from broken cells in a temperature-, energy-, and cytosol-dependent manner in sealed membranes. (A) Cells expressing H1 were surface labeled with sulfo-NHS-SS-biotin on ice, washed, and then incubated with reduced glutathione (+GSH) to release the coupled biotin (lanes 1 and 2) or without glutathione (–GSH; lanes 3 and 4). On cell lysis, biotinylated proteins were isolated with avidin-agarose. Twenty and 80% of each sample were separated by SDS-gel electrophoresis and analyzed by immunoblotting by using anti-myc antibody. Stripping of surface-bound biotin by glutathione is essentially complete. (B) Surface-biotinylated cells were incubated at 37°C for 10 min to allow internalization, chilled in ice, and surface biotin was stripped with reduced glutathione. The cells were then broken and incubated for 30 min at 4 or 37°C, with or without cytosol (1.2 mg/ml) or nucleotides and energy-regenerating system as indicated. Cells were pelleted and the supernatant was lysed and analyzed by avidin precipitation of biotinylated proteins, SDS-gel electrophoresis, and immunoblotting by using anti-myc antibody. Values were normalized to the amount of total labeled H1 by analyzing 10% of a sample before centrifugation as a standard (10% std; lane 5). Average and SD of at least four independent determinations are shown. (C) The supernatant of cells treated as in B, lane 4, was incubated at 4°C for 45 min with or without trypsin in the presence or absence of detergent (NP-40). Trypsin was stopped with soybean trypsin inhibitor. Biotinylated proteins were then isolated, separated by SDS-gel electrophoresis, and analyzed by immunoblotting for myc-tagged H1. The positions of molecular weight markers are indicated (in kilodaltons).

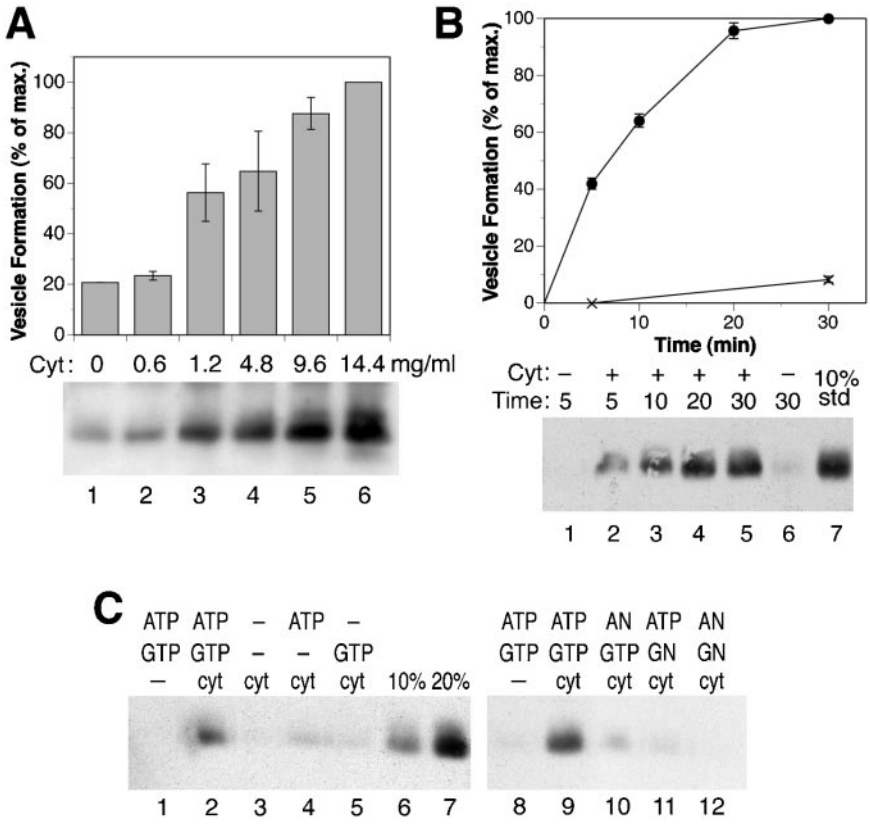


Figure 2. Vesicle formation depends on cytosol concentration, time, and hydrolyzable ATP and GTP. (A) The assay for the formation of endosomal vesicles was performed using increasing concentrations of cytosol at 37°C in the presence of ATP, GTP, and an ATP-regenerating system. Recovery of biotinylated H1 in the supernatant as determined by immunoblot analysis was quantified and plotted relative to the maximal value. The data represent the average and SD of three independent determinations. (B) Biotinylated broken cells were incubated in the presence of ATP, GTP, and an ATP-regenerating system at 37°C with or without cytosol (12.5 mg/ml) for increasing times. Recovery of biotinylated H1 in the supernatant was plotted relative to the maximal value (●, with cytosol; ×, without cytosol). (C) Vesicle formation was assayed in the presence of cytosol (12.5 mg/ml) with GTP or GMP-PNP (GN), and ATP or AMP-PNP (AN) or without nucleotides. Lanes 1–7 and 8–12 are from separate experiments.

Roche Diagnostics (Indianapolis, IN); sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin) and avidin-Sepharose were from Pierce Chemical (Rockford, IL); protein A-Sepharose was from Zymed Laboratories (South San Francisco, CA), protein G-Sepharose was from Gerbu (Gaiberg, Germany); and LY294002 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Mouse monoclonal anti-γ-adaptin (100/3) and anti-α-adaptin (100/2) antibodies, horseradish peroxidase-coupled anti-mouse IgG and anti-rabbit IgG antibodies, reduced glutathione, and BFA were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-myc (9E10) and anti-clathrin (X22) antibodies were purified using protein A-Sepharose from culture media of hybridomas obatained from American Type Culture Collection (Manassas, VA); anti-α-adaptin (AP6) was from Affinity Bioreagents (Golden, CO); and anti-rabaptin-5 antibodies were from BD Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-rab5 antibody (CL621.3) was a kind gift from Reinhard Jahn (Max Planck Institute, Göttingen, Germany) and Jean Gruenberg (University of Geneva, Geneva, Switzerland), and anti-rab4 antibody was from Bruno Goud (Institut Curie, Paris, France). A rabbit antiserum was raised against a glutathione *S*-transferase fusion construct with residues 22–756 of δ-adaptin (a gift of Margaret Robinson, University of Cambridge, Cambridge, United Kingdom) (Simpson *et al.*, 1997).

In Vitro Assay for the Formation of Recycling Vesicles

The MDCK (strain II) cell line stably expressing the human ASGP receptor subunit H1 with a C-terminal myc-tag has been described previously (Leitinger *et al.*, 1995). Semiconfluent cells of four 15-cm plates were washed three times with ice-cold phosphate-buffered saline (PBS) supplemented with 0.7 mM $CaCl_2$, 0.25 mM $MgCl_2$ (PBS^{++}), and surface-biotinylated with 1 mg/ml sulfo-NHS-SS-biotin in the same buffer at 4°C for 30 min. The reaction was quenched by washing the cells three times with PBS^{++}, by a 5-min incubation with 50 mM glycine in PBS, and by another three washes with PBS^{++}. The cells were then incubated in prewarmed serum-free medium (minimal essential medium; Invitrogen) containing 20 mM HEPES (pH 7.4) for 10 min at 37°C to allow internalization of biotinylated surface proteins. Cells were rinsed with ice-cold PBS^{++}. Biotin at the cell surface was stripped by two 20-min incubations with 50 mM reduced glutathione, 75 mM NaCl, 75 mM NaOH, 1 mM EDTA, with 1% bovine serum albumin. The cells were rinsed twice with PBS^{++} and incubated for 5 min with 5 mg/ml iodoacetamide in PBS^{++} to quench any residual glutathione. After two additional rinses with PBS^{++}, the cells were permeabilized by incubation in swelling buffer (15 mM HEPES/KOH, pH 7.2, 15 mM KCl) for 15 min at 4°C, scraped in transport buffer [20 mM HEPES/KOH, pH 7.2, 90 mM KOAc, 2 mM $Mg(OAc)_2$], and sedimented at 800 × *g* for 5 min. The broken cells were resuspended twice in stripping buffer [20 mM HEPES/KOH, pH 7.2, 500 mM KOAc, 2 mM $Mg(OAc)_2$] for 10 min on ice, pelleted again, and resuspended in transport buffer.

In a standard assay, permeabilized cells at 0.5 mg/ml protein were incubated with 1.2 mg/ml cytosol and an ATP-regenerating system (2 mM ATP, 4 mM GTP, 12 mM creatine phosphate, 320 μg/ml creatine kinase) in a total volume of 200 μl. The reaction mixture was left on ice for 5 min and then incubated at 37°C for 30 min. Reactions were stopped on ice and centrifuged at 800 × *g* for 5 min. The supernatants were carefully aspirated and solubilized for 1 h at 4°C with lysis buffer (1% Triton X-100, 0.5% deoxycholate in PBS, 2 mM phenylmethylsulfonyl fluoride) containing protease inhibitor cocktail (500-fold diluted from 5 mg/ml benzamidine, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 1 mg/ml antipain, 1 mg/ml chymostatin in 40% dimethyl sulfoxide and 60% ethanol) for 1 h. Insoluble material was removed by centrifugation in a microcentrifuge at 14,000 rpm for 10 min. Supernatants were recovered and rotated end over end for 1 h at 4°C with 40 μl of avidin-Sepharose. The beads were washed three times with lysis buffer and boiled in SDS-sample buffer. Proteins were separated by SDS-gel electrophoresis and transferred to polyvinylidene difluoride membrane that was then incubated in blocking buffer (PBS with 0.1% Tween 20 and 5% nonfat dry milk) for 30 min and with anti-myc antibody in blocking buffer for 1 h at room temperature or overnight at 4°C. Antibody was detected using horseradish peroxidase-conjugated anti-mouse secondary antibody and the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Cytosol and Immunodepletion

Cytosol was obtained from calf brain as the high-speed supernatant after homogenization (Campbell *et al.*, 1984), supplemented with protease inhibitors. For immunodepletion, a 1:1 mixture of protein A- and protein G-Sepharose beads, was first saturated with 5 mg/ml bovine serum albumin overnight at 4°C, washed with transport buffer, incubated overnight with anti-α-adaptin (AP6), anti-δ-adaptin, anti-rabaptin-5, or anti-rab5 antibody in transport buffer, and washed extensively with transport buffer. Fifty microliters of packed beads was then incubated with 200 μl (0.75 mg) of brain cytosol for 3 h at 4°C with gentle rocking. For depletion of AP-1 and rab4, the cytosol was first incubated overnight at 4°C with anti-γ-adaptin or anti-rab4 antibody, respectively, before albumin-saturated protein A/G-Sepharose beads were added for 3 h at 4°C. After centrifugation, the supernatant was collected and the beads were washed. Bound and unbound proteins were analyzed by immunoblotting by using the corresponding specific antibodies

(to detect α-adaptin on blots, antibody 100/2 was used). As a control, cytosol was mock treated with beads without antibody.

Protein Purification

Clathrin-coated vesicles were isolated from calf brains as described previously (Campbell *et al*., 1984). The coats were released and fractionated on a Superose 6 column as described previously (Crottet *et al*., 2002). Clathrin-containing fractions were collected and dialyzed against 20 mM ethanolamine, pH 8.9, 2 mM EDTA, 1 mM dithiothreitol, and loaded onto a Mono-Q HR 5/5 column (Amersham Biosciences). Elution with a 5-ml linear gradient of 0–150 mM NaCl, followed by a 50-ml gradient of 150–450 mM NaCl in the same buffer yielded pure clathrin (adapted from Ahle *et al*., 1988). AP-1 was purified from bovine adrenal medulla as described previously (Crottet *et al*., 2002). Native rabaptin-5/rabex-5 complex was purified from calf brain cytosol according to Horiuchi *et al*. (1997), followed by immunodepletion of AP-1, which was a major contaminant. His_6-tagged rabaptin-5 in complex with rabex-5 purified from Sf9 insect cells infected with recombinant baculovirus as described previously (Lippe *et al*., 2001), was a generous gift by Marino Zerial (Max Planck Institute, Dresden, Germany).

Electron Microscopy

F(ab′) fragments of anti-ASGP receptor antibodies were covalently attached to Nanogold (Nanoprobes, Yaphank, NY) to minimize steric effects essentially as described by Hainfeld (1987). The IgG fraction of a polyclonal antiserum was isolated with protein A-Sepharose, digested with 4% pepsin in Na-citrate, pH 4.5, for 1 h at 37°C, dialysed against 0.1 M Na-phosphate, pH 6, 0.5 mM EDTA, and reduced for 1 h at room temperature with mercaptoethylamine hydrochloride (13.3 mg/mg antibody). The mixture was desalted on a Sephadex PD-10 gel filtration column equilibrated with 20 mM Na-phosphate, 150 mM NaCl, 1 mM EDTA, pH 6.5, and incubated with threefold molar excess of monomaleimido-Au1.4 nm solution for 18 h at 4°C. Unbound gold particles were separated from antibody conjugates by gel filtration on a Superose-12 column.

Cells were incubated with nanogold-coupled F(ab′) fragments in PBS at 4°C for 1 h, allowed to internalize at 37°C for 10 min, and processed for in vitro vesicle formation. The resulting vesicle supernatant was fixed with 3% formaldehyde and 0.2% glutaraldehyde for 2 h at room temperature and then pelleted by centrifugation at 100,000 × *g* for 1 h. The pellet was washed with 0.1 M phosphate buffer, pH 7.4, and free aldehyde groups were quenched by incubation in 50 mM NH_4Cl for 30 min at room temperature. After three rinses with phosphate buffer, the sample was processed for cryosectioning according to Liou *et al*. (1996). Briefly, the pellet was mixed with 10% gelatin, cooled on ice, cut into small pieces and infiltrated with 2.3 M sucrose overnight at 4°C, frozen in liquid nitrogen on cutting pins, and cryosectioned at −120°C by using a Leica Ultracut UCT ultramicrotome. Sections were thawed and transferred to Formvar-coated nickel grids. The nanogold marker was enhanced by silver (HQ Silver enhancement kit; Nanoprobes). To localize AP-1 complexes, they were labeled with monoclonal mouse anti-γ-adaptin antibodies followed by goat anti-mouse IgG conjugated to 10-nm colloidal gold (British Biocell International, Cardiff, United Kingdom). Grids were stained and dried as described (Liou *et al*., 1996) and viewed with a Philips CM10 electron microscope.

RESULTS

In Vitro Formation of Recycling Vesicles Is Dependent on Temperature, Energy, and Cytosol

The ASGP receptor is a hepatic transport receptor responsible for the rapid clearance of galactosyl-terminal glycoproteins from the circulation (Ashwell and Harford, 1982; Spiess, 1990). The cytoplasmic domain of its major subunit H1 contains a tyrosine-based sorting signal for efficient endocytosis and basolateral sorting (Geffen *et al*., 1993; Fuhrer *et al*., 1994). To study receptor recycling from endosomes, we used an MDCK cell line stably expressing myc-tagged H1 and monitored the release of vesicles containing H1 from endosomes in broken cells under various conditions. To obtain specificity for early endosomes as the starting compartment for vesicle formation, surface proteins of intact cells were first biotinylated at 4°C with sulfo-NHS-SS-biotin, an impermeant amine-specific reagent coupled to biotin by a disulfide bond. The cells were then incubated for 10 min at 37°C to allow biotinylated H1 to be endocytosed to early/recycling endosomes. Back at 4°C, biotin was stripped from the cell surface by incubation with reduced glutathione, whereas internalized biotinylated H1 was protected. Cells were then permeabilized by swelling and scraping and washed with high-salt buffer to remove cytosol, proteins peripherally associated with the cytosolic face of membranes, and free transport vesicles. These permeabilized, washed cells were incubated with or without added bovine brain cytosol, ATP, and GTP at 37°C for 30 min. Any vesicles released during this incubation were recovered in the supernatant after low-speed centrifugation. On lysis, biotinylated molecules were isolated with avidin-Sepharose and sepa-

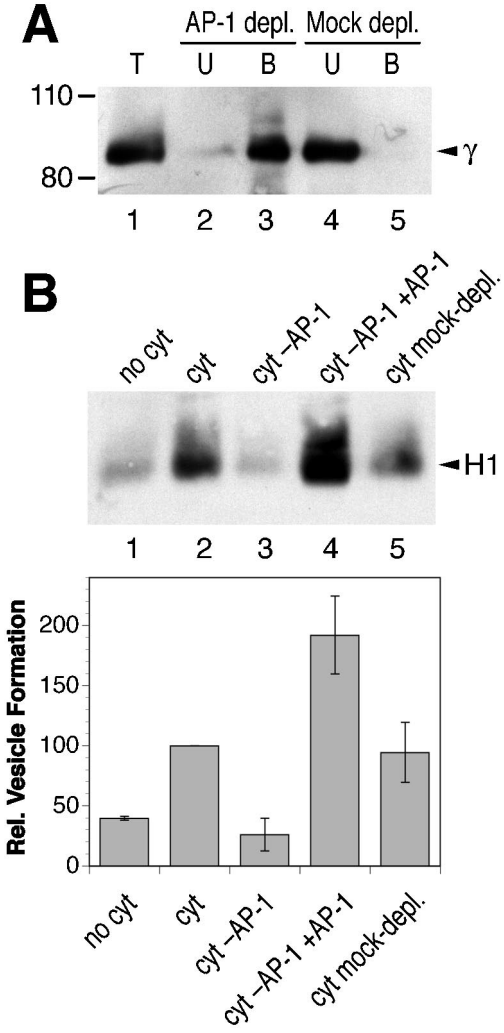


Figure 3. AP-1 adaptors are necessary for endosomal vesicle formation in vitro. (A) Bovine brain cytosol was immunodepleted of AP-1 by using anti-γ-adaptin antibody and protein A/G-Sepharose beads. Total cytosol (T) and corresponding aliquots of the depleted cytosol (unbound fraction, U) and the bound material (B) were analyzed by immunoblotting with anti-γ-adaptin antibody. Depletion efficiency was >80%. The positions of molecular weight markers are indicated (in kilodaltons). (B) Biotinylated permeabilized cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol (no cyt), with untreated cytosol (cyt; 1.2 mg/ml), with AP-1–depleted cytosol (cyt–AP-1), with AP-1–depleted cytosol that had been supplemented with 12 μg/ml purified AP-1 (cyt–AP-1+AP-1), or with mock-depleted cytosol. Immunoblot analysis of biotinylated H1 in the supernatant after cell pelleting is shown for a representative experiment. Quantitation of three independent experiments (average with SD) is presented below.

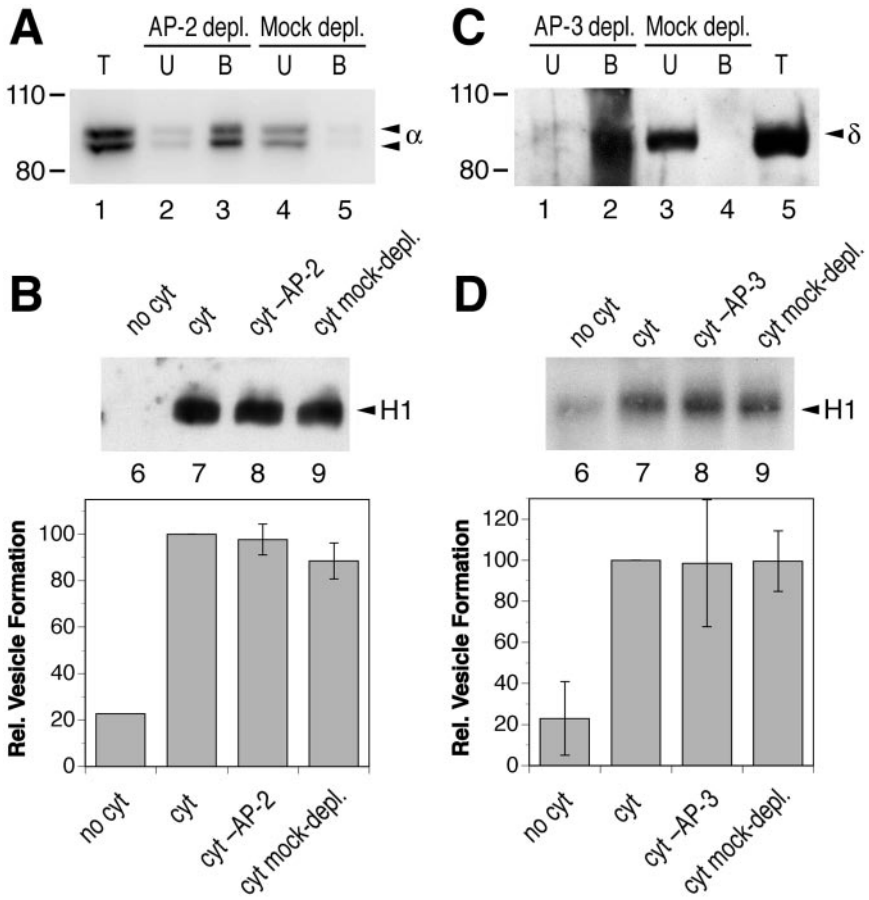


Figure 4. Depletion of AP-2 or AP-3 adaptors does not affect in vitro vesicle formation from labeled endosomes. Bovine brain cytosol was immunodepleted for AP-2 (A) or AP-3 adaptors (C) by using anti-α- and anti-δ-adaptin antibodies, respectively, as in Figure 3A for AP-1. Depletion efficiency was >90% in both cases. Biotinylated permeabilized cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol (no cyt), with untreated cytosol (cyt; 1.2 mg/ml), with adaptor-depleted cytosol (cyt–AP-2 or -3), or with mock-depleted cytosol (B for AP-2 and D for AP-3). Immunoblot analysis of biotinylated H1 in the supernatant after cell pelleting is shown for a representative experiment. Quantitation of three independent experiments (average with SD) is presented below.

rated by SDS-gel electrophoresis, and H1 was visualized by immunoblot analysis.

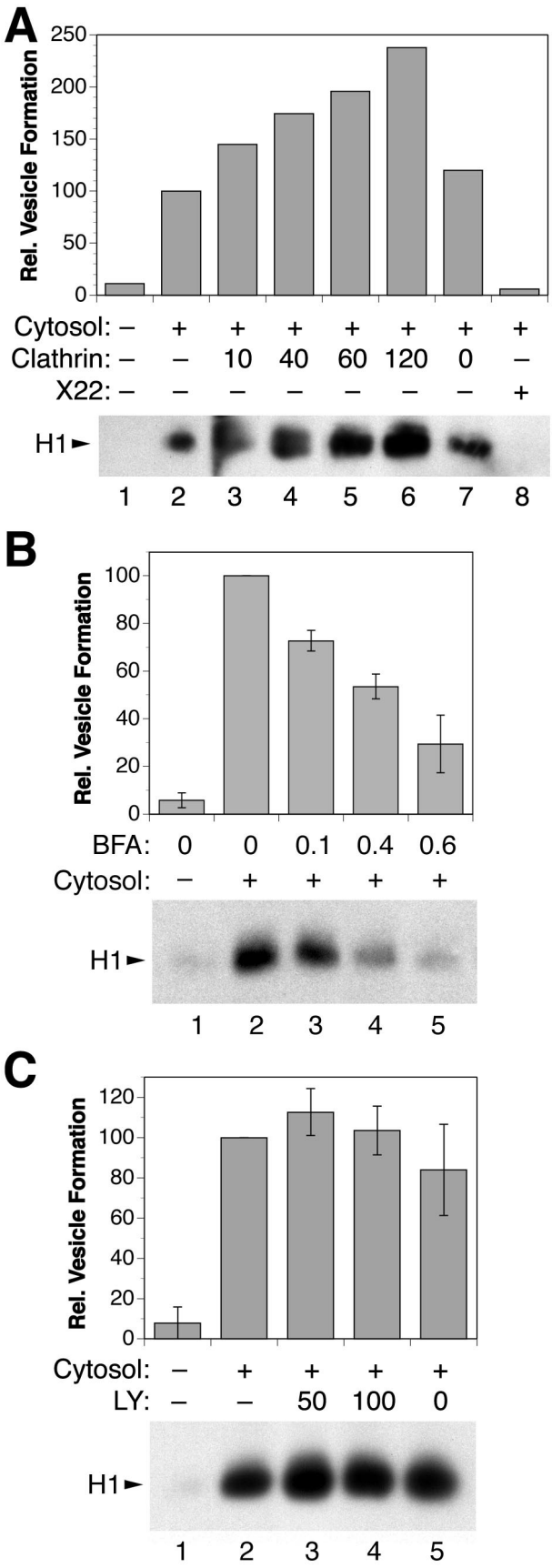
As is shown in Figure 1A in a control experiment without internalization, removal of biotin from the labeled cell surface by reduced glutathione is essentially complete, excluding the possibility that vesicles containing biotinylated H1 generated in the assay may have been derived from the plasma membrane directly. When labeled cells were allowed to internalize before surface-stripping and permeabilization, no vesicles were released upon incubation at 37°C without additions (Figure 1B, lane 2) or when incubated with nucleotides and cytosol at 4°C (lane 1). However, in the presence of ATP, GTP, and 1.2 mg/ml cytosol at 37°C, typically ~10% of biotinylated H1 in the starting material was recovered in the supernatant (lane 4). In the absence of added cytosol, nucleotides supported a basal release of H1 into the supernatant of typically ~20% of that in the presence of cytosol (lane 3), most likely due to residual cytosolic proteins and/or coat proteins already recruited to the membranes that had not been removed by the high-salt washes. The amount of H1 released independently of added cytosol was somewhat variable between experiments, most likely because of differences in the removal of cytosolic proteins from the broken cells.

To test whether H1 was released in sealed vesicles, the supernatant of permeabilized cells incubated with exogenous cytosol and nucleotides was subjected to a protease protection assay (Figure 1C). Released H1 was resistant to added trypsin except for the 40-amino acid cytoplasmic portion of H1, resulting in an electrophoretic shift of ~4 kDa. Only upon solubilization of membranes by detergent was H1 completely digested by trypsin.

The amount of H1-containing vesicles generated in the in vitro reaction depended on the concentration of cytosol used (Figure 2A). It increased with higher cytosol concentrations up to ~10 mg/ml protein. In most of the following experiments, we used a limiting amount of cytosol of 1.2 mg/ml to more sensitively detect effects of depletion or addition of individual components. In a time-course experiment, formation of H1-containing vesicles was detectable already within 5 min of incubation and reached a maximum after ~20 min (Figure 2B). Unless specified, vesicles were harvested after 30-min incubation at 37°C in all subsequent experiments. Efficient formation of H1-containing vesicles required the presence of both ATP and GTP (Figure 2C, lanes 1–5), indicating the involvement of ATPase(s) and GTPase(s). The nonhydrolyzable analogues AMP-PNP and GMP-PNP did not substitute for ATP and GTP, respectively (Figure 2C, lanes 8–12), suggesting that nucleotide hydrolysis is essential.

Clathrin and AP-1 Adaptors, but Not AP-2 or AP-3, Are Involved in Generating Recycling Vesicles

To investigate whether the formation of H1-containing recycling vesicles is mediated by clathrin adaptors, we analyzed the effect of cytosol immunodepleted for AP-1, AP-2, or AP-3 adaptors. On depletion of >80% of AP-1 by using an anti-γ-adaptin antibody (Figure 3A, lane 2), vesicle formation was strongly inhibited by ~75% compared with the level obtained with control cytosol or mock-depleted cytosol (Figure 3B). Readdition of purified AP-1 isolated from cloth-



rin-coated vesicles of bovine adrenal glands to the depleted cytosol fully restored vesicle formation even beyond the initial extent (Figure 3B, lane 4). This confirms that the inhibition by immunodepletion was due to the removal of AP-1 itself, rather than a component that might have been associated with cytosolic AP-1.

In contrast to AP-1, depletion of AP-2 or AP-3 did not affect formation of H1-containing vesicles (Figure 4). AP-2 was depleted to <10% by using an anti-α-adaptin antibody (Figure 4A). Generation of recycling vesicles was unchanged by AP-2 depletion (Figure 4B). Because AP-2 complexes function in endocytosis, this confirms that the H1-containing vesicles generated in our assay were not derived from the plasma membrane. AP-3 adaptors have been localized to endosomal membranes and are involved in protein sorting toward lysosomes (Dell'Angelica *et al*., 1998). AP-3 was depleted to ~10% by using an anti-δ-adaptin antibody (Figure 4C). Compared with control cytosol, no significant variation of vesicle formation was observed with AP-3–depleted cytosol (Figure 4D).

If AP-1 adaptors are part of the coat to form recycling vesicles, clathrin is likely to be involved as well. Supplementing purified clathrin to the cytosol enhanced the formation of H1-containing endosomal vesicles up to more than twofold in a dose-dependent manner (Figure 5A, lanes 1–7). Preincubation of the cytosol with the anti-clathrin antibody X22 (Brodsky *et al*., 1987) strongly inhibited vesicle formation (Figure 5A, lane 8). X22 has been shown to bind to an epitope in the clathrin heavy chain and to inhibit in vivo both fluid-phase and receptor-mediated endocytosis (Doxsey *et al*., 1987; Draper *et al*., 1990). Furthermore, AP-1 recruitment generally involves the small GTPase ARF1 and guanine nucleotide exchange factors sensitive to BFA. Indeed, increasing concentrations of BFA added to the cytosol resulted in increasing inhibition of vesicle formation (Figure 5B), although only at high concentrations. These two findings support the notion that H1-containing recycling vesicles were generated by AP-1/clathrin coats.

In vivo, transferrin receptor recycling was shown to be partially sensitive to wortmannin and LY294002, inhibitors of phosphatidyl inositol 3-kinase (van Dam *et al*., 2002). Addition of 50 or 100 μM LY294002, however, had no inhibitory effect on the in vitro formation of endosome-derived vesicles containing biotinylated H1 (Figure 5C).

Figure 5. Clathrin stimulates and BFA inhibits in vitro formation of recycling vesicles. (A) Biotinylated permeabilized cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol, with cytosol (1.2 mg/ml), or with cytosol supplemented with up to 120 μg/ml purified clathrin (0 indicates the addition of solvent only). In a further reaction, cytosol preincubated for 15 min at 4°C with 40 μg/ml anti-clathrin antibody X22 was used. Immunoblot analysis and quantitation of biotinylated H1 in the supernatant after cell pelleting is shown for a representative experiment. (B) Biotinylated and permeabilized cells were incubated with nucleotides, ATP-regenerating system with and without cytosol, and the indicated amount of BFA first at 4°C for 15 min and then for 10 min at 37°C. Immunoblot analysis of biotinylated H1 in the supernatant after cell pelleting is shown together with the quantitation of three independent experiments (average with SD). (C) Biotinylated and permeabilized cells were incubated with nucleotides, ATP-regenerating system with and without cytosol, and up to 100 μM phosphatidyl inositol 3-kinase inhibitor LY294002 (LY; 0 indicates the addition of solvent only). Immunoblot analysis of biotinylated H1 in the supernatant after cell pelleting is shown together with the quantitation of two independent experiments (average with range).

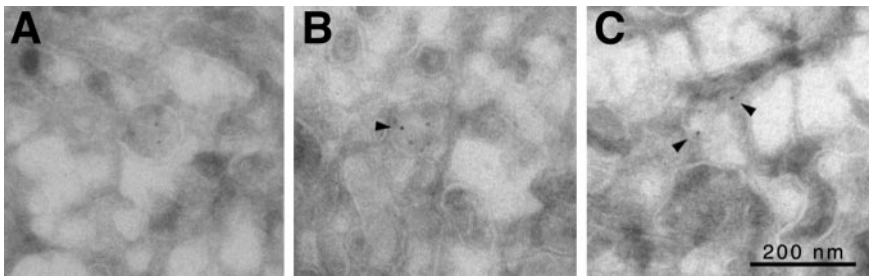


Figure 6. Electron microscopic visualization of H1-containing endosomal vesicles. (A) H1 at the cell surface was decorated with nanogold-coupled anti-H1 F(ab') fragments and allowed to internalize for 10 min. Cells were stripped of surface antibody; permeabilized; incubated with cytosol, nucleotides, and ATP-regenerating system; and sedimented. Membranes in the supernatant were collected by high-speed centrifugation and subjected to cryoelectron microscopy and silver enhancement of nanogold. (B and C) As in A, but in addition decorated with anti-γ-adaptin and a secondary anti-mouse IgG antibody coupled to 10-nm colloidal gold. Arrowheads indicate colloidal gold particles, which have a more dense appearance than silver-enhanced nanogold particles. Bar, 200 nm.

Visualization of In Vitro-generated Vesicles Containing H1

To morphologically characterize the recycling vesicles generated in our assay, H1 at the cell surface was first decorated at 4°C with nanogold-labeled F(ab') fragments generated from rabbit anti-ASGP receptor immunoglobulins and then allowed to internalize for 10 min. F(ab') at the cell surface was removed by acid washes, and the cells were then permeabilized and incubated with nucleotides and cytosol as described above. Membranes recovered in the low-speed supernatant were collected, concentrated by high-speed centrifugation, and processed for cryoelectron microscopy. Nanogold particles were enhanced with silver. The presence of nanogold indicated the presence of H1 that had been internalized from the plasma membrane to endosomes before permeabilization of the cells. Labeled membranes generally had a vesicular appearance with a diameter of ~100 nm (Figure 6).

Coat structures were not obvious on any of the membranes in the sample. To test for the presence of AP-1, the cryosections were in addition decorated with anti-γ-adaptin antibody and protein A coupled to 10-nm colloidal gold. Vesicles could be found that were positive for both colloidal gold and nanogold (Figure 6, B and C), consistent with the biochemical evidence that H1 is released from endosomes in vesicles involving AP-1–containing coats.

Rab4 and Rabaptin-5/Rabex-5 Regulate the Formation of Recycling Vesicles

Rab proteins are well-known regulators of endosomal functions (Zerial and McBride, 2001). We tested the requirement of rab4 and rab5, which both are localized to early endosomes, for in vitro vesicle formation by depleting the cytosol by using specific antibodies (Figure 7, A and B). Whereas the formation of H1-containing vesicles was not affected by depletion of rab5, it was strongly inhibited by the removal of rab4 (Figure 7C). An involvement of rab4 in the generation of recycling vesicles is in agreement with previous observations in living cells (van der Sluijs *et al.*, 1992).

A candidate to connect rab4 function to AP-1/clathrin coat formation on endosomes is the rabaptin-5/rabex-5 complex. Initially, rabaptin-5 was identified as an interactor of rab5 (Stenmark *et al.*, 1995) and rabex-5 as a rab5 guanine nucleotide exchange factor (Horiuchi *et al.*, 1997). Rabaptin-5 in addition was shown to bind to rab4 and AP-1 (Vitale *et al.*, 1998; de Renzis *et al.*, 2002; Deneka *et al.*, 2003). On immunodepletion of rabaptin-5/rabex-5 from the cytosol with an anti-rabaptin-5 antibody (Figure 8A), formation of endosomal vesicles was reproducibly stimulated (Figure 8B), suggesting an inhibitory role of the complex. To test the effect of increased rabaptin-5/rabex-5 concentrations on vesicle formation, the native complex was partially purified from calf brain cytosol according to the procedure by Horiuchi *et al.* (1997). Because we observed that AP-1, which stimulates vesicle formation, was also enriched together with rabaptin-5/rabex-5, AP-1 was depleted from the preparation by anti-γ-adaptin antibody coupled to protein A/G-Sepharose before use. Addition of enriched rabaptin-5/rabex-5 consistently inhibited in vitro vesicle formation (Figure 8C). To exclude that this effect was due to contaminants, the complex of rabex-5 and His6-tagged rabaptin-5 was expressed using the baculovirus/Sf9 cell system and purified as described previously (Lippe *et al.*, 2001). Addition of 12 or 24 μg/ml rabaptin-5/rabex-5 (corresponding approximately to once or twice the amount already present in the added cytosol, respectively) clearly inhibited the formation of H1-containing vesicles (Figure 8D). The rabaptin-5/rabex-5 complex thus negatively regulates vesicle generation.

DISCUSSION

AP-1A/Clathrin Coats Mediate Receptor Recycling

One important finding of our study is that in vitro reconstitution of recycling vesicles is clearly dependent on AP-1 adaptors: the formation of H1-containing endosome-derived vesicles is blocked upon AP-1 depletion of the cytosol and restored upon readdition of purified protein. The involvement of AP-1/clathrin coats is further substantiated by the stimulating effect of increased clathrin concentration and by the sensitivity to BFA. These results thus support the earlier observations that transferrin receptor recycling in living cells at least in part was affected by BFA and involved dynamin (van Dam and Stoorvogel, 2002; van Dam *et al.*, 2002). Perturbation of clathrin function in living cells was always found to affect endocytosis more effectively than receptor recycling (Bennett *et al.*, 2001; Wettey *et al.*, 2002; Moskowitz *et al.*, 2003). The reduced recycling rates thus could be interpreted as an indirect result of disturbing clathrin-dependent pathways from the plasma membrane, and from and to the TGN, rather than the result of a direct involvement of clathrin in recycling. However, the existence of at least two major pathways from early endosomes to the plasma membrane (Sheff *et al.*, 1999; Hao and Maxfield, 2000; van Dam *et al.*, 2002) provides an explanation why interference with just one transport mechanism only partially blocks recycling. In addition, compensatory mechanisms are likely to make up for the inhibited route in long-term experiments. In the reconstitution assay used here, normal permeabilized cells, loaded with internalized biotinylated proteins were used for a single round of vesicle formation. This process is unlikely to be significantly affected by other transport events. Consistent with this notion, depletion of AP-2

(which is involved in endocytosis) or AP-3 (which mediates lysosomal sorting from endosomal tubules positive also for AP-1; Peden *et al*., 2004), or of rab5 (involved in the formation of sorting endosomes, endosome fusion and organization), did not affect the formation of H1-containing endosome-derived vesicles.

Kinetic, pharmacological, and temperature-shift experiments provided evidence for two distinct recycling pathways (Sheff *et al*., 1999; Hao and Maxfield, 2000; Hunyady *et al*., 2002; van Dam *et al*., 2002): a rapid recycling pathway from sorting endosomes that is sensitive to phosphatidyl inositol 3-kinase inhibitors (wortmannin and LY294002), and a slow one from recycling endosomes that is sensitive to BFA and involves dynamin. Because the formation of H1-containing vesicles is insensitive to LY294002 but sensitive to higher concentrations of BFA, it seems plausible that the vesicles generated in our assay correspond to the slow pathway from recycling endosomes. However, there is no evidence on the precise distribution of internalized H1 between sorting and recycling endosomes during the in vitro incubation. In a similar in vitro assay by using CHO cells, endosome-derived vesicles containing transferrin receptor and GLUT4 were generated by a BFA-insensitive but neomycin-sensitive mechanism (Lim *et al*., 2001). Because endocytic proteins had been internalized at 15°C, a temperature at which transport from sorting to recycling endosomes is blocked (Ren *et al*., 1998; van Dam *et al*., 2002), the starting compartment was predominantly sorting endosomes and the vesicles generated thus might have represented the fast recycling pathway.

Basolateral sorting of a subset of transport receptors, among them the receptors of transferrin, LDL, and ASGPs, was recently shown to depend on AP-1B, i.e., AP-1 adaptor complexes containing the epithelial-specific subunit isoform μ1B (Fölsch *et al*., 1999, 2003; Sugimoto *et al*., 2002), in particular also in basolateral recycling (Gan *et al*., 2002). Polarized sorting of these receptors is defective in the kidney epithelial cell line LLC-PK1, which lacks μ1B, but is restored upon μ1B expression. This mechanism was shown to be operative also in MDCK cells (Ang *et al*., 2003). We have used MDCK cells in our experiments in combination with cytosol from calf brain lacking μ1B. As a result, we reconstituted vesicle formation in the absence of the specifically basolateral coat proteins, a situation like that in LLC-PK1 cells. This mechanism involving the ubiquitous isoform AP-1A is likely to correspond to such a nonpolarized recycling and recycling in nonpolarized cells.

How can the apparent signal-independence of receptor recycling (Jing *et al*., 1990; Johnson *et al*., 1993) be reconciled with the involvement of AP-1A/clathrin coats? It has been observed that bafilomycin A1, a blocker of the endosomal proton pump, inhibited recycling of the wild-type, but not of a mutant transferrin receptor lacking its cytoplasmic sorting signals (Johnson *et al*., 1993). This might suggest that proteins with and without sorting signals depart from different endosomal subcompartments and use different recycling pathways.

Rab4 and Rabaptin-5/Rabex-5 Regulate Recycling Vesicle Formation

Rab GTPases are established regulators of various aspects of endosomal functions and trafficking pathways (Zerial and McBride, 2001). Three rab proteins have been implicated in regulating receptor recycling. Rab4 was implicated in regulating recycling from endosomes to the cell surface in nonpolarized and to the apical surface in polarized cells (van der Sluijs *et al*., 1992; Mohrmann *et al*., 2002; Deneka *et al*., 2003). In vitro budding of transferrin receptor-containing vesicles from PC12 cells expressing GTPase-deficient or GDP-locked rab4 mutants was stimulated or inhibited, respectively, in comparison with untransfected cells or cells expressing wild-type rab4 (de Wit *et al*., 2001). For rab11, there is evidence for an involvement in several pathways, from the

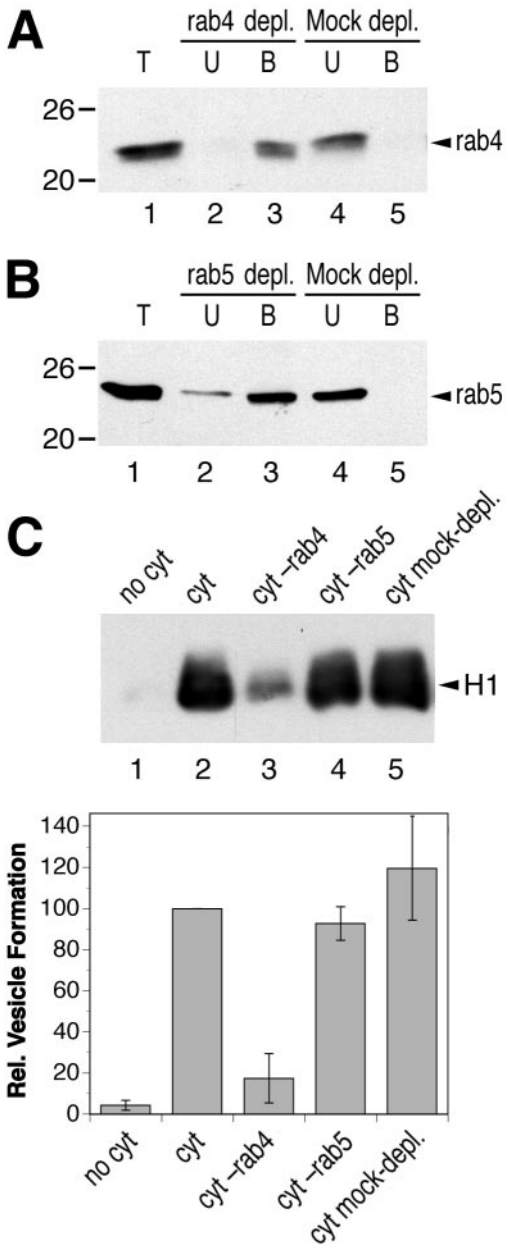


Figure 7. Rab4, but not Rab5, is required for in vitro formation of recycling vesicles. Bovine brain cytosol was immunodepleted for rab4 (A) or rab5 (B) by using specific antibodies as in Figure 3A for AP-1. Depletion efficiency was >90% in both cases. The positions of molecular weight markers are indicated (in kilodaltons). Biotinylated permeabilized cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol (no cyt), with untreated cytosol (cyt; 1.2 mg/ml), with rab-depleted cytosol (cyt–rab4; cyt–rab5), or with mock-depleted cytosol (C). Immunoblot analysis of biotinylated H1 in the supernatant after cell pelleting is shown for a representative experiment. Quantitation of three independent experiments (average with SD) is presented below.

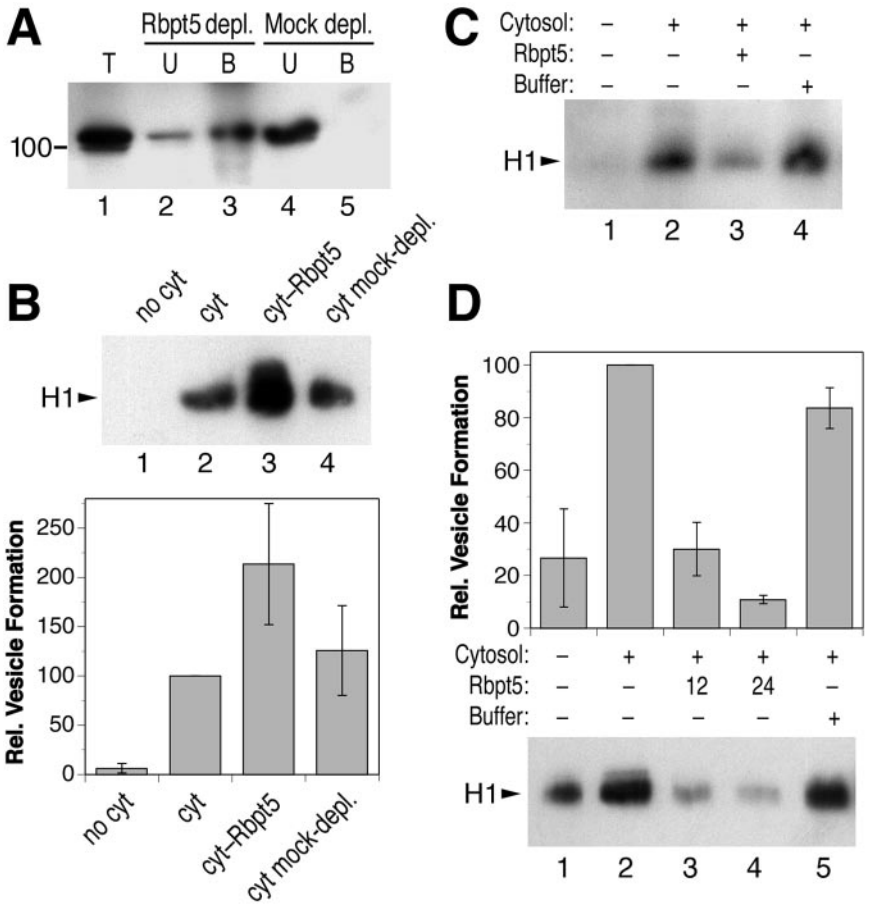


Figure 8. Rabaptin-5/rabex-5 inhibits in vitro formation of recycling vesicles. Bovine brain cytosol was immunodepleted for rabaptin-5/rabex-5 (Rbpt5) by using an antibody directed against rabaptin-5 as in Figure 3A for AP-1 (A). Biotinylated permeabilized cells were incubated in the presence of ATP, GTP, and ATP-regenerating system without cytosol, with untreated cytosol (cyt; 1.2 mg/ml), and with modified cytosol. In B, the effect of the rabaptin-5/rabex-5-depleted cytosol was tested, in C the effect of cytosol supplemented with rabaptin-5/rabex-5 partially purified from calf brain and immunodepleted for AP-1 (approximately doubling the amount of rabaptin-5 already in the cytosol), and in D the effect of adding 12 or 24 μg/ml purified rabaptin-5/rabex-5 produced by the baculovirus system. As controls, cytosol containing the buffer of the corresponding rabaptin-5/rabex-5 preparation was analyzed in parallel. Immunoblot analysis of biotinylated H1 in the supernatant after cell pelleting is shown for a representative experiment, as well as the quantitation of three independent experiments (average with SD) in B and D.

TGN to the plasma membrane (Chen *et al*., 1998), and from endosomes to the plasma membrane (Ren *et al*., 1998) and to the TGN (Wilcke *et al*., 2000). A constitutively active rab8 mutant was found to interfere specifically with AP-1B localization and function, disturbing basolateral sorting (Ang *et al*., 2003). With the antibodies available to us, we were able to efficiently remove rab4 and rab5 from cytosol. Only the depletion of rab4 blocked the production of H1-containing vesicles in the in vitro assay, suggesting a rather direct role of this GTPase in the formation of recycling vesicles.

Rab proteins are believed to exert their activity by recruiting specific effector proteins to the membrane domains in which they are localized (Zerial and McBride, 2001; de Renzis *et al*., 2002). Rabenosyn-5 and rabaptin-5/rabex-5 have been shown to be interaction partners of rab4 as well as rab5 (Vitale *et al*., 1998; de Renzis *et al*., 2002). The fact that these proteins can simultaneously bind to both active rab GTPases suggested that they regulate endosomal protein sorting and recycling by defining and connecting endosomal subdomains (de Renzis *et al*., 2002). In our assay, addition of rabaptin-5/rabex-5 inhibited and depletion stimulated the formation of recycling vesicles. This is not likely to be the result of an indirect effect on rab5 function (e.g., altered activation of rab5 by increased or decreased amounts of its exchange factor rabex-5), because depletion of rab5 itself had no effect on vesicle formation.

Interestingly, rabaptin-5 had been shown to interact also with the γ ear domains of AP-1 and GGAs, a family of ARF1-dependent clathrin adaptors (Hirst *et al*., 2000; Shiba *et al*., 2002; Deneka *et al*., 2003; Mattera *et al*., 2003). A possible role of rab4 might thus be to recruit rabaptin-5/rabex-5 to recycling endosomes as a docking site for AP-1 adaptors. Our finding that rabaptin-5 depletion stimulates vesicle formation, argues against this model. The rabaptin-5 interaction with GGA was shown to inhibit clathrin binding to GGA in vitro, suggesting a possible role of rabaptin-5 in releasing clathrin from GGA-coated membranes (Mattera *et al*., 2003). Similarly, rabaptin-5/rabex-5 binding to AP-1 may block the interaction of clathrin with AP-1, which has clathrin binding sites in the hinge regions of β1- and γ-adaptins. Because rab4 is necessary for vesicle formation, i.e., plays a positive role, it might do so by counteracting the inhibitory effect of its interaction partner rabaptin-5/rabex-5. It is therefore conceivable that rab4 triggers the release of rabaptin-5/rabex-5 from AP-1 to free the clathrin binding site of γ-adaptin and to allow the completion of the coat and the formation of a vesicle. In an alternative model, rabaptin-5/rabex-5 might inhibit rab4 function while bound to AP-1. On its displacement by clathrin binding to AP-1, rab4 might be derepressed to perform its function, e.g., the recruitment of the machinery to pinch off the coated bud. These hypotheses remain to be tested experimentally both in vivo and in vitro.

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