An Endosomal βCOP Is Involved in the pH-dependent Formation of Transport Vesicles Destined for Late Endosomes

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Abstract. In this paper, we show that βCOP is present on endosomes and is required for the formation of vesicles which mediate transport from early to late endosomes. Both the association of βCOP to endosomal membranes as well as transport vesicle formation depend on the lumenal pH. We find that εCOP, but not γCOP, is also associated to endosomes, and that this association is also lumenal pH dependent. Our data, thus, indicate that a subset of COPs is part of the mechanism regulating endosomal membrane transport, and that membrane association of these COPs is controlled by the acidic properties of early endosomes, presumably via a trans-membrane pH sensor.

THE cytoplasmic surface of some subcellular compartments and vesicles is known to be coated by specific sets of peripheral membrane proteins (Kreis and Pepperkok, 1994), including clathrin and adaptor proteins (Pearse and Robinson, 1990), COP coatomers (Rothman and Orci, 1992) and COPII proteins (Barlowe et al., 1994). Although the proteins which constitute each coat are clearly distinct, sequence homologies have been found between some clathrin-associated adaptor proteins and COP coatomers (Duden et al., 1991; Kuge et al., 1993).

Clathrin is part of the coat of at least two distinct populations of vesicles, which mediate transport from the plasma membrane and from the TGN to endosomes (Robinson, 1992). In addition to clathrin, the coat of each vesicle population is formed by a complex of specific, but homologous, adaptor proteins, AP1 and AP2 on TGN- and plasma membrane-derived vesicles, respectively. Both COP and COPII are involved at early stages of the biosynthetic pathway. COPs are required for the formation of Golgi-derived vesicles (Orci et al., 1986; Ostermann et al., 1993) and for membrane transport from the intermediate compartment to the Golgi complex (Pepperkok et al., 1993). In yeast, however, formation of ER-derived vesicles requires COPII (Barlowe et al., 1994), as well as the small GTP-binding protein Sar1p (D'Enfert et al., 1991). In addition, Sar1p also promotes vesicle budding from the ER, but not the Golgi, in mammalian cells (Kuge et al., 1994). Thus, the COP and COPII coats may be associated to either two parallel or two sequential transport steps from the ER to the Golgi. These coats may also support transport in opposite directions, as suggested by recent studies showing that retrieval of di-lysine tagged proteins to the ER depends on COPs (Letourneur et al., 1994).

Except for the AP2/clathrin coat at the plasma membrane, relatively little is known about the possible involvement of coat proteins in the endocytic pathway. At the electron microscope level, early endosomal membranes were shown to contain clathrin-coated pits with a diameter smaller than on the plasma membrane; these pits were proposed to mediate membrane recycling back to the cell surface (Killisch et al., 1992). Nonclathrin-coated domains have also been observed on early endosomal membranes (Parton, R., unpublished result), but the identity and role of these putative coat proteins are unknown. Whereas GTP-binding proteins of the ARF/sar family regulate coat assembly at different steps of the biosynthetic pathway (D'Enfert et al., 1991; Orci et al., 1993*a*; Stammes and Rothman, 1993), their role in the endocytic pathway is not clear. In vitro studies may suggest that an ARF protein is involved in endosome fusion (Lenhard et al., 1992), while overexpression of a mutant ARF6 defective in nucleotide binding caused accumulation of nonclathrin-coated structures on peripheral tubules, and reduced transferrin–receptor recycling (D'Souza-Schorey et al., 1994; Peters et al., 1994). Brefeldin A, a drug which inhibits the Golgi-associated ARF1 exchange factor (Donaldson et al., 1992; Helms and Rothman, 1992), was shown to affect the morphology of endosomes (Lippincott-Schwartz et al., 1991; Tooze and Hollinshead, 1991; Wood and Brown, 1992), but the molecular events causing these changes are not clear. Finally, expression of εCOP, a component of the COP coat (Hara-Kuge et al., 1994), could correct pleiotro-

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pic transport defects, including in the endocytic pathway, in a mutant CHO cell line (Guo et al., 1994).

In previous studies, we have observed that tracers endocytosed in BHK cells first appear in early endosomes, then in intermediate vesicles with the typical morphology of multivesicular endosomes, and finally in late endosomes (Gruenberg et al., 1989). Our in vitro studies indicate that these vesicles, which we term endosomal carrier vesicles (ECVs)[1], are obligatory transport intermediates between early and late endosomes, since ECVs are only fusogenic with late endosomes, but not with early endosomes nor with each other (Gruenberg et al., 1989; Bomsel et al., 1990; Aniento et al., 1993*a*). We also observed that neutralization of the vacuolar pH with bafilomycin A_1, a specific inhibitor of the vacuolar ATPase (Bowman et al., 1988), blocks ECV formation in vivo (Clague et al., 1994). The former treatment had no effect on internalization into and recycling from early endosomes in vivo, or on endosome fusion properties in vitro. In the present paper, we have investigated the mechanisms of ECV formation from early endosomes using an in vitro assay. Our data show that βCOP is associated to early endosomes and is involved in ECV formation. Our observations also suggest that some, but not all, COPs are involved in this process, and that coat formation is controlled by the acidic properties of early endosomes, presumably via a trans-membrane pH sensor.

Materials and Methods

Cells, Viruses, and Immunological Reagents

Monolayers of BHK-21 cells were grown and maintained as described (Gruenberg et al., 1989). For each experiment, a minimum of 6 × 10 cm Petri dishes were seeded 16 h before use. Vesicular stomatitis virus (VSV) was produced as described (Gruenberg et al., 1989). All manipulations of the cells were at 4°C, except when indicated. The P5D4 monoclonal antibody against the cytoplasmic domain of the spike glycoprotein G of VSV (Kreis, 1986) was a gift of T. Kreis (University of Geneva, Geneva, Switzerland), as well as the M3A5, maD, A1, E1, and D1 antibodies against βCOP peptides (Allan and Kreis, 1986; Pepperkok et al., 1993). The CM1A10 anti-coatomer antibody (Orci et al., 1993*b*) and the anti-εCOP antibody (Hara-Kuge et al., 1994) were gifts from J. Rothman (Sloan-Kettering Institute, New York, NY). The antibody against γCOP (Stenbeck et al., 1992) was a gift from F. Wieland (Ruprecht Karls University, Heidelberg, Germany). The antibody against ERGIC53 was a gift from H.-P. Hauri (Biocenter, Basel, Switzerland). The monoclonal antibodies against rab5a and rab3a were gifts from R. Jahn (Yale University, New Haven, CT), and the monoclonal antibody against the transferrin receptor was a gift from I. Trowbridge (Salk Institute, San Diego, CA). The antibody against annexinII was a gift from V. Gerke (University of Münster, Münster, Germany).

Subcellular Fractionation of Endosomes

Endosomes were separated from each other and from the plasma membrane using a step flotation gradient, as described (Chavrier et al., 1991; Gorvel et al., 1991; Aniento et al., 1993*a*; Emans et al., 1993). Briefly, cells were homogenized gently to limit damage that may be caused to endosomes, and a postnuclear supernatant (PNS) was prepared. The PNS was adjusted to 40.6% sucrose, 3 mM imidazole, pH 7.4, loaded at the bottom of an SW60 tube, and then overlaid sequentially with 35 and 25% sucrose solutions in 3 mM imidazole, pH 7.4, and then with homogenization buffer (HB; 250 mM sucrose, 3 mM imidazole, pH 7.4). The gradient was centrifuged for 60 min at 35,000 rpm using an SW60 rotor. Early endosomes were then collected at the 35%/25% interface and both ECVs and late endosomes at the 25%/HB interface. In immunoisolation experiments, endosomal fractions were prepared using the same gradient, except that 16% sucrose and 10% sucrose in D_2O were used instead of 35 and 25% sucrose, respectively (Gorvel et al., 1991).

Early and late endosomes were immunoisolated using as antigen the cytoplasmic domain of the spike glycoprotein G of VSV (Gruenberg et al., 1989; Howell et al., 1989; Aniento et al., 1993*a*; Emans et al., 1993). Briefly, the G protein was implanted into the plasma membrane by low pH-mediated fusion of the viral envelope with the plasma membrane and then internalized for 5 min at 37°C to label early endosomes or for 45 min at 37°C to label late endosomes. The cells were then homogenized and fractionated in the gradient, as described above. Early and late endosomal fractions were collected and used as input in immunoisolation experiments. For immunoisolation, we used magnetic beads (M-450 Dynabeads) with coupled antibodies against mouse IgG as solid support and the P5D4 antibody against the cytoplasmic domain of the G protein as specific antibody (Kreis, 1986). Alternatively, endosomes were immunoisolated using the maD or the M3A5 antibody against βCOP as specific antibody.

In Vitro Formation of ECVs from Early Endosomes

To provide a marker of the early endosomal content (Aniento et al., 1993*a*; Emans et al., 1993; Gruenberg et al., 1989), cells were incubated for 5 min in the presence of 5 mg/ml HRP. The cells were then homogenized and early endosomes were separated from ECVs and late endosomes after flotation on the gradient (see above). The early endosomal fraction was then diluted to ≈0.2 mg protein/ml with HB, adjusted to 12.5 mM Hepes, pH 7.0, 1 mM DTT, 1.5 mM MgOAc, 60 mM KCl, and supplemented with an ATP regenerating system (Gruenberg and Howell, 1986) and 4 mg/ml rat liver cytosol (Aniento et al., 1993*b*). In the assay, the mixture, containing 300–500 μg of early endosomal protein in a final volume of 1.4–2.3 ml, was incubated for 30 min at 37°C. After the incubation, the mixture containing both donor early endosomes and vesicles formed in vitro was brought to 25% sucrose, 3 mM imidazole, pH 7.4, loaded at the bottom of an SW60 tube and overlaid with HB. After 1 h centrifugation at 35,000 rpm, donor early endosomes (pelleted fraction; PF) and budded vesicles (floated fraction; FF) were recovered from the pellet and the 25% sucrose/HB interface, respectively. Both fractions were re-centrifuged for 30 min at 100,000 *g* to sediment membranes, and the HRP activity was quantified in the pellets. For SDS gel electrophoresis, membranes were washed during a second centrifugation step in HB containing 150 mM NaCl to remove loosely bound cytosolic factors, before solubilization in gel sample buffer.

In some experiments, the cytosol was depleted of coatomer using the CM1A10 anti-coatomer antibody, as reported by Orci et al. (1993*b*). The cytosol was incubated with the antibody for 90 min at 4°C and then for an additional 60-min time period with protein A–Sepharose coupled to rabbit anti–mouse IgG. After removal of the immune complex, both β and εCOP were reduced to ≈15–20% of the original amounts. In controls, the anti-βCOP antibody was replaced by an irrelevant antibody (P5D4). A fraction highly enriched in coatomer was prepared by high speed centrifugation (200,000 *g*, 2 h) of rat liver cytosol, as established by F. Wieland (Ruprecht Karls University, Heidelberg, Germany). The high-speed supernatant then retained only ≈20% of both β and εCOP. The high-speed pellet (HSP) enriched in coatomer was resuspended in HB and used in the assay.

In Vitro Fusion of ECVs with Late Endosomes

The fusion properties of vesicles formed in vitro were measured using an assay we have established (Gruenberg et al., 1989; Bomsel et al., 1990; Gorvel et al., 1991; Aniento et al., 1993*a*). Formation of vesicles in vitro was as above, except that biotinylated HRP (bHRP) was used to label the vesicle content, instead of HRP. Then, ECVs formed in vitro were separated from donor membranes by flotation as described above, except that the reaction mixture was loaded onto a 35% sucrose cushion in 3 mM imidazole, pH 7.4, to avoid pelleting of the donor membranes. Thus, donor early endosomal membranes (PF) were collected at the interface between 35 and 25% sucrose, and ECVs formed in vitro (FF) at the interface between 25% sucrose and homogenization buffer. In control experiments, early endosomes labeled with bHRP internalized for 5 min at 37°C were prepared as above using the gradient. The total bHRP activity of each fraction used in the fusion assay was always identical, so that experiments

1. *Abbreviations used in this paper*: bHRP, biotinylated HRP; ECV, endosomal carrier vesicle; FF, floated fraction; HB, homogenization buffer; HSP, high-speed pellet; PF, pelleted fraction; PNS, postnuclear supernatant; VSV, vesicular stomatitis virus.

could be directly compared. In parallel, early or late endosomes were labeled with avidin after incubation at 37°C for 5 min, or 5 min followed by a 40-min chase in marker-free medium, respectively (Gruenberg et al., 1989; Gorvel et al., 1991; Aniento et al., 1993*a*). After homogenization, a PNS was prepared and used in the fusion assay, as described (Gruenberg et al., 1989). In the assay, bHRP-labeled vesicles formed in vitro (50 μl containing 5–10 μg protein) were mixed with 70 μl PNS containing avidin-labeled endosomes, and then supplemented with 30 μl rat liver cytosol (20 mg protein/ml), an ATP-regenerating or -depleting system, 0.05 mg/ml biotinylated insulin, 60 mM KOAc, 1.5 mM MgOAc, 1 mM DTT, and 12.5 mM Hepes, pH 7.4, and the mixture was incubated for 45 min at 37°C. In some experiments, endogenous tubulin was polymerized in the assay using 20 μM taxol (Bomsel et al., 1990; Aniento et al., 1993*a*). Then the avidin–bHRP complex formed upon membrane fusion was extracted in detergent, immunoprecipitated with anti-avidin antibodies and the enzymatic activity of bHRP was quantified. To calculate fusion efficiency, this value was expressed as a percentage of the total amount of avidin–bHRP complex formed in the presence of detergent and in the absence of biotinylated insulin.

Electron Microscopy

For electron microscopic localization studies, BHK cells were grown on polylysine-coated coverslips for 2 d before the experiment. The cells were washed and incubated with 10 mg/ml HRP for 10 min at 37°C to label early endosomes. They were then washed with cold PBS and transferred to ice. Rip-off of the dorsal surface was performed as described by De Curtis and Simons (1989). Briefly, a nitrocellulose filter was laid on top of the coverslips in contact with the cells. After applying slight pressure to the nitrocellulose using a bent glass pipette, the nitrocellulose was removed. All the following steps, before fixation, were performed on ice. The cells were then incubated for 10 min in 12.5 mM Hepes, pH 7.0 buffer, containing 75 mM KCl, 1.5 mM MgOAc and 0.1% BSA, and then incubated in the same buffer for 30 min with either the M3A5 anti-βCOP antibody or with a control monoclonal antibody at exactly the same concentration. After washing the cells over 30 min, they were then incubated with gold-labeled second antibodies (Aurion, Wageningen, The Netherlands). The cells were washed to remove unbound gold, fixed in 2.5% glutaraldehyde in 50 mM cacodylate, and then processed for Epon embedding as previously described (Parton et al., 1992). Sections were cut parallel to the substratum and viewed without further contrasting.

When analyzed in plastic sections, fractions containing HRP-labeled donor membranes (PF) or vesicles formed in vitro (FF) were prepared as described above, and centrifuged for 30 min at 100,000 *g*. The membrane pellets were fixed, reacted with diaminobenzidine, and processed for electron microscopy as described (Parton et al., 1989; Bomsel et al., 1990). In some experiments, the assay measuring vesicle formation was carried out in the presence of GTPγS and M3A5 anti-βCOP antibody. Then, rabbit anti–mouse IgG and protein A–gold were added. Membranes were collected by centrifugation in a sucrose step flotation gradient at the 35–10% interface and then re-centrifuged for 30 min at 100,000 *g*. The membrane pellets were fixed, reacted with diaminobenzidine, and processed for electron microscopy.

Analytical Techniques

Quantification of protein was according to (Bradford, 1976). Western blot analysis was carried out using peroxidase-conjugated sheep anti–mouse or goat anti–rabbit IgG as secondary antibodies and detected by chemiluminescence using the ECL reagent (Amersham Corp., Arlington Heights, IL). Western blots exposed in the linear range of detection, as established with controls, were quantitated using a GS300 transmitance/reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Results

βCOP (or a Closely Related Homologue) Is Present in Endosomal Fractions

We have tested whether βCOP could be detected on endosomes of BHK cells. As a first step, we have analyzed endosomal fractions obtained by flotation in a step gradient which we have established. In this gradient, early endosomes, containing annexinII and the small GTPase rab5, equilibrate at the interface between two cushions of 35 and 25% sucrose, whereas late endosomes, containing the rab7 protein as well as the mannose-6-phosphate receptor and lysosomal glycoproteins, are found at a lighter position corresponding to the interface between 25 and 8.5% sucrose (Chavrier et al., 1991; Gorvel et al., 1991; Aniento et al., 1993*a*; Emans et al., 1993). A detailed analysis of the in vitro fusion properties and protein composition of these fractions revealed that ECVs equilibrate at the same position as late endosomes (Aniento et al., 1993*a*).

A Western blot analysis revealed the presence of proteins migrating at the position of βCOP and εCOP in both early and late endosomal fractions (Fig. 1 *A*, *EE* and *LE*, respectively). In contrast, these fractions did not contain detectable amounts of γCOP. Previously, the bulk of βCOP was shown to localize to early stages of the biosynthetic pathway after immunogold labeling of cryosections using an antibody raised against a βCOP peptide (Duden et al., 1991; Pepperkok et al., 1993). We therefore analyzed the distribution of the intermediate compartment between ER and Golgi in our gradient, using antibodies against ERGIC53 (Schweizer et al., 1988). The bulk of ERGIC53, βCOP, and εCOP was recovered, as expected, in the same fraction (heavy membranes, Fig. 1 *A*, *HM*), at the interface between the load and the 35% sucrose cushion, well separated from endosomes. Quantification of the gels showed that the early endosomal fraction contained only ≈10% of the amounts of ERGIC53 present in the HM fraction and <5% of γCOP, but 55–70% of ε and βCOP (Fig. 1 *B*). It is, however, not clear whether these values really reflect the intracellular distribution of β and εCOP, or whether the endosomal forms of these proteins are preferentially recognized by the antibodies we used. Moreover, both ε and βCOP were also present in late endosomal fractions, but neither ERGIC53 nor γCOP (even after long exposures of the gels). To ensure that the presence of COPs in endosomal fractions did not reflect spurious membrane association of the protein after homogenization, we prepared mitochondria and Golgi/endosome fractions from rat liver (Aniento et al., 1993*b*). A Western blot analysis showed that βCOP was indeed present in the latter fraction, as expected, but absent from the mitochondrial fraction (not shown). These observations may suggest that β and εCOP, but not γCOP, are associated to endosomes.

Immunoisolation of Early Endosomes

We then used immunoisolation to determine whether β and εCOP were indeed present on early endosomes (Gruenberg et al., 1989; Howell et al., 1989; Aniento et al., 1993*a*; Emans et al., 1993). The spike glycoprotein G of VSV was implanted into the plasma membrane by low pH-mediated fusion of the viral envelope with the plasma membrane, and then internalized for 5 min at 37°C to label early endosomes. After homogenization, endosomes were fractionated on the gradient and then immunoisolated on magnetic beads (Dynabeads M-450) with bound antibodies against the cytoplasmic domain of the G protein (Kreis, 1986). As shown in Fig. 1 *A*, both β and εCOP, but neither ERGIC53 nor γCOP, were specifically associated to im-

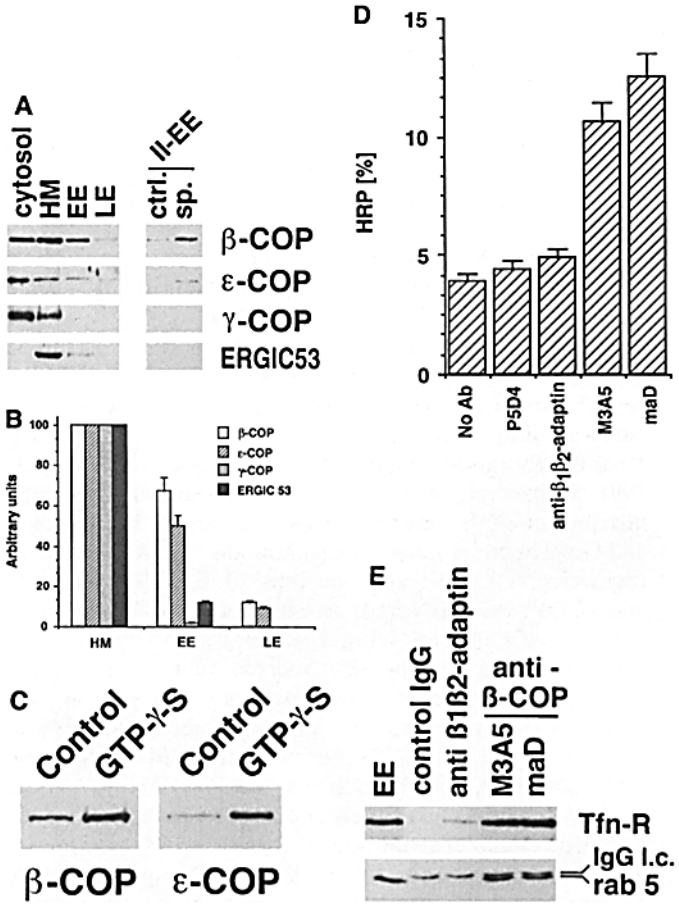

Figure 1. COPs in endosomal fractions. (*A*) The distribution of COPs and ERGIC53 was studied in BHK cytosol and in BHK subcellular fractions by SDS-PAGE followed by Western blotting, using antibodies against ERGIC53, ε or γCOP, and the M3A5 antibody against βCOP. The PNS was loaded at the bottom of a sucrose flotation gradient, and, after centrifugation, fractions were collected: heavy membranes (*HM*), 40.6%/35% interface; early endosomes (*EE*) 35%/25% interface; late endosomes (*LE*) 25% sucrose/homogenization buffer interface. In all lanes, 15 μg protein was loaded. Early endosomes were further purified from 60 μg EE fraction by immunoisolation (*II-EE*). Then, the VSV-G glycoprotein was implanted into the plasma membrane and then internalized for 5 min at 37°C. After the gradient, early endosomes were retrieved using anti–mouse magnetic beads without (control, *ctrl.*) or with the P5D4 antibody against VSV-G cytoplasmic domain (specific, *sp.*) (*B*) The distribution of ERGIC53, β, ε, and γCOP in the gradient fractions (*A*) was quantified by densitometric scanning of the blots. To facilitate comparison, the amounts of each marker present in the HM fraction was normalized to 100%. (*C*) Immunoisolated early endosomes (*II-EE* in *A*) were incubated in vitro in the presence of 1 mg/ml BHK cytosol with (*GTPγS*) or without (*control*) 50 μM GTPγS, washed, and analyzed by SDS-PAGE followed by Western blotting with antibodies against β or εCOP, as in *A*. (*D*) HRP was internalized for 5 min at 37°C and then cells were fractionated as in *A*, but immunoisolation was carried out with the following antibodies: *No Ab*, no antibody; *P5D4*, control with P5D4 antibody; *anti-$\beta_1\beta_2$-adaptin*, antibody against both β_1 and β_2 adaptins; *M3A5* and *maD*, antibodies against different βCOP peptides. The HRP activity associated to the beads was quantified and expressed as a percentage of the total HRP activity originally present in the fraction. (*E*) As in *D*, but analysis was by SDS-PAGE and Western blotting with antibodies against the transferrin receptor (*Tfn-R*) or against rab5. For comparison, the starting early endosomal fraction (*EE*) was also analyzed. rab5 migrates at a position close to the light chain of the IgG used in immunoisolation (*IgG* l.c.).

munoisolated early endosomal membranes. We then tested whether β and εCOP association to endosomes was, as is the case in the biosynthetic pathway, sensitive to GTPγS. As shown in Fig. 1 *C*, the amounts of β and εCOP present on immunoisolated early endosomes were significantly increased after incubation in the presence of cytosol and GTPγS, indicating that COP recruitment onto endosomal or biosynthetic membranes share some mechanistic similarities.

As a next step, we investigated whether early endosomes could also be immunoisolated using two different anti-βCOP antibodies (M3A5 or maD). The antibodies were bound to the same solid support and the complex was added to endosomal fractions which had been collected from the gradient, as above. In these experiments, we used horseradish peroxidase internalized for 5 min at 37°C as marker of the early endosomal content, and the transferrin receptor (Hopkins and Trowbridge, 1983; Trowbridge et al., 1993) as well as the small GTPase rab5 (Chavrier et al., 1990), as markers of early endosomal membranes. Fig. 1 (*D* and *E*) shows that the three markers were specifically retrieved with either anti-βCOP antibody, but not with control antibodies nor with antibodies recognizing the clathrin-associated β_1 adaptin as well as the β_2 adaptin, which has some homology to βCOP (Duden et al., 1991). Altogether, these experiments show that β and εCOP, but not γCOP, are present on early endosomal membranes.

Electron Microscopy Analysis of βCOP on Early Endosomes

We then investigated whether βCOP could be detected on early endosomal membranes by electron microscopy. Since neither the M3A5 nor the maD antibody recognized their antigen on cryosections, we used a pre-embedding labeling technique. Cells were first incubated in the presence of HRP for 10 min at 37°C, to label the early endosomal lumen, and then the dorsal surface of the cells was ripped off with nitrocellulose (De Curtis and Simons, 1989) to gain access to the cytoplasm. The opened cells were then treated with the M3A5 antibody against βCOP followed by gold-labeled second antibody, and then the cells were processed for electron microscopy. Fig. 2 shows that the anti-βCOP antibody, but not the control antibody, specifically labeled early endosomes containing internalized HRP.

In Vitro Budding of Endosomal Carrier Vesicles

The fact that β and εCOP were present in both early and late endosomal fractions (Fig. 1 *A*) suggested that these

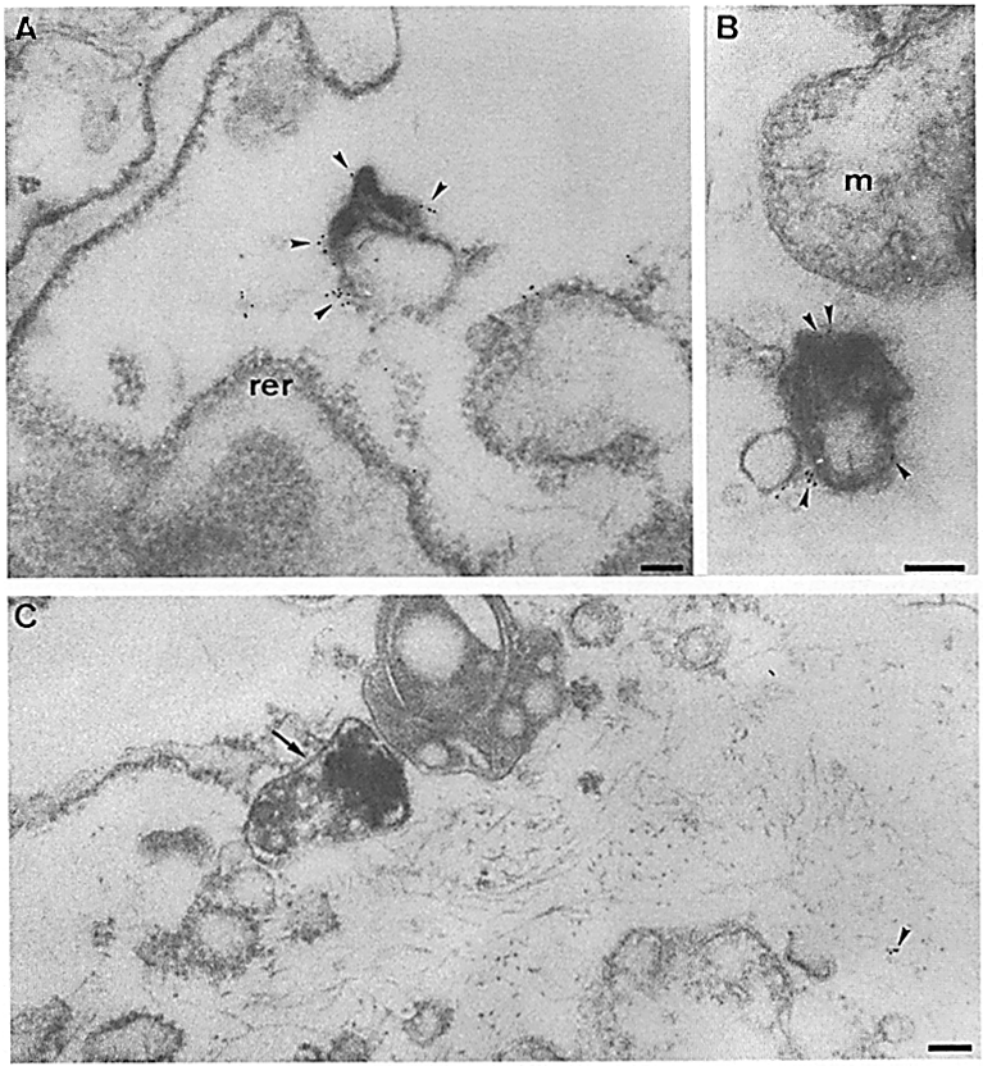

Figure 2. Endosome localization of βCOP in permeabilized cells. Cells were incubated with HRP for 10 min at 37°C to label early endosomes, and were then permeabilized. The permeabilized cells were then incubated with the M3A5 anti-βCOP antibody (*A* and *B*) or an irrelevant control antibody (*C*) followed by gold-labeled second antibodies. HRP-labeled endosomes are labeled by the anti-βCOP antibody (*A* and *B*, *arrowheads*) but not by control antibodies (*C*); gold particles indicated by *arrowheads*, HRP-labeled endosome by an *arrow*. The rough endoplasmic reticulum (*rer*) and mitochondria (*m*) show lower labeling with the M3A5 antibody (*A* and *B*). Bars, 100 nm.

proteins may be involved in membrane transport between early and late endosomes. We, therefore, decided to establish an in vitro assay measuring ECV formation and to make use of the large panel of antibodies available against βCOP to test whether the protein was involved in this process.

Cells were incubated for 5 min at 37°C in the presence of HRP, to provide a marker of the early endosomal content. Early endosomes were then separated from the lighter ECVs and late endosomes, using the flotation gradient. In the assay, these early endosomal fractions were incubated at 37°C in the presence of ATP and cytosol, to allow formation of ECVs from early endosomes to occur in vitro. The mixture was then loaded on a similar flotation gradient, to separate ECVs formed in vitro from the denser donor membranes (early endosomes). After centrifugation, the HRP activity of the FF (containing vesicles formed in vitro) and PF (containing donor membranes) was quantified. Thus, the assay measures the percentage of the total early endosomal content entrapped within vesicles formed in the assay.

As shown in Fig. 3 *A*, vesicle formation in vitro occurred at 37°C, but not at 4°C, and required both cytosol and ATP. The process was rapid (Fig. 3 *B*), like other budding events in vitro (Salamero et al., 1990; Tooze and Huttner, 1990; Rexach and Schekman, 1991): within 5–10 min ≈10% of the original early endosomal volume was entrapped within vesicles formed in vitro. We also observed that vesicle formation was inhibited by low concentrations of GTPγS (Fig. 3 *A* and see Discussion). Addition of GTPγS, or any one of the other treatments, had no effect on the sedimentation properties of early or late endosomal membranes in continuous sucrose gradients (data not shown). Western blotting of SDS gels with two different anti-βCOP antibodies showed that vesicles formed in vitro contained βCOP, as predicted (Fig. 3 *C*). The low amounts of ERGIC53 originally present in the early endosomal fraction (Fig. 1) remained associated to donor membranes after the assay (Fig. 3 *C*). Finally, ECVs formed in vitro contained <5% of the amounts of rab5 and transferrin receptor remaining in donor membranes after the assay (Fig. 3 *C*). These observations indicate that the floated fraction was not contaminated with early endosomal vesicles, and that both rab5 and the transferrin receptor were excluded from ECVs formed in vitro, as in vivo. These experiments also show that the floated fraction did not contain recycling vesicles destined to carry the transferrin receptor back to the plasma membrane.

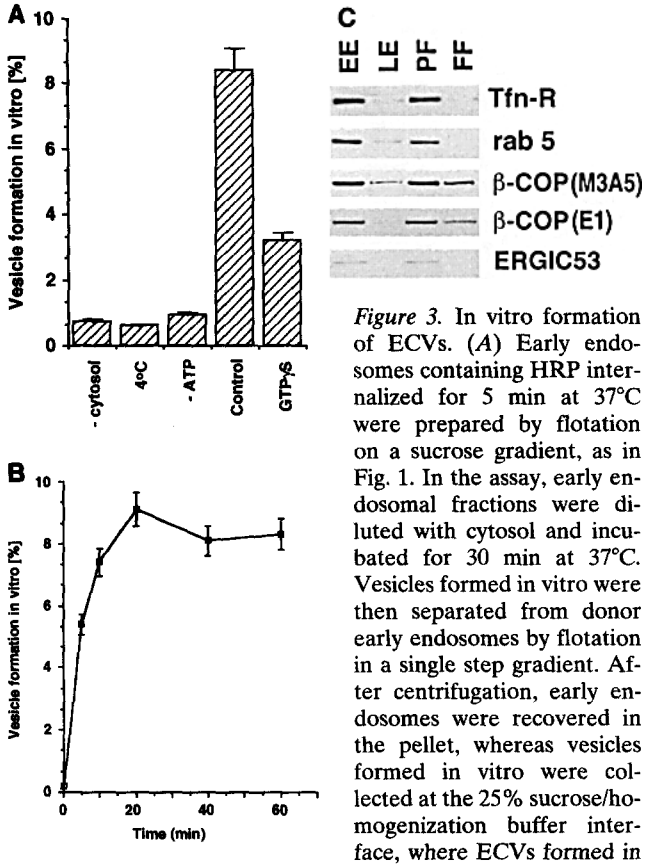

Figure 3. In vitro formation of ECVs. (*A*) Early endosomes containing HRP internalized for 5 min at 37°C were prepared by flotation on a sucrose gradient, as in Fig. 1. In the assay, early endosomal fractions were diluted with cytosol and incubated for 30 min at 37°C. Vesicles formed in vitro were then separated from donor early endosomes by flotation in a single step gradient. After centrifugation, early endosomes were recovered in the pellet, whereas vesicles formed in vitro were collected at the 25% sucrose/homogenization buffer interface, where ECVs formed in vivo equilibrate (Aniento et al., 1993*a*). Then, HRP activity was quantified in the fractions, and the efficiency of vesicle formation is expressed as a percentage of the total HRP activity originally present in the donor early endosomal fraction. *Control*, assay carried out at 37°C in the presence of cytosol and ATP; −ATP, ATP was omitted; −cytosol, cytosol was omitted; *4°C*, the assay was at 4°C; *GTPγS*, 10 μM GTPγS was present in the assay. (*B*) The assay was carried out as in *A* for the indicated time periods. (*C*) The assay was as in *A*, but the fractions were analyzed by SDS-PAGE followed by Western blotting with antibodies against the transferrin receptor (*TfnR*), rab5 (*rab5*), ERGIC53 and two different anti-βCOP antibodies (*M3A5* and *E1*). *FF*, the floated fraction containing ECVs formed in vitro; *PF*, the pelleted fraction containing donor early endosomes. For comparison, endosomal fractions prepared as in Fig. 1 are also shown. *EE*, early endosomal fraction; *LE*, late endosomal fraction containing both late endosomes and ECVs (Aniento et al., 1993*a*). Each lane contained 15 μg protein, corresponding to ≈2× as much HRP in LE and FF fractions, when compared to EE and PF fractions, respectively.

A morphological analysis showed that the HRP-labeled structures present in the floated fraction were predominantly multivesicular with the characteristic appearance of ECVs, as expected, whereas the donor fraction contained a large proportion of typical tubular- and ring-shaped early endosomal elements (Fig. 4, *A* and *B*, see Table I). The complete assay mixture was then incubated without or with GTPγS, which increases βCOP association to endosomes (Fig. 1 *C*) but blocks vesicle formation (Fig. 3 *A*), and then analysed by immunoelectron microscopy using anti-βCOP antibodies (Fig. 4, *C* and *D*). Under both conditions, βCOP was detected on HRP-labeled early endosomal membranes, including at the neck of multivesicular structures resembling forming ECVs. The epitope may be less accessible on the ECVs, since ECV labeling with these antibodies was low. Small (<100 nm) βCOP-coated vesicles with the characteristic appearance of biosynthetic COP-coated vesicles (see Kreis and Pepperkok, 1994) were not seen in these preparations. Nor did we observe an endosomal coat with the typical appearance of the Golgi COP coat, even after tannic acid treatment of the samples to enhance coat visualization (Orci et al., 1986). These experiments confirm the presence of βCOP on endosomes, but also suggest that the endosomal COP coat does not only differ in composition but also in morphology and/or appearance from the biosynthetic COP coat.

Endosomal Carrier Vesicles Formed In Vitro Are Fusogenic with Late Endosomes

We had previously shown that ECVs formed in vivo undergo fusion with late endosomes in vitro, in contrast to early endosomes (Bomsel et al., 1990; Aniento et al., 1993*a*). Here, we tested whether ECVs which were formed in vitro from early endosomes had acquired the capacity to undergo fusion with late endosomes. Fusion was measured by the formation of a fusion-specific complex between bHRP and avidin (Gruenberg et al., 1989; Aniento et al., 1993*a*). ECVs were formed in vitro exactly as described above (see Fig. 3, *FF*), except that bHRP was used instead of HRP to label the endosomal content. As a control, we also measured the fusion properties of the bHRP-labeled donor early endosomes before (*EE*) and after (*PF*) budding in vitro. In the fusion assay, the total bHRP activity of each fraction was identical to that of the ECV fraction, so that different experiments could be directly compared. Then, the bHRP-labeled vesicles were mixed with endosomes containing internalized avidin (Aniento et al., 1993*a*), cytosol and ATP, and then incubated for 45 min at 37°C. If fusion occurred, a complex was formed between bHRP and avidin. At the end of the experiment, the complex was immunoprecipitated with anti-avidin antibodies in the presence of detergent and the enzymatic activity of bHRP was quantified.

As shown in Fig. 5, ECVs formed in vitro have acquired the capacity to undergo fusion with late endosomes (FF-LE), fusion efficiency being similar to the value measured with freshly prepared ECVs (Aniento et al., 1993*a*). As previously reported for freshly prepared ECVs (Bomsel et al., 1990; Aniento et al., 1993*a*), interactions between ECVs formed in vitro and late endosomes were facilitated by the presence of polymerized microtubules (not shown). The donor membranes recovered after in vitro budding (PF) retained the typical capacity of the original early endosomes (EE) to undergo homotypic fusion with other early endosomal elements (PF-EE and EE-EE) (Gruenberg et al., 1989). In addition, the direct fusion of donor membranes with late endosomes was comparatively low, whether the donor was tested before (EE-LE) or after (PF-LE) the budding assay, in agreement with our previous observations (Gorvel et al., 1991; Aniento et al., 1993*a*). The low fusion signal which was then detected may be due to a low contamination of the early endosomal fraction with ECVs, or to the formation of new ECVs during the fusion assay (≈10% of the early endosomal vol-

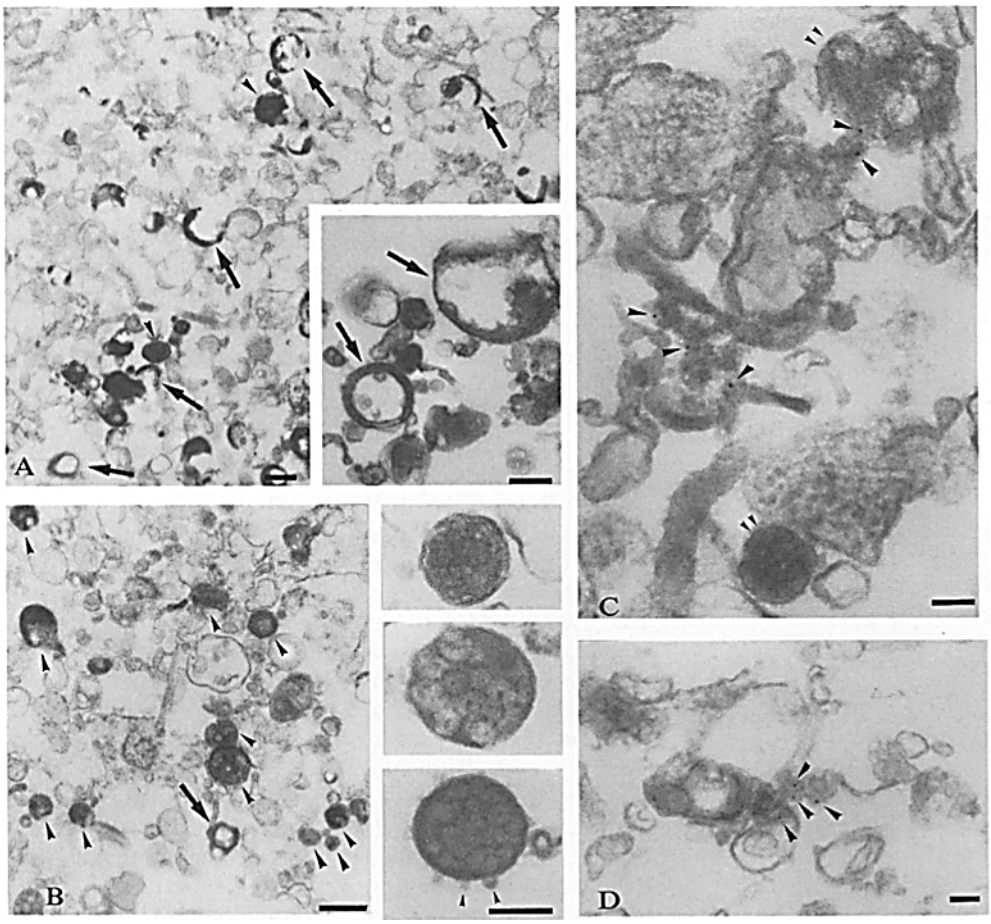

Figure 4. Electron microscopy analysis of donor membranes (PF) and vesicles formed in vitro (FF). (*A* and *B*) The assay was carried out as in Fig. 3 *A*, and then analyzed after Epon embedding. Main panels show low magnification overviews of HRP-labeled elements in the pelleted (*A*) and floated (*B*) fractions. The HRP-labeled profiles comprise tubules, ring-shaped structures (*arrows*) and spherical multivesicular bodies (*arrowheads*). Early endosomal elements with a typical ring-shaped appearance are present in the donor pelleted fraction (higher magnification in inset to *A*). When compared to the donor pelleted fraction, the floated fraction is enriched in spherical multivesicular structures (higher magnification in insets to *B*, see Table I). Use of tannic acid in an attempt to stain coats associated with the large vesicles revealed no significant COP-like coat. Some electron dense material was however associated with some multivesicular vesicles (*B*, *inset*, *small arrowheads*). Bars, 0.5 μm (*A–D*) and 0.2 μm (*insets*). (*C* and *D*) The assay was carried out as in Fig. 3 *A* in the presence of the M3A5 anti-βCOP antibody with (*D*) or without (*C*) 10 μM GTPγS. The mixture containing donor membranes was then incubated with second antibodies and protein A–gold, and embedded in Epon using a method to reveal any coat material. Specific labeling is associated with HRP-labeled buds and tubules (*large arrowheads*) but rarely with the HRP-labeled multivesicular domains of early endosome (*small double arrowheads*). However, panels C (*top right corner*, no GTPγS) and D (with GTPγS) show examples of βCOP labeling at the neck of structures resembling forming ECVs. Bars, 100 nm.

ume is entrapped within newly formed ECVs in vitro; Fig. 3 *A*). Our data thus indicate that the fusion properties of both ECVs formed in vitro and donor endosomes (recovered after in vitro budding) are characteristic of the corresponding compartments (Gorvel et al., 1991; Aniento et al., 1993*a*) and establish that functional ECVs are formed from early endosomes in vitro.

Formation of ECVs Depends on βCOP

We then tested whether βCOP was involved in ECV formation from early endosomes. In these experiments, vesicles were formed in vitro as described above, except that the reaction mixture was preincubated for 60 min on ice with antibodies against βCOP, before raising the temperature to 37°C. As shown in Fig. 6 *A*, formation of ECVs was inhibited by a polyclonal (D1) or by a monoclonal (maD) antibody against the same βCOP peptide, or by an anti-coatomer antibody (CM1A10). The process was also completely abolished by the M3A5 monoclonal antibody against βCOP. The specificity of inhibition observed with these antibodies is entirely consistent with our other biochemical and morphological observations. The maD and M3A5 could also be used for endosome immunoisolation (Fig. 1, *D* and *E*) and immunolabeling (Figs. 2 and 4), whereas the CM1A10 could be used for immunodepletion of cytosolic COPs (see Fig. 6 *C*), as previously shown by

Table I. Morphological Analysis of Donor Early Endosomes and Vesicles Formed In Vitro

	Tubular	Ring-shaped	Spherical	Others
	%	%	%	%
Donor EE	25	31	40	4
Budded vesicles	3	19	75	3

Cells were incubated with HRP for 5 min at 37°C to label early endosomes, and homogenized. Then, early endosomes were purified by flotation in the step gradient. In the assay, these early endosomes were incubated at 37°C in the presence of cytosol and ATP, to allow formation of ECVs to occur in vitro (as in Fig. 3). Donor early endosomes were separated from ECVs after centrifugation in a similar gradient. Membranes were then sedimented, fixed, and processed for electron microscopy, as in (Gorvel et al., 1991). The profiles containing internalized HRP were analyzed. According to their morphology in thick sections, HRP-positive structures were counted as tubular, ring-shaped, or spherical elements (see Fig. 4). The number of profiles for each category is indicated as a percentage of the total number of labeled structures in each fraction, donor early endosomes present in the pelleted fraction (*Donor EE*) or vesicles formed in vitro present in the floated fraction (*Budded vesicles*).

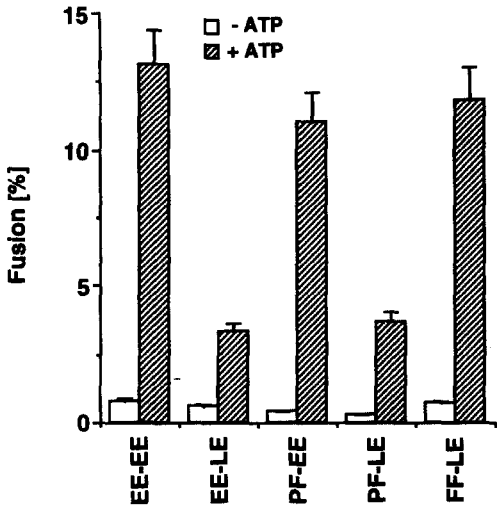

Figure 5. ECVs formed in vitro are fusogenic with late endosomes. The assay measuring vesicle formation in vitro was carried out essentially as in Fig. 3, except that the reaction mixture (adjusted to 25% sucrose) was loaded onto a 35% sucrose cushion to avoid possible damage to the donor membranes after pelleting. Thus, after centrifugation, donor early endosomal membranes (PF) were recovered at the 35/25% sucrose interface, whereas ECVs formed in vitro (*FF*) were collected at the interface between 25% sucrose and homogenization buffer. In a second step, the fusion activity of FF and PF fractions with late endosomes (*LE*, see Fig. 1) was quantified using our in vitro fusion assay (Aniento et al., 1993*a*). The results are compared with the fusion activity of freshly prepared early endosomes (*EE*, see Fig. 1). The fusion assay itself is described in the text and in the Materials and Methods section. The homotypic fusion activity of donor early endosomes with fresh early endosomes (*PF-EE*) is in the same range as the fusion activity of fresh early endosomes with each other (*EE-EE*). Neither donor early endosomes (*PF-LE*), nor fresh early endosomes (*EE-LE*) can undergo direct fusion with late endosomes, as expected (Aniento et al., 1993*a*). However, vesicles formed in vitro and present in the FF fraction have acquired the competence to undergo fusion with late endosomes (*FF-LE*), like bona fide ECVs (Aniento et al., 1993*a*). *Hatched bars*, with ATP; *open bars*, control without ATP.

Orci et al. (1993*b*). In contrast, the A1 antibody had no effect in the assay, suggesting that this epitope is not accessible on endosomal membranes.

As a control, we also tested in the assay antibodies against the small GTPase rab5, which inhibit early endosome fusion in vitro (Gorvel et al., 1991), and against the small GTPase rab3a, which is present on synaptic vesicles (Fischer von Mollard et al., 1991). We also tested antibodies against clathrin-associated α adaptin, and against both β_1 and β_2 adaptins (Robinson, 1992). None of these antibodies had any effect on ECV formation from early endosomes (Fig. 6 *A*). Finally, as an additional control, we also tested the same antibodies in our assay measuring homotypic fusion of early endosomes (Gruenberg et al., 1989). Whereas fusion was inhibited by anti-rab5 antibodies, as expected (Gorvel et al., 1991), none of the other antibodies, including those against βCOP, had any effect on endosome fusion (not shown). The fact that anti-βCOP antibodies block ECV formation from early endosomes but not early endosome fusion, in contrast to anti-rab5 antibodies, suggest that βCOP is involved in the formation of ECVs from early endosomes.

As a next step, we tested the effects of COP depletion on vesicle formation in the assay. The cytosol was immunodepleted using the CM1A10 anti-coatomer antibody (Fig. 6 *C*), which has been previously used to immunodeplete coatomer (Orci et al., 1993*b*). After immunodepletion, other cytosolic properties required for vesicle formation were not altered, since depleted cytosol supported the formation of ECVs from control untreated membranes (not shown). Since COPs are also present on endosomal membranes, cells were treated with nigericin before fractionation to release endosome-associated COPs (Fig. 6 *C*; see below and Fig. 7 *A*). Membrane depletion was reversible, since vesicle formation then occurred at control levels in the presence of untreated cytosol when nigericin was omitted in the assay (Fig. 6 *B*, *controls*).

Fig. 6 *B* shows that depletion of both cytosolic and membrane pools of βCOP significantly inhibited vesicle formation in the assay. To ensure that these treatments were reversible, low amounts of complete cytosol containing COPs were added to the assay. Then, vesicle formation from early endosomes was fully restored (Fig. 6 *B*). This effect was not due to the presence of some factors with general stimulating activity, since addition of extra cytosol to the controls did not further increase vesicle formation. To test the involvement of COP proteins more directly, a high speed pellet highly enriched in COPs was prepared after centrifugation of the cytosol (not shown), as established by F. Wieland (personal communication). Centrifugation did not alter other cytosolic properties, since the high speed supernatant still supported both ECV formation from undepleted membranes as well as endosome fusion in vitro (not shown). As shown in Fig. 6 *B*, the COP-enriched fraction could restore ECV formation after depletion of both cytosolic and membrane pools of COPs. Altogether, these depletion–recomplementation experiments, together with the effects of anti-βCOP antibodies in the assay, demonstrate that COPs, in particular βCOP, are necessary for the formation of vesicles mediating transport from early to late endosomes.

ECV Formation Is Sensitive to the Vacuolar pH

Our previous observations had shown that ECV formation is abolished after inhibition of the vacuolar ATPase in vivo, whereas internalization into and recycling from early endosomes in vivo, or the fusion properties of early endosomes in vitro are not affected (Clague et al., 1994). We therefore measured whether in vitro budding also required an active vacuolar ATPase. In our assay, ECV formation was inhibited to the same extent in the presence of 10 μM bafilomycinA_1 or 100 nM concanamycinB (Fig. 7 *A*), two specific inhibitors of the vacuolar ATPase (Bowman et al., 1988; Woo et al., 1992; Villa et al., 1993). The extent of inhibition was similar when cells were pretreated with either drug in vivo, or when the drug was directly added to the assay in vitro. However, a combination of both treatments caused a more pronounced inhibition, presumably because of a more efficient neutralization of the endosomal pH (Yoshimori et al., 1991; Clague et al., 1994). In addition, replacement of KCl in the budding as-

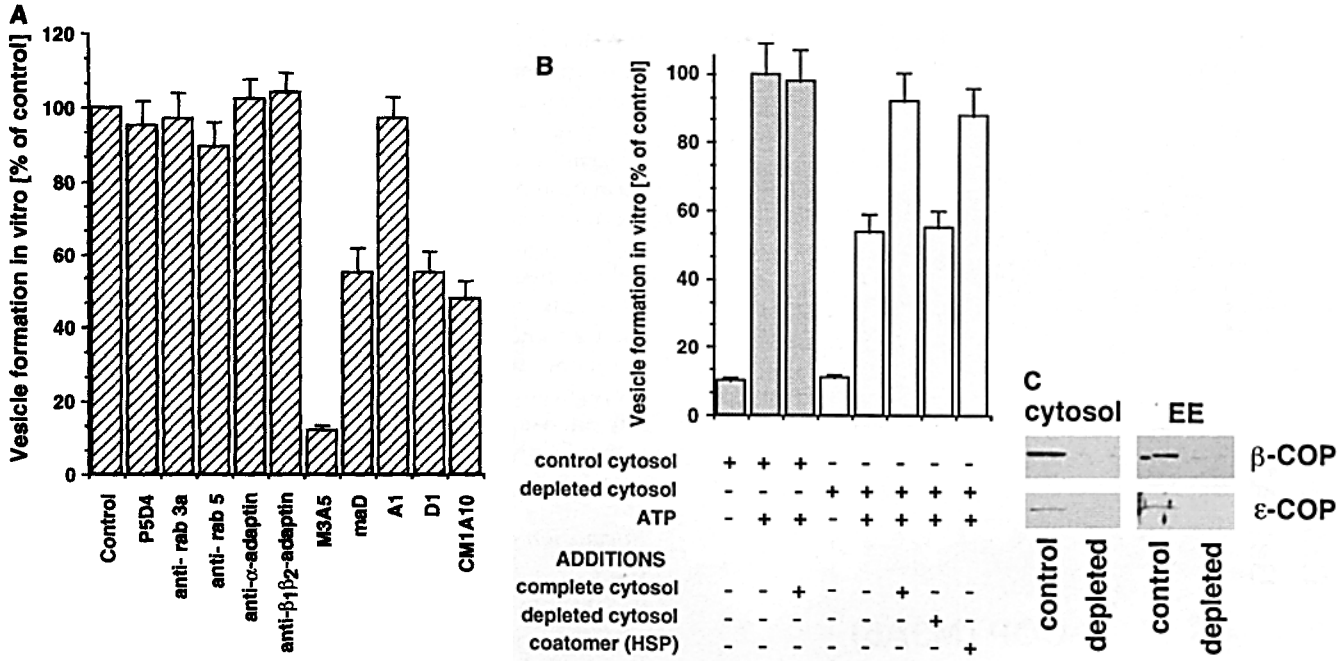

Figure 6. ECV formation depends on βCOP. (*A*) The assay measuring ECV formation in vitro was carried out as described in the legend of Fig. 3, except that the mixture was preincubated for 60 min on ice with the indicated antibodies. *Control*, no addition; *P5D4*, control antibody as in Fig. 1 (*D* and *E*); *anti-rab3a*, antibody against the small GTPase rab3a; *anti-rab5*, antibody against the small GTPase rab5; *anti-α adaptin*, antibody against α adaptin; *anti-β1 β2 adaptin*, antibody against both β_1 and β_2 adaptins; *M3A5*, *maD*, *A1*, *D1*, antibodies against βCOP; *CM1A10*, anti-coatomer antibody. Vesicle formation is expressed as a percentage of the controls. (*B*) Vesicle formation in vitro (as in Fig. 3) was measured after COP depletion. To deplete endogenous COPs associated to endosomes, cells were pretreated with 10 μM nigericin (see panel *C* and Fig. 7). This treatment was reversible, since ECV formation in vitro was restored by control rat liver cytosol, which had been incubated for 90 min with an irrelevant antibody (P5D4) and then for 1 h with protein A–Sepharose coupled to rabbit anti–mouse IgG (control, see *C*). To deplete cytosolic coatomers, the cytosol was incubated as above, but with the CM1A10 anti-coatomer antibody (depleted, see *C*). ECV formation was inhibited after depletion of both endosomal and cytosolic pools of COPs. ECV formation could be restored by the addition of 25% (μg/μg) untreated cytosol to the assay (complete cytosol), but not by COP-depleted cytosol (depleted cytosol). Addition of extra cytosol had no effects on the controls. ECV formation could also be restored after addition of a coatomer-enriched fraction prepared by high speed centrifugation of the cytosol (coatomer HSP). (*C*) SDS-gel and Western blotting analysis of β and εCOP distribution in cytosol and early endosomes (*EE*) prepared as in *B*.

say with KOAc decreased the efficiency of vesicle budding, in agreement with the role of Cl^- channels in maintenance of an acidic lumenal pH (Mellman et al., 1986), and with the finding that loss of function of the cystic fibrosis transmembrane regulator, which causes defective Cl^- conductance, delays α_2-macroglobulin degradation (Barasch et al., 1991). Finally, we used nigericin, an ionophore which exchanges protons and K^+ ions, to dissect the acidogenic and electrogenic properties of the vacuolar ATPase (Mellman et al., 1986). The inhibition observed in the presence of the drug (Fig. 7 *A*) demonstrates that vesicle formation in vitro was inhibited by neutralization of the lumenal pH.

βCOP Association to Endosomes Is Sensitive to the Vacuolar pH

We then investigated whether βCOP association to endosomal membranes was also sensitive to the lumenal pH. In these experiments, endosomes immunoisolated using the VSV-G protein as antigen were treated in the presence of cytosol under the same conditions as in the in vitro assay. A quantitative Western blot analysis using different anti-βCOP antibodies showed that the amounts of βCOP associated to early endosomal membranes were reduced to 55 ± 5% of the control value by bafilomycinA1, to 35 ± 5% by concanamycinB, and to 15 ± 3% by nigericin (Fig. 7 *B*). We also used immunogold labeling and electron microscopy to quantify the amounts of βCOP present on early endosomes after neutralization of the lumenal pH with concanamycinB or bafilomycinA_1 (not shown). These studies also showed that only 30 ± 8% of the amounts of βCOP present on control membranes remained associated to early endosomes after either drug treatment. The drugs did not cause endosome fragmentation, since 100 ± 5% of the transferrin receptor present in the control remained associated to immunoisolated endosomes after each treatment (Fig. 7 *B*). Neither did the drugs release rab5, which normally cycles between cytosol and membranes (Zerial and Stenmark, 1993), or annexinII, a peripheral protein of the early endosomal membrane (Emans et al., 1993); for both proteins, 95 ± 5% of the amounts present in the control remained associated to endosomes after each treatment. This lumenal pH dependence was specific to endosomes, since COP association, including γCOP, to ERGIC53-containing membranes (*HM* in Fig. 1 *A*) was not affected by the drugs (not shown). Finally, these drugs also caused COPs to be released from endosomal membranes in vivo, as illustrated by the effects of nigericin on β and εCOP (Fig. 6 *C*). Therefore, we conclude that the for-

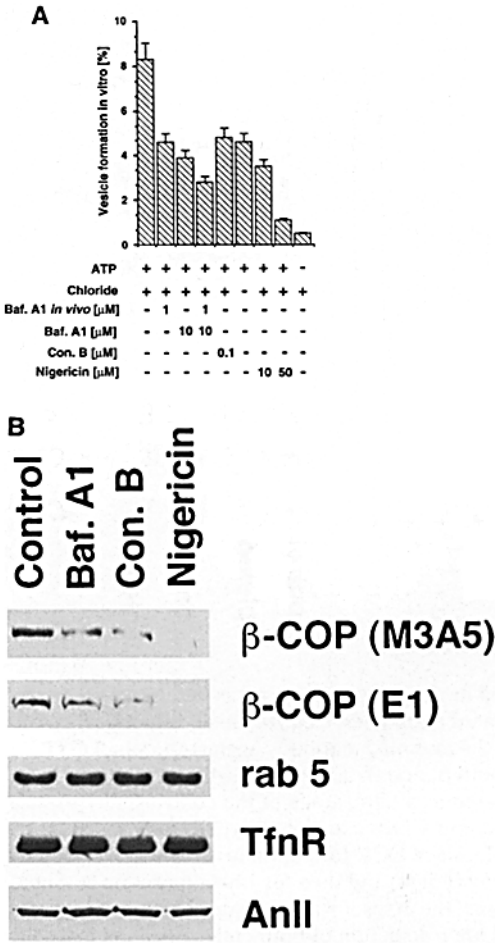

Figure 7. ECV formation depends on an acidic endosomal pH. (*A*) The assay measuring ECV formation in vitro was carried out as described in the legend of Fig. 3. *ATP*, in the presence of an ATP regenerating system (+) or an ATP-depleting system (−); *Chloride*, when indicated (−) KCl was replaced with KOAc; *Baf A1 in vivo*, the cells were pre-treated with 1 μM bafilomycin A1 for 30 min at 37°C, before HRP internalization; *Baf A1*, *Con. B*, and *Nigericin*, the donor early endosomal fraction was treated in vitro with the indicated concentrations of bafilomycin A1, concanamycin B or nigericin for 15 min at 4°C in the absence of ATP, and then for 15 min in the presence of ATP and cytosol, before raising the temperature to 37°C. (*B*) Immunoisolated early endosomes, prepared as in Fig. 1 *B*, were treated in vitro with 10 μM bafilomycin A1, 100 nM concanamycin B, or 50 μM nigericin as in *A*. Then, endosomal membranes were collected by centrifugation and analyzed by SDS-PAGE followed by Western blotting with the M3A5 or E1 antibodies against βCOP, or antibodies against rab5, the transferrin receptor and annexinII. Each lane contained ≈10 μg protein.

mation of ECVs from early endosomes depends on βCOP or a closely related βCOP homologue, and that this process is regulated by the acidification properties of early endosomes.

Discussion

We have previously shown that, both in polarized and nonpolarized cells, membrane transport from early to late endosomes occurs via vesicular intermediates which we termed ECVs (Gruenberg et al., 1989; Bomsel et al., 1990; Aniento et al., 1993*a*). Similar vesicles also mediate transport from early to late endosomes both in the axons and in the dendrites of cultured neurons (Parton et al., 1992). In this paper we show that some, but not all, COPs are present on endosomal membranes and are involved in the formation of ECVs from early endosomes in vitro. We observe that neutralization of the endosomal pH either with specific inhibitors of the vacuolar ATPase or with the ionophore nigericin causes both a decrease in the amounts of COPs associated to early endosomes and an inhibition of the formation of ECVs from early endosomes. These experiments suggest that a COP subcomplex is part of the mechanism regulating membrane transport from early to late endosomes, and that membrane association of endosomal COPs depends on the acidic properties of the endosomal milieu.

Formation of ECVs from Early Endosomes

We have used an in vitro assay to reconstitute the formation of ECVs from early endosomes. We find that ≈10% of a fluid phase tracer internalized for 5 min at 37°C in vivo is packaged within vesicles formed in vitro with a $t_{1/2}$ ≈5 min. Our previous studies indicated that a similar fraction of the early endosomal content is packaged within ECVs over a 5–10-min time period in vivo (Gruenberg et al., 1989). These observations may indicate that a single round of vesicle formation was reconstituted in the assay. This process depends on the presence of cytosol and ATP and is inhibited at low temperature. Vesicles formed in vitro do not originate from the simple fragmentation of early endosomes, since they do not contain the small GTPase rab5, which is restricted to the plasma membrane and early endosomes in vivo even after 10–50-fold overexpression (Chavrier et al., 1990, 1991; Gorvel et al., 1991). Nor are these vesicles recycling endosomal vesicles, since they lack the transferrin receptor, a marker of the recycling pathway (Hopkins, 1983; Stoorvogel et al., 1987; Mayor et al., 1993; Ghosh and Maxfield, 1995). Additional evidence that vesicles formed in vitro are not recycling vesicles comes from our observations that vesicle formation is inhibited after neutralization of the lumenal pH, as is ECV formation in vivo. In contrast, recycling of the endosomal content back to the cell surface is not sensitive to the lumenal pH (Clague et al., 1994). Finally, evidence that functional ECVs are formed from early endosomes in vitro comes from our observations that these vesicles are fusogenic with late endosomes, like ECVs formed in vivo (Aniento et al., 1993*a*; Bomsel et al., 1990).

Our data argue against a gradual change of the membrane composition during passage from early to late endosomes (Stoorvogel et al., 1991). Indeed, ECVs, but not donor early endosomes, have acquired the capacity to dock onto and fuse with late endosomes, and these ECVs exclude both rab5 and the transferrin receptor. Our data, thus, suggest that a strict compartment boundary exists on early endosomal membranes at the site of ECV formation.

Coats and Endosomes

With the exception of plasma membrane clathrin-coated pits, little is known about the role of coat proteins in the

endocytic pathway. Morphological studies have shown the presence of small clathrin-coated pits on early endosomal membranes, presumably involved in receptor recycling back to the cell surface (Killisch et al., 1992). Non clathrin coats have also been observed on early endosomes (Parton, R., unpublished observations), but the function of these coats is unknown. In addition, expression of ϵCOP, a component of the COP coat required at early stages of the biosynthetic pathway (Kreis and Pepperkok, 1994), was recently shown to correct pleiotropic membrane transport defects, including in the endocytic pathway, in the CHO ldlF mutant cell line (Guo et al., 1994; Hobbie et al., 1994).

Our studies show that βCOP, another component of the COP coat, is present on early endosomes and is required for the formation of ECVs, the vesicular intermediates destined for late endosomes. Consistent with the findings of Guo et al. (1994), we find that ϵCOP is also present on endosomes. However, all COP subunits do not appear to be shared by the endocytic and the biosynthetic pathways, since γCOP is not detected in any of our different endosome preparations. These observations are also in good agreement with the finding that β, ϵ, and ζCOP, but not γCOP, are associated to endosomes prepared by free-flow electrophoresis (Whitney, J.A., M. Gomez, T.E. Kreis, and I. Mellman, manuscript submitted for publication). Although the precise function of endosomal COPs is not established, our data, examined in the light of the known roles of coat proteins (Kreis and Pepperkok, 1994; Robinson, 1994), suggest that endosomal COPs contribute to coat formation during vesicle transport from early to late endosomes.

Endosomal and Biosynthetic COPs

We find that some, but not all, antibodies against βCOP can inhibit ECV formation in vitro, suggesting that some epitopes only are accessible to antibodies when the protein is bound to endosomal membranes. These observations may also suggest that endosomal and biosynthetic forms of βCOP are, at least to some extent, immunologically distinct. Indeed, several βCOP isoforms have been found in high resolution two-dimensional gels (Celis et al., 1994), presumably reflecting the existence of different post-translational modifications. More importantly, the presence of β, ζ, and ϵCOP, but not γCOP, on endosomes suggests that coatomer subcomplexes can exist, and that β, ζ, and ϵCOP may be part of the same subcomplex on endosomal membranes. Presumably, this subcomplex is associated to other, as yet unknown, components on endosomal membranes.

The mechanisms regulating both COP association to endosomes and ECV formation differ, at least to some extent, from those regulating the formation of COP-coated vesicles in the biosynthetic pathway. Both ECV formation and COP association to endosomes are sensitive to the lumenal pH. In contrast, biosynthetic COP-coated vesicles form on the membranes of nonacidic compartments (Mellman et al., 1986), and COP association to ERGIC53-enriched membranes is pH insensitive. We also find that ECV formation is inhibited by GTPγS, as is the formation of plasma membrane-derived clathrin-coated vesicles (Carter et al., 1993) and secretory granules (Tooze et al., 1990), but in contrast to the budding of COP- or COPII-coated vesicles (Ostermann et al., 1993; Barlowe et al., 1994). However, COP binding to endosomes is increased by GTPγS, as is the case for Golgi membranes (Donaldson et al., 1990; Donaldson et al., 1992; Helms and Rothman, 1992; Palmer et al., 1993). On Golgi membranes, both COP recruitment and vesicle formation are stimulated by GTPγS via the small GTP-binding protein ARF1 (see Kreis and Pepperkok, 1994). Since we find that GTPγS stimulates COP association to endosomes but inhibits ECV formation, it appears that these two processes are uncoupled in the endocytic pathway, presumably because they are regulated by more than one GTP-binding protein. Alternatively, COP association to endosomes may be regulated by an ARF with properties distinct from ARF1. Indeed, membrane association of ARF6, which has been implicated between plasma membrane and early endosomes, does not depend on the bound nucleotide state and is insensitive to brefeldinA, in contrast to ARF1 (Peters et al., 1994).

Altogether, our observations indicate that the association of endosomal and biosynthetic COPs to membranes shares similar GTP dependence but is regulated, at least in part, by different mechanisms. Since COP subunits are only partially shared by endosomal and biosynthetic membranes, one may speculate that mechanistic similarities and differences reflect the presence of common and specific coat components, respectively.

Acidification, Membrane Transport, and COPs

Our data show that COPs are directly involved in the formation of ECVs. Neutralization of the endosomal pH reduces the amounts of COPs associated to endosomal membranes, and concomitantly inhibits the formation of ECVs in vitro, as we had observed in vivo (Clague et al., 1994). These observations suggest that the acidification properties of BHK early endosomes may control the formation of vesicles destined for late endosomes by controlling coat recruitment onto the membranes. This mechanism is consistent with the fact that the lumenal pH drops from early to late endosomes (Mellman et al., 1986) and with the observation that ECVs can be more acidic than early endosomes (Killisch et al., 1992). It is not clear how information on the lumenal pH may then be transferred to the cytoplasmic face of the membrane. The simplest view is that pH differences may be sensed by the conformational change of a membrane protein. Indeed, the conformation of trans-membrane proteins can be sensitive to the endosomal pH (Watts, 1984). We, therefore, speculate that the conformation of an endosomal trans-membrane protein, possibly a COP receptor, is pH sensitive, and thus serves as a sensor of the endosomal pH.

In conclusion, formation of transport vesicles at the early endosomal membrane appears to be regulated by COP recruitment on the membrane in a process dependent on the acidic milieu of early endosomes. This mechanism, which is also regulated by GTP-binding proteins, but not by rab5 present on early endosomes, may provide an appropriate means to regulate the formation of these relatively large and multivesicular carriers (Gruenberg et al., 1989; Bomsel et al., 1990; Aniento et al., 1993*a*). One of

our future goals will be to make use of our approach to study in more detail the formation of these vesicular intermediates, and, in particular, the precise role of coat proteins in this process.

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