

Cytoplasmic Coat Proteins Involved in Endosome Function

J. Andrew Whitney,^{*†} Marie Gomez,^{‡†} David Sheff,^{*}
Thomas E. Kreis,[‡] and Ira Mellman^{*}

^{*}Department of Cell Biology
Yale University School of Medicine
New Haven, Connecticut 06520-8002

[‡]Department of Cell Biology
University of Geneva
Sciences III
30, quai Ernest-Ansermet
CH-1211 Geneva 4
Switzerland

Summary

Endosomes are intermediates for a complex series of sorting and transport events that occur during receptor-mediated endocytosis. These involve the recognition of targeting determinants on the cytoplasmic domains of many membrane proteins as well as the formation of specific transport vesicles. Accordingly, endosome function is likely to be governed by the regulated assembly of cytoplasmic coat complexes. We have found that, *in vitro*, endosomes recruit a characteristic set of cytoplasmic proteins in a GTP γ S-stimulated and brefeldin A-sensitive fashion. Among these are members of the COP-I and ARF families of coat proteins. In addition, endosomes were also found to assemble distinct, clathrin-like coats. Since microinjection of antibodies to β -COP inhibits the entry of enveloped viruses via the endocytic pathway, it is apparent that the recruitment of COP-I or COP-I-related proteins plays an important role in the function of endosomes in intact cells.

Introduction

Receptors and ligands internalized during endocytosis are delivered to endosomes, where they are sorted and then transferred to their final destinations. In general, ligands are dissociated from receptors in early endosomes and transported to late endosomes and lysosomes for degradation, while receptors are transferred back to the plasma membrane via a distinct population of recycling vesicles (Kornfeld and Mellman, 1989; Trowbridge et al., 1993). In different cell types, receptors and other membrane proteins may also be targeted into one of several specialized postendosomal vesicles, such as transcytotic vesicles in epithelial cells, synaptic vesicles in neuronal cells, GLUT4 vesicles in adipocytes, and possibly major histocompatibility complex class II-containing vesicles in B lymphocytes (Matter and Mellman, 1994). Thus, early endosomes distinguish multiple types of cargo and target each into vesicular carriers specific for different pathways.

Although little is known about the formation of endosome-derived transport vesicles, three classes of transport vesicles, derived from the endoplasmic reticulum (ER), Golgi apparatus, and plasma membrane, have been well described. In each case, vesicle formation is associated with the assembly of distinct coats from cytosolic precursors. The coats may be responsible for the budding of nascent transport vesicles, the inclusion of specific membrane components into the buds, or both (Kreis et al., 1995). First described was the formation of clathrin-coated vesicles from the plasma membrane (Robinson, 1994). Clathrin assembly occurs following the recruitment of a complex of proteins termed AP-2 adaptors, which recognize coated pit localization determinants on the cytoplasmic domains of endocytic receptors. Clathrin-coated vesicles also form on the secretory pathway following the recruitment of a second adaptor complex (AP-1) to the *trans*-Golgi network (TGN) and transport newly synthesized lysosomal components to endosomes.

Two other types of coat complexes mediate transport between the ER and the Golgi complex (Barlowe et al., 1994; Orci et al., 1986; Waters et al., 1991). COP-I vesicles are formed following the recruitment of a precursor complex (coatomer) to the ER intermediate compartment membrane, the Golgi membrane, or both and have been associated with transport between these organelles (Pepperkok et al., 1993; Peter et al., 1993). Coatomer consists of seven subunits (α , β , β' , γ , δ , ϵ , and ζ) and binds to target membranes in association with ARF (for ADP-ribosylation factor), a small GTPase (Donaldson et al., 1992; Palmer et al., 1993; Serafini et al., 1991). GDP–GTP exchange on ARF occurs concomitant with binding; inhibition of exchange by the drug brefeldin A (BFA) blocks the assembly of COP-I coats and inhibits normal transport (Donaldson et al., 1992; Helms and Rothman, 1992). COP-I coats interact with the KKXX motif found on many resident ER proteins and have been implicated as serving a sorting function in the return of ER proteins from the Golgi complex (Letourneau et al., 1994). ER-to-Golgi transport also involves the formation of a second class of vesicles, designated COP-II (Barlowe et al., 1994). COP-II coats bind to ER membranes in conjunction with an ARF-like GTPase (Sar1p) and may have the ability to discriminate ER proteins from cargo destined for forward transport.

It is not yet clear whether known or novel coat proteins mediate the analogous events on endosomes. Several considerations suggest that such coat proteins exist, however. The sorting of receptors in epithelial cells is determined by cytoplasmic domain targeting signals localized on proteins destined for the basolateral plasma membrane (Matter and Mellman, 1994). These targeting signals may be related to determinants involved in clathrin-coated pit localization and are decoded both in endosomes and in the TGN. They are found on proteins expressed in polarized and nonpolarized cells, suggesting that they play a general role in transport. In addition, BFA inhibits polarized sorting in endosomes and induces a tubular morphology

[†]The first two authors contributed equally to this work.

reminiscent of BFA-treated Golgi and ER membranes (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Matter et al., 1993), suggesting that endosome function may be dependent on an ARF. Various early reports also described clathrin or unidentified coats associated with endosomes (Killisch et al., 1992; Rabinowitz et al., 1992).

We now find that a distinct subset of cytosolic proteins binds selectively to endosomes, including members of the ARF and COP-I families. Their functional involvement is strongly implied by the fact that the entry of vesicular stomatitis virus (VSV), which requires endocytosis for infection, is blocked by microinjection of antibodies to β -COP. A direct role for COP-I proteins in endosome function would explain why CHO cells with a mutation in ϵ -COP exhibit defects in the recycling of plasma membrane receptors (Guo et al., 1994; Hobbie et al., 1994).

Results

GTP γ S Enhances Recruitment of Distinct Cytosolic Proteins to Endosomes

To determine whether potential coat proteins could be selectively recruited to endosomes, we used free flow electrophoresis (FFE) to produce highly purified endosome fractions devoid of contamination by ER and Golgi membranes (Amigorena et al., 1994; Marsh et al., 1987; Schmid et al., 1988). After labeling of early endosomes by incubation for 10 min in horseradish peroxidase (HRP), CHO cells were homogenized and fractionated on a discontinuous sucrose gradient to generate an enriched endosome/Golgi fraction. The fraction contained 40% of the total HRP activity (endosomes), 20% of the cisternal Golgi marker mannosidase II, but <5% of lysosomal marker β -hexosaminidase. After separation by FFE, the major peak of membrane protein migrated in an unshifted position and was coincident with markers for the plasma membrane, the intermediate compartment (p53; ERGIC-53) (Schindler et al., 1993), and the ER (Figure 1). The bulk of the Golgi membranes, as measured by mannosidase II and the TGN marker UDP-galactosyltransferase, was shifted slightly toward the anode. Endosomes (HRP) were more sharply shifted and comigrated with residual β -hexosaminidase activity. The profile allowed pooling of an unshifted fraction containing ER, Golgi, and plasma membrane and a shifted fraction containing endosomes and remaining lysosomes (<10% by protein) and depleted of markers for all other organelles (the detection limit of each assay was <4%).

To determine whether the isolated membranes could recruit cytosolic proteins, both the shifted (endosome) and unshifted (Golgi/ER/plasma) membranes were next incubated with [35 S]methionine-labeled CHO cell cytosol in the presence of 1 mM ATP with or without 25 μ M GTP γ S. As shown in Figure 2, GTP γ S enhanced the binding of several labeled proteins to both the unshifted and shifted membrane fractions. Several had molecular weights reminiscent of known COP-I subunits: 160 kDa (α -COP), 100 kDa (β -COP, β' -COP, γ -COP), 60 kDa (δ -COP), and 21 kDa (ARF). The identities of these proteins were confirmed by Western blot (see below), suggesting that COP-I compo-

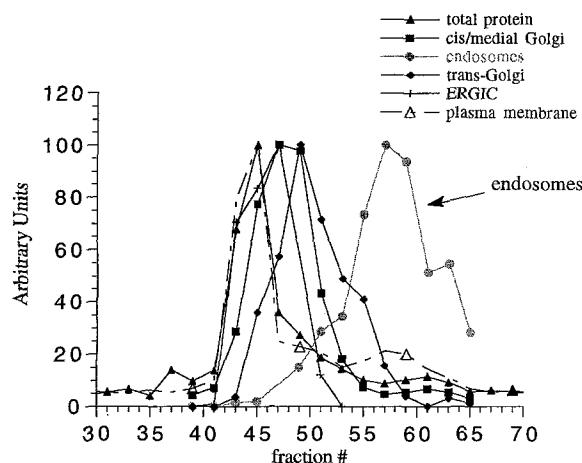


Figure 1. Fractionation of Endosomes in CHO Cells by Use of FFE
CHO cells were incubated with HRP for 10 min and fractionated by FFE. Fractions were collected and analyzed for presence of various organelle markers, either by enzymatic assay or immunoblot. Endosomes routinely shifted 15 fractions from the bulk of remaining protein and 10 fractions from the bulk of Golgi membranes. Fractions were selected and pooled so as to minimize contamination of the endosomes by Golgi membranes.

nents were among the major species recruited to both ER/Golgi and endosome membranes. Western blots also revealed that major bands of 45 kDa and 35 kDa were actin and tubulin, respectively (data not shown). However, significant amounts of both proteins were also recovered in the absence of membranes, suggesting that they sedimented as free microtubules or actin filaments. Several unidentified species were also recruited selectively to either the endosomal or ER/Golgi fractions, as indicated by isoelectric focusing or SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

β -COP Binds to Endosomes in a GTP γ S-Stimulated, BFA-Sensitive Fashion

We first asked whether the best-characterized COP-I component, β -COP, bound to endosomes in vitro. Unshifted and shifted membranes were incubated with unlabeled CHO cytosol and then probed with the anti- β -COP anti-peptide antibody KLVE (Pepperkok et al., 1993). Little if any immunoreactive β -COP was detectable in either membrane fraction if incubated in the absence of cytosol, or if cytosol was added in the absence of membranes (Figure 3A). However, incubation of membranes in cytosol and ATP resulted in the recruitment of β -COP to ER/Golgi-containing unshifted fractions and to endosome-containing shifted fractions. We found that 10%–35% as much β -COP was recruited to the shifted as to the nonshifted fractions (normalized per microgram of membrane protein). The binding to both fractions was inhibited by 200 μ M BFA and stimulated severalfold by 25 μ M GTP γ S (Figures 3A and 3B). In contrast, the G protein agonist ALF4, shown to enhance β -COP binding to ER/Golgi membranes (Donaldson et al., 1991), did not affect the recruitment of β -COP to endosomes (Figure 3A).

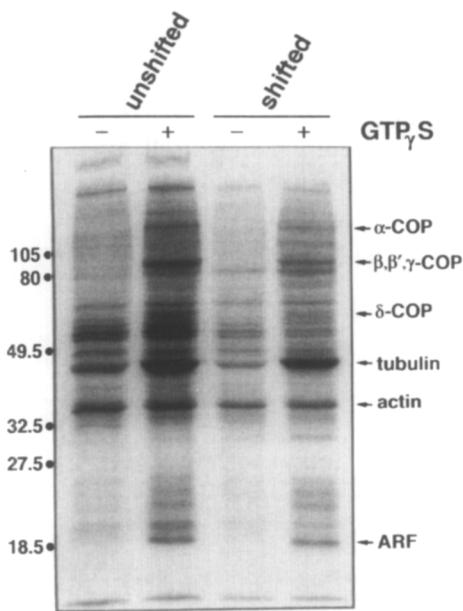


Figure 2. GTP γ S Stimulates Binding of a Discrete Set of Proteins to Endosomes

FFE-enriched endosomes were incubated with 35 S-labeled cytosol for 15 min at 37°C in the absence or presence of 25 μ M GTP γ S. Membranes and bound proteins were then isolated by sequential spins onto a 1 M sucrose cushion and through a 0.75 M cushion. Pellets were resuspended in appropriate sample buffer and analyzed by gel electrophoresis and autoradiography or phosphorimaging. The migration of various known components is indicated.

β -COP could be recruited to endosomes even when purified coatomer and ARF were used instead of crude cytosol. Rat liver coatomer (Waters et al., 1991; Sheff et al., 1995) was incubated in the presence of crude or FFE-fractionated membranes in the presence or absence of recombinant ARF1, GTP γ S, or both. As shown in Figure 3B, total Golgi/ER/endosome membranes (prior to FFE) as well as both the electrophoretically shifted (endosome) and nonshifted (Golgi/ER) membrane fractions recruited β -COP in a GTP γ S- and ARF-dependent fashion.

Differential Binding of Individual COP-I Subunits to Endosome Membranes In Vitro

We next compared the recruitment of β -COP with that of the other COP-I subunits. For these experiments, FFE fractions were combined into three pools, with pools 1 and 2 corresponding to the trailing and leading edges of the unshifted peak and pool 3 the region of the endosome peak that was devoid of Golgi markers. Following incubation with cytosol, equal amounts of each fraction were probed with antibodies to COP-I components as well as other coat-type proteins.

As expected, all COP-I subunits were recruited to the ER/Golgi fractions in the presence of GTP γ S (pools 1 and 2) (Figure 4). Many of the subunits also recruited to the highly purified endosome membranes (pool 3). Quantitation of Western blots exposed in the linear range indicated that α -COP, β -COP, β' -COP, and ε -COP bound to endosomal membranes at levels 10%–35% of those of their bind-

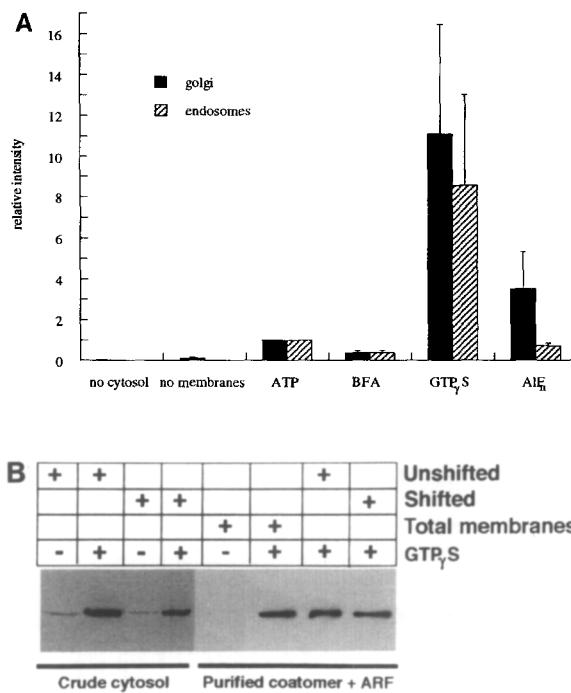


Figure 3. Recruitment of β -COP to ER/Golgi and Endosome Membranes In Vitro

(A) Quantitation of immunoreactive β -COP binding (detected by using the anti-peptide antibody KLVE) to ER/Golgi membranes (closed bars) or endosome membranes (stippled bars). In the first pair of bars (membranes alone), ER/Golgi or endosome membranes were incubated in the absence of added cytosol in ATP at 37°C prior to washing by repeated centrifugation. Cytosol alone refers to cytosol incubated in the absence of added membranes and then processed by centrifugation. This control demonstrates that aggregated β -COP-containing coatomer created negligible background. The remaining pairs of bars represent ER/Golgi and endosome membranes incubated in cytosol in the presence of 1 mM ATP and the absence (ATP) or presence of either 200 μ M BFA (BFA), 25 μ M GTP γ S (GTP γ S), or AlF₃ (AlF₃). (B) Left lanes show quantitative Western blots of β -COP binding to ER/Golgi (unshifted) versus endosome (shifted) membranes in the absence or presence of 25 μ M GTP γ S. Right hand lanes show β -COP binding to total (membranes prior to FFE) as well as to unshifted and shifted membranes following incubation under the same conditions, except using purified rat liver coatomer (10 μ g/ml) and recombinant ARF1 (<1 μ g/ml) in the presence or absence of GTP γ S.

ing to ER/Golgi membranes. On the other hand, binding of γ -COP and δ -COP to pool 3 was not detected, despite the fact that these subunits bound as well as the other COP-I subunits to pools 1 and 2. The 21 kDa subunit, ζ -COP, was recruited nearly as well to endosome membranes as it was to ER/Golgi membranes (>50%).

Recruitment of all COP-I subunits to each membrane fraction was enhanced by GTP γ S and inhibited by BFA (data not shown). That δ -COP and γ -COP failed to bind endosomes even in GTP γ S could mean that coatomer can exist as smaller subcomplexes that bind differentially to different membranes. Subcomplexes of coatomer can be generated in vitro, with a trimer consisting of α -COP, β -COP, and ε -COP (Lowe and Kreis, 1995). Alternatively, antibodies to individual subunits may exhibit differential

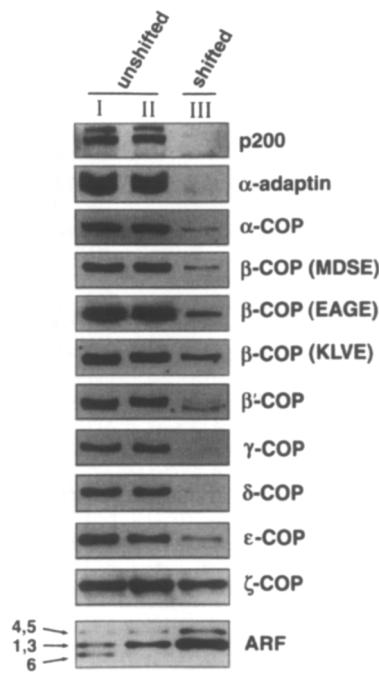


Figure 4. Recruitment of Known Cytoplasmic Coat Proteins to ER/Golgi or Endosome Membranes In Vitro

FFE fractions were divided into three pools corresponding to the trailing and leading edges of the ER/Golgi peak (pools 1 and 2, respectively) and an ER/Golgi-depleted endosome peak (pool 3). Membranes were then incubated in cytosol in the presence of 25 μ M GTP γ S, washed, separated by SDS-PAGE, and probed by Western blot using monospecific antibodies to each of the indicated coat proteins. Individual ARF species were identified by using isoform-specific polyclonal antibodies provided by R. Kahn (Cavanagh et al., submitted).

reactivities to distinct COP-I species that bind to ER/Golgi membranes versus endosomes.

Evidence for differential reactivities was apparent in the case of β -COP. Using a panel of six anti-peptide antibodies directed against epitopes that span the β -COP sequence (Pepperkok et al., 1993), we found that antibodies that exhibited equal reactivity to β -COP that bound to fractions 1 and 2 exhibited different reactivities to β -COP that bound to fraction 3. For example, the anti-peptide antibodies KLVE and EAGE detected the β -COP recruited to fraction 3 3-fold more efficiently than the anti-peptide antibody MDSE (Figure 4). The basis for the differential immunoreactivity is not known. Despite extensive PCR, low stringency cDNA cloning, expression cloning, and database searching, we have been unable to detect β -COP homologs that could explain the antibody cross-reactivity (unpublished data). Since β -COP is phosphorylated, it is possible that such posttranslational modifications may be responsible for differential recruitment, antibody reactivity, or subcomplex formation (Sheff et al., 1995). The fact that purified coatomer can support β -COP binding to both ER/Golgi and endosome membranes suggests that the β -COP comes from a common pool. However, distinct coatomer complexes may copurify despite containing different β -COP species, or novel forms of δ -COP and γ -COP, or both possibilities.

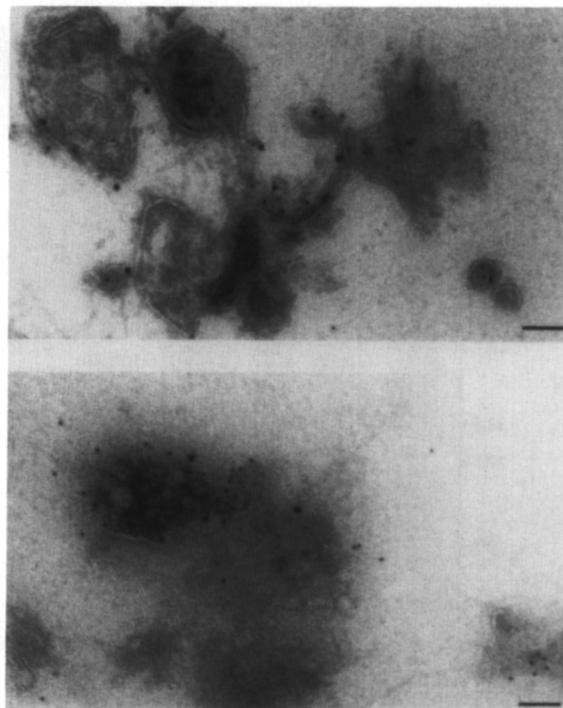


Figure 5. Immunoelectron Microscopy of ζ -COP Binding to Isolated Endosomes

CHO cells were labeled for 15 min with HRP, fractionated by FFE, and incubated with cytosol in the presence of 25 μ M GTP γ S. Samples were then fixed in 8% paraformaldehyde in 100 mM HEPES (pH 7.0) and processed for cryoimmunoelectron microscopy. Cryosections were double-labeled with rabbit anti-HRP followed by 5 nm gold-labeled protein A and with rabbit anti- ζ -COP followed by 10 nm gold-labeled protein A. Scale bars, 100 nm.

It is unlikely that the COP recruitment to endosome-containing fraction 3 reflected contamination by Golgi, ER, or intermediate compartment membranes. First, markers of these organelles, including the intermediate compartment-specific marker ERGIC-53 (p53), were not detectable in fraction 3. Second, the differential immunoreactivity of β -COP recruited to fraction 3 is inconsistent with the presence of membranes equivalent to those in fractions 1 and 2. Third, the cytosolic protein p200, which associates with TGN membranes in a BFA-sensitive fashion (Narula et al., 1992), was not recruited to fraction 3 even in GTP γ S, despite binding well to fractions 1 and 2 (Figure 4). Fourth, the plasma membrane adaptor protein α -adaptin was found in fractions 1 and 2 but not in fraction 3 (Figure 4). Finally, examination of fraction 3 by electron microscopic immunocytochemistry revealed that HRP-containing endosomes bound ζ -COP in the presence of GTP γ S (Figure 5). Qualitatively similar results were found by using antibodies to β -COP and ARF5 (data not shown).

The observation that binding of COP-I components and unidentified proteins to endosome membranes in vitro was stimulated by GTP γ S suggested that one or more ARF proteins may also bind. Probing each of the three membrane fractions with a monoclonal antibody (1D9) that detects all known ARF proteins demonstrated that ARF re-

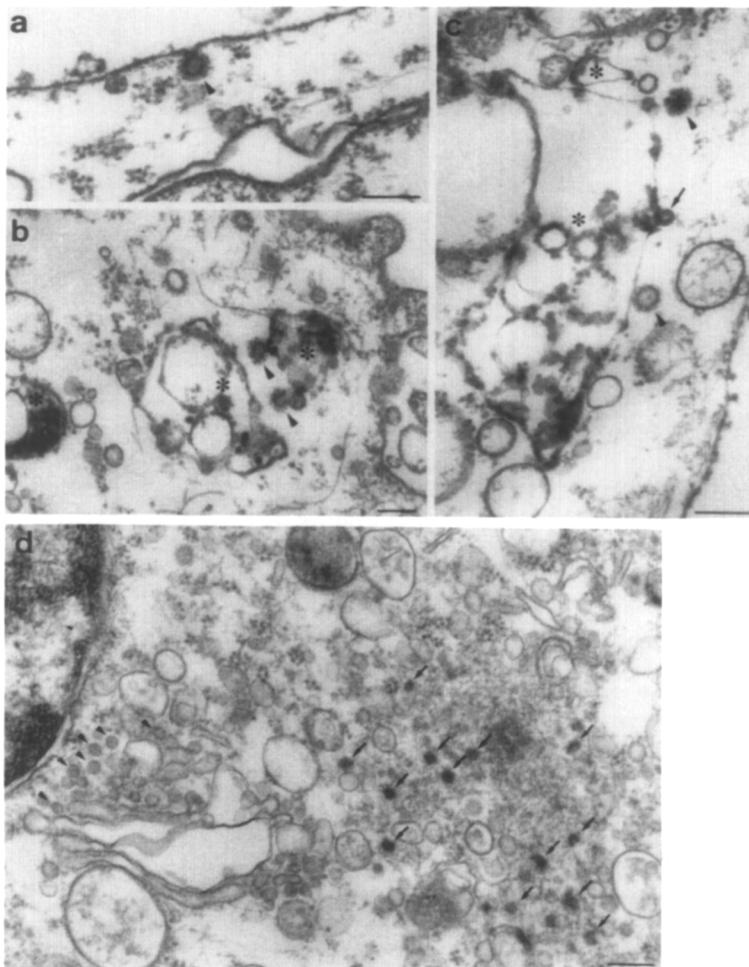


Figure 6. Identification of Coat Structures on Endosomes

CHO cells expressing human transferrin receptor were allowed to internalize Tfn–HRP for 30 min and permeabilized with SLO while in the presence of cytosol and GTP γ S. The cells were then fixed, processed for HRP cytochemistry using DAB, and embedded in Epon.

(a) Clathrin-coated pit at the plasma membrane. Scale bar, 200 nm.

(b and c) Coat-like structures on endosomes. Asterisks mark endosomes containing Tfn–HRP DAB reaction product. Arrowheads point to clearly identifiable clathrin-like structures at the plasma membrane and on endosomes. The arrow points to a Tfn–HRP-positive bud whose cytoplasmic surface contains amorphous dense material different from clathrin. Note that the clathrin-coated pit at the plasma membrane is about twice the overall diameter of the otherwise similar coated bud on the Tfn–HRP-containing endosome. Scale bars, 200 nm.

(d) Endosomal vesicles can be distinguished from Golgi vesicles. Arrows point to Tfn–HRP-positive endosome-derived vesicles in the pericentriolar region, while arrowheads point to presumptive Golgi-derived vesicles. Scale bars, 200 nm.

cruitment to fraction 3 was equal to or greater than recruitment to fractions 1 and 2 (see Figure 4, bottom row). Using a panel of ARF type-specific polyclonal antibodies (Cavanagh et al., submitted), we were able to identify each of these bands and to conclude that ARF1–ARF5 were all avidly recruited to endosomes. Only ARF6, the one ARF that does not exhibit a free cytosolic pool but is stably associated with the plasma membrane, was not associated with endosomes.

Visualization of Endosome Coats

We next determined whether conditions that led to the recruitment of cytoplasmic coat proteins correlated with the appearance of morphologically detectable coats. CHO cells were allowed to internalize transferrin (Tfn)–HRP for 20 min at 37°C to label early endosomes and recycling vesicles. The cells were permeabilized with streptolysin O (SLO) and incubated in CHO cytosol \pm 50 μ M GTP γ S; HRP was visualized with diaminobenzidine (DAB). Although the SLO treatment resulted in a swelling of endosomal vesicles, Tfn–HRP-positive structures were clearly identified (Figure 6, asterisks). Often, the endosomes displayed coated budding structures. The buds, which typically contained HRP reaction product, fell into two classes. The first was characterized by a spiked coat array (arrow-

heads) similar to clathrin-coated pits at the plasma membrane (Figure 6a versus Figures 6b and 6c). However, the endosome-associated buds were approximately half the diameter of cell surface-coated pits (0.2 μ m). These structures were observed both in the presence and in the absence of GTP γ S.

The other type of coated bud was less frequently observed and had a less distinct cytoplasmic coat although appearing to contain HRP reaction product (Figure 6c, arrow). More common were small Tfn–HRP-containing vesicles (60–90 nm diameter) that accumulated in the vicinity of the Golgi complex (Figure 6d), a region occupied by early endosomes and recycling vesicles (Trowbridge et al., 1993). Although the same diameter as and interspersed with presumptive Golgi-derived vesicles, they could easily be distinguished by DAB staining and by the apparent presence of an amorphous coat on their cytoplasmic surfaces (Figure 6d). The small Tfn–HRP-containing endosomal vesicles accumulated most abundantly in permeabilized cell preparations incubated in GTP γ S, although observed at lower frequency following incubation in ATP.

To determine whether these vesicle populations contained COP-I components, the permeabilized cells were reacted with anti- ζ -COP antibodies. As shown in Figures

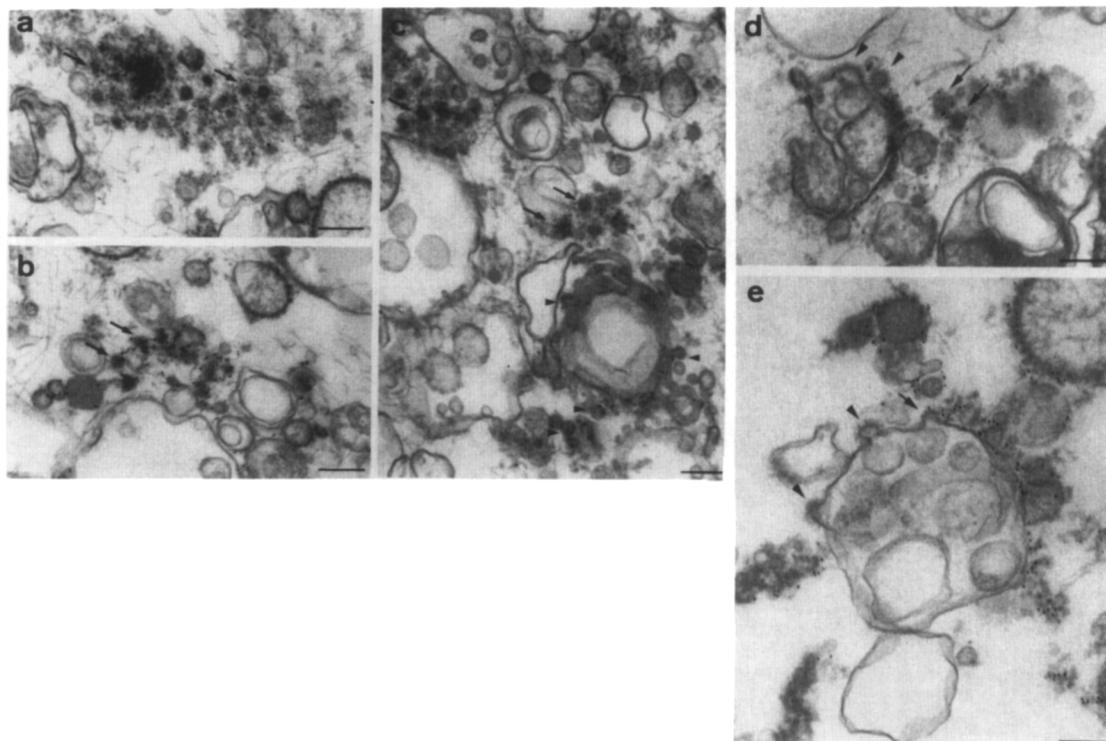


Figure 7. TfN-Containing Endosomes Recruit ζ -COP

Tfn receptor-expressing CHO cells were labeled with TfN-HRP and permeabilized with SL-O as in Figure 6. They were then fixed briefly with paraformaldehyde, labeled with ζ -COP antibodies followed by 5 nm gold-labeled protein A, reacted for HRP using DAB, and embedded in Epon as before.

(a, b, and c) ζ -COP labels endocytic vesicle structures. Arrows point toward endosomal vesicles containing TfN-HRP as well as labeling for ζ -COP. Arrowheads point toward presumptive ER- or Golgi-derived vesicles labeled with ζ -COP antibodies. Scale bars, 200 nm.

(d and e) ζ -COP labels endosomes, but does not label clathrin-like coated buds. Arrows point toward endosomal buds and vesicles labeled with ζ -COP antibodies, and arrowheads point toward clathrin-like buds on endosomes. Scale bars, 200 nm.

7a, 7b, and 7c, the small TfN-HRP-positive vesicles in the Golgi region, as well as elements of the Golgi complex and ER (but not other membranes), labeled strongly for ζ -COP. While it is possible that the amorphous material outside these vesicles was comprised of a COP-I subunit-containing coat, the fact that the material remained in the absence of GTPyS while the ζ -COP labeling decreased argues against this possibility. Indeed, under these fixation conditions, it was not possible to visualize COP-I coats, even on Golgi or ER membranes. The clathrin-like coated buds were not labeled by the anti- ζ -COP (Figures 7d and 7e). These results demonstrate that TfN receptor-containing early endosomes, recycling vesicles, or both give rise to the formation of two types of coated buds or vesicles, one of which labels with the COP-I component ζ -COP.

COP-I Components Are Involved in Endosome Function

We next asked whether the association of COP-I components with endosomes had any functional significance. For this purpose, we modified the microinjection approach used to establish a role for β -COP in transport between the ER and the Golgi complex (Pepperkok et al., 1993). A single-cell assay was devised to measure the entry of

VSV, an enveloped virus that infects cells by endocytosis (O. Rosarius and T. E. K., unpublished data). Infection by VSV of Vero cells was monitored by the accumulation of newly synthesized viral glycoprotein (ts-O45-G) in the ER at nonpermissive temperature. VSV infection requires arrival of viral particles in endosomes with sufficiently acidic pH (presumably late endosomes, pH < 6.0), triggering fusion and release of nucleocapsid into the cytoplasm (Matlin et al., 1982). Thus, any inhibitory effect of injected antibodies on transport to or function of this endosomal compartment would result in an inhibition of virus entry and prevent the synthesis of G protein.

Microinjection of antibody against the EAGE peptide of β -COP, previously shown to block transport between the ER and Golgi, blocked infection of ts-O45 VSV; antibodies that failed to inhibit ER-to-Golgi transport (anti- β' -COP, nonimmune IgG) were without effect. As shown in Figure 8, injection of anti-EAGE prior to exposing Vero cells to VSV prevented synthesis and accumulation of ts-O45-G in the ER (Figure 8b, cells marked by asterisks). As expected, anti-EAGE decorated Golgi/intermediate compartment membranes in the perinuclear cytoplasm (Figure 8a). Microinjection of control IgG resulted in the antibody simply filling the cytoplasm (Figure 8c) and had no effect on virus infection and subsequent synthesis of G protein

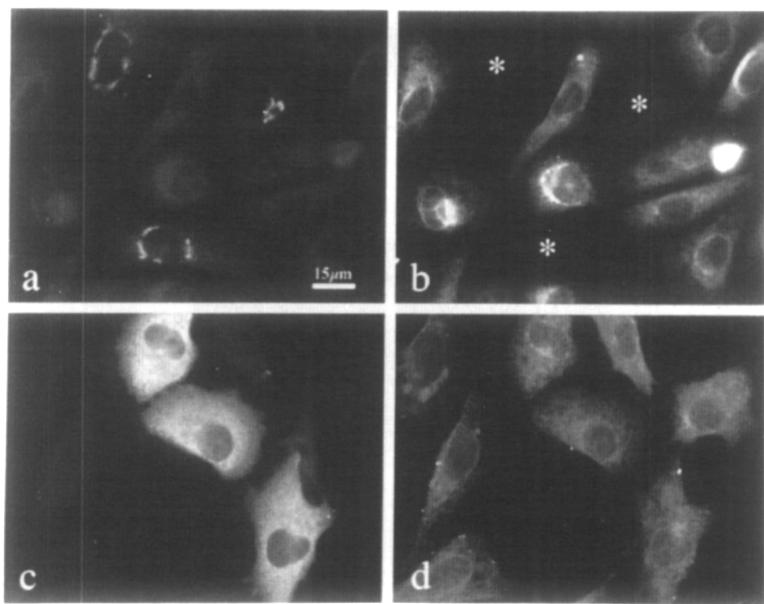


Figure 8. Inhibition of VSV Infection by Microinjection of Antibodies against β -COP

Vero cells were microinjected with affinity-purified anti- β -COP anti-peptide antibody EAGE (a) or rabbit IgG (c) and 2 hr later infected with ts-O45-VSV. Cells were permitted to endocytose virus for 2.5 hr at the nonpermissive temperature and were fixed, and the viral membrane glycoprotein (G protein) was labeled with a monoclonal antibody (P5D4) (Kreis, 1986) against the cytoplasmic domain of ts-O45-G (b and d). The primary antibodies were visualized with fluorescein-conjugated (a and c) or rhodamine-conjugated (b and d) secondary antibodies, respectively. Asterisks (b) indicate the anti-EAGE-injected cells; note the absence of viral protein in these cells. Cells injected with IgG showed no inhibition of G protein synthesis when compared with neighboring uninjected cells.

(Figure 8d). Importantly, anti-EAGE was one of the antibodies that reacted well with the β -COP species recruited to endosomes in vitro.

The results of the microinjection experiments were dramatic. Of cells injected with anti-EAGE, <5% exhibited detectable accumulation of ts-045-G in the ER, whereas 95% of cells injected with control IgG or an antibody to β -COP exhibited normal levels of the viral glycoprotein (Figure 9). NH₄Cl, which neutralizes endosomal pH and thus inhibits fusion of viral and endosomal membranes, completely inhibited ts-045-G synthesis, suggesting that infection was via the endocytic pathway. Finally, the protein synthesis inhibitor cycloheximide (CHX), as expected, blocked G protein synthesis when present throughout the assay. However, if CHX was removed following the initial period of virus uptake (2 hr), ts-045-G was readily detected in the ER. Thus, virus entry from endosomes did not require new protein synthesis. Microinjection also had no obvious effect on the organization of TfN-containing early endosomes or on late endosomes and lysosomes visualized by acridine orange staining (data not shown). Thus, inhibition of virus entry was not simply due to a disruption of the endosomal system. Microinjection of anti-EAGE similarly arrests transport between the ER and the Golgi complex without causing obvious alterations in Golgi morphology (Pepperkok et al., 1993).

To confirm that anti-EAGE acted by inhibiting virus entry from endosomes and not by interfering with nucleocapsid function or G protein translation in the cytosol, we coinjected infectious viral nucleocapsids together with the antibody, conditions which permit VSV infection but which bypass the endocytic pathway. As shown in Figures 10a and 10b, coinjection of anti-EAGE and detergent-treated wild-type VSV resulted in the efficient production of G protein. As expected, the G protein accumulated in perinuclear structures (Figure 10b) that were also labeled by the anti-EAGE (Figure 10a) (Pepperkok et al., 1993). In contrast, coinjection of permeabilized virus with control

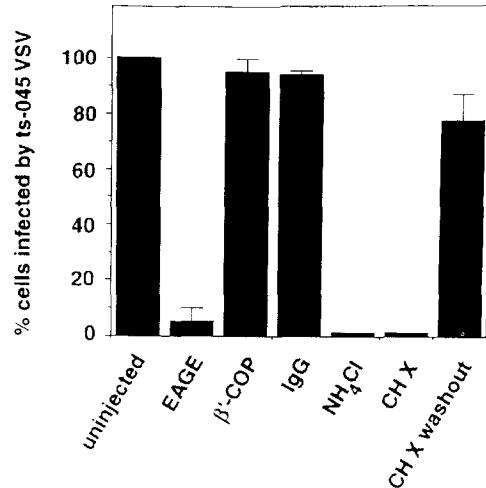


Figure 9. Quantitation of Inhibition of VSV Infection by Microinjection of Anti- β -COP Antibodies

Vero cells were microinjected with antibodies and infected with ts-O45 VSV (conditions that yielded 100% infected cells) as described in the legend to Figure 8. The percentage of cells where significant levels of G protein could be detected in the ER was determined out of a total of 150 cells injected with anti- β -COP (EAGE) or rabbit IgG and 80 cells injected with an affinity-purified antibody to β -COP and compared with uninjected cells in three experiments. Injection with anti-EAGE prevented G protein synthesis in 95% of the injected cells, while injection of rabbit IgG or anti- β -COP had negligible effects. Ts-O45-G in the ER could not be observed when 20 mM NH₄Cl or 10 mg/ml of CHX was present in the culture medium during the infection. On the other hand, washout of CHX in control cells microinjected with IgG restored G protein synthesis in 80% of the cells.

IgG resulted not only in the expected Golgi accumulation of G protein, but also its apparent transport to the cell surface (Figures 10c and 10d). These results strongly suggest that microinjected anti-EAGE acts to prevent the synthesis of G protein by blocking the entry of VSV from endosomes into the cytosol, where viral replication is initiated.

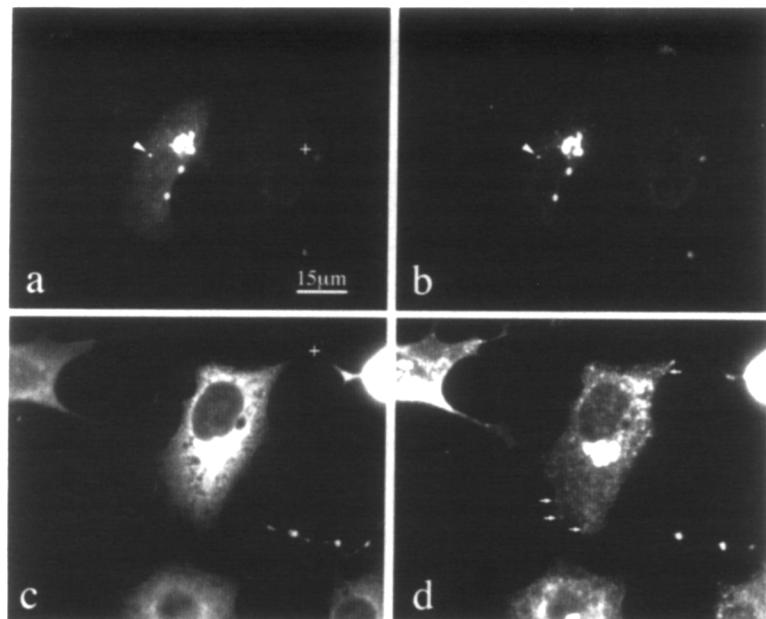


Figure 10. Microinjection of Antibodies against β -COP Has No Effect on Viral Glycoprotein Production from Cytosolic Nucleocapsids

To determine whether delivery of infectious nucleocapsids directly into the cytoplasm bypassed inhibition of VSV entry by microinjection of anti- β -COP antibodies, Vero cells were coinjected with VSV-particles and anti-EAGE (a) or a rabbit IgG fraction (c). Cells were kept in medium containing 20 mM NH₄Cl for 4 hr at 37°C, fixed, and G protein-labeled with P5D4 (b and d). The primary antibodies were visualized with fluorescein-conjugated (a and c) or rhodamine-conjugated (b and d) secondary antibodies, respectively. G protein was synthesized in comparable fractions of cells injected with either antibody fraction. In the cells injected with anti-EAGE, but not IgG, transport of VSV-G to the cell surface was blocked, as no virus particles can be observed at the cell periphery (arrows; compare [b] with [d]). VSV-G accumulated in the region of the Golgi complex and punctate perinuclear structures (arrowheads in [a] and [b]). Neighboring uninjected cells are indicated by a plus symbol.

Therefore, we conclude that β -COP, or a β -COP-related protein, is essential for endocytic transport leading to acidic endosomes in intact cells.

Discussion

Our results suggest that cytosolic proteins that form membrane-associated coat complexes involved in transport through the early stages of the secretory pathway also play a role in transport through the endocytic pathway. While we have not established precisely what step, or steps, during endocytosis are mediated by COP-I components, the finding that COP-I proteins, or their closely related relatives, may be directly involved in endosome function is significant and emphasizes the likelihood that all vesicular transport events are governed by common principles (Rothman, 1994).

Intracellular Distribution and Function of COP-I

β -COP has been found by immunofluorescence and quantitative immunoelectron microscopy to be concentrated in the ER, intermediate compartment, and *cis*-face of the Golgi complex (Griffiths et al., 1995; Oprins et al., 1993; Orci et al., 1993). However, reduced amounts of β -COP have also been observed throughout the Golgi stack and the TGN and with vesicles not associated with Golgi elements. Since COP-I is also thought to mediate vesicular transport between Golgi cisternae (Orci et al., 1986), the sites of greatest concentration of COP-I at equilibrium may not define the only sites of COP-I function. The relative lack of COP-I associated with endosomes in intact cells under normal conditions may reflect a limited number of COP-I binding sites or a rapid turnover of endosome-bound COP-I.

The fact that the immunoreactivity of the β -COP that bound to endosomes was different from that which bound to ER/Golgi membranes may contribute to the difficulty

in detecting endosome-associated β -COP in intact cells. Certain anti-peptide anti- β -COP antibodies detected the endosome-binding form of β -COP better than other antibodies, despite the fact that these reagents had identical reactivities to β -COP that bound to ER/Golgi membranes. Given our inability thus far to identify cDNAs encoding closely related β -COP relatives, it is possible that differences in immunoreactivity reflect posttranslational modifications such as phosphorylation (Sheff et al., 1995). Conceivably, phosphorylation of β -COP controls its site of membrane attachment or the assembly of possible organelle-specific subcomplexes.

Support for the functional association of β -COP with endosomes comes from two independent lines of evidence. Krieger and colleagues have identified a temperature-sensitive CHO cell mutant (*ldlf*) that not only inhibits transport through the secretory pathway but also inhibits the normal recycling of plasma membrane low density lipoprotein (LDL) receptors (Guo et al., 1994; Hobbie et al., 1994). At 40°C, surface LDL receptors are cleared from the plasma membrane and at least partially degraded. While the cellular basis of the *ldlf* phenotype has yet to be established, the mutation itself affects a single gene for ϵ -COP, one of the COP-I components recruited to endosomes *in vitro*. Conceivably, defective COP-I function alters the ability of early endosomes to sort incoming receptors, lysosomal enzymes, or both, thus exposing LDL receptors to damaging proteases long enough to result in degradation of the receptor. Our initial results suggests that endosome sorting is impaired in *ldlf* cells. Even more importantly, we have found that, like wild-type cells injected with anti-EAGE, *ldlf* cells at the nonpermissive temperature are not infected by endocytosed VSV. Importantly, both genetic and immunologic inactivation of COP-I subunits interfere with endosome function.

Second, Gruenberg and colleagues (Aniento et al., submitted) have recently found that addition of an anti- β -COP

antibody reduces the ability of isolated endosomes to exhibit a characteristic vesicle budding event in vitro, presumably corresponding to the formation of late endosomes or carrier vesicles. Irrespective of the precise step reconstituted in this assay, it is clear that an activity intrinsic to endosomes can again be blocked by an antibody to a COP-I component, which we find binds to endosomes in vitro and is required for endosome function in intact cells.

Diversity and Function of Endosome Coat Proteins

The conditions required for fixation and immunocytochemistry combined with the extremely amorphous morphology of endosomes have thus far precluded the visualization of identifiable COP-I-containing coats on endocytic organelles. In vitro, FFE prevented the identification of clear coat structures even on ER/Golgi membranes, despite the fact that the presence of abundant COP-I components could be demonstrated by immunocytochemistry. Similarly, owing to the fixation conditions required, clear coats were not seen on ER/Golgi or endosome membranes of permeabilized cells. As a result, it was impossible to associate the formation of COP-I-containing coats on endosomes with the formation of a specific class of nascent coated vesicles. The presence of Tf_n-HRP in COP-coated structures does, however, demonstrate that they are, or are derived from, early endosomes.

The microinjection experiments clearly demonstrated that interfering with β -COP function blocked VSV infection, but only of virions internalized by endocytosis. Direct microinjection of nucleocapsids into the cytosol bypassed the β -COP-sensitive step. Although the pH threshold of VSV is broad, it must reach endosomal compartments of pH < 6.0 to activate its membrane fusion activity and penetrate from endosomes into the cytosol (Matlin et al., 1982). Therefore, it is likely that the virus must reach late endosomes for entry, as we previously found for a Semliki Forest virus mutant that also activates membrane fusion activity at pH < 5.7 (Schmid et al., 1989). These considerations suggest that COP-I function is required for the transfer of endocytic tracers from early to late endosomes, a suggestion consistent with the in vitro results of Gruenberg and colleagues (Aniento et al., submitted). Furthermore, there is no obvious inhibition in the loss of FITC-Tf_n from recycling vesicles in the microinjected cells (data not shown). Anti- β -COP antibodies may act to inhibit the formation of vesicles involved in early to late endosome transport, or they may simply derange the overall sorting activities of endosomes. We can also not eliminate the possibility that injection of anti- β -COP blocked VSV entry by inhibiting endosome acidification.

It is important to note that endosomes were also found to assemble at least one additional type of coat, morphologically recognizable as containing clathrin. Although immunoreactive clathrin and clathrin coats have long been observed on endosomes, their function has remained unknown. The presence of clathrin adaptor-binding sites on endosomes may simply reflect their transient presence due to receptor recycling. However, it is also possible that the formation of clathrin coats play a critical role in endoso-

mal sorting. Cytoplasmic domain targeting determinants that specify the polarized recycling of internalized receptors to the basolateral surface of MDCK cells often possess tyrosine- or dileucine-based sequence motifs similar to those that specify coated pit localization on the plasma membrane (or in the TGN) (Matter and Mellman, 1994). Thus, it is conceivable that clathrin coats on endosomes participate in the sorting of such recycling receptors.

A role for COP-I coats in sorting must, however, also be considered, particularly given the recruitment of COP-I-related proteins to early endosome-derived Tf_n-containing vesicles. Although COP-I coats have classically been associated with nonselective forward transport from the ER to the Golgi, recent evidence has illustrated that they specifically interact with proteins bearing the KKXX ER retrieval motif (Letourneur et al., 1994). Interestingly, when KKXX-containing proteins are expressed on the plasma membrane, they are rapidly internalized, presumably via clathrin-coated pits (Kappeler et al., 1994). Such a situation would suggest that clathrin adaptors can recognize a ligand for COP-I-dependent sorting. If so, it may also be true that COP-I can decode sorting determinants structurally related to clathrin-coated pit localization signals, such as those involved in basolateral recycling.

Experimental Procedures

Cell Fractionation

CHO cells were grown and fractionated by FFE using a modified Vap21/22 (G. Weber, Munich) essentially as described (Schmid et al., 1988; Amigorena et al., 1994). CHO cells (2×10^7) were incubated with HRP (10 mg/ml in α MEM with 5% FCS) for 10 min at 37°C, followed by three washes in α MEM with 5% FCS at 4°C. Labeled cells were combined with 1×10^9 carrier CHO cells and homogenized, and the resulting postnuclear supernatant was brought to 1.15 M sucrose and loaded below a discontinuous sucrose gradient containing layers of 0.25 M, 0.86 M, 1.00 M, and 1.15 M sucrose. The gradient was centrifuged for 90 min at 40,000 rpm in an SW-41 rotor, and the 0.86 M/1.00 M interface was harvested, treated with 2 μ g of trypsin per milligram of membrane protein for 5 min at 37°C, quenched with a 10-fold excess of soybean trypsin inhibitor on ice, and then subjected to FFE. Trypsin treatment did not affect COP or other protein binding to unfractionated membranes.

Fractions collected from the FFE were assayed for specific organelles as described (Schmid et al., 1988; Amigorena et al., 1994) or by Western blot. Fractions were combined into unshifted and shifted pools that were concentrated by centrifugation for 30 min at 34,000 rpm in an SW41 rotor onto a 1 M sucrose cushion. Cushions were collected and frozen in liquid nitrogen until use for biochemical experiments. For electron microscopy, samples were used immediately.

Antibodies to Cytoplasmic Coat Proteins

Antibodies against β -COP and β' -COP have been previously characterized (Pepperkok et al., 1993; Lowe and Kreis, 1995). Antibodies to all other COP-I components were the gifts of J. Rothman, C. Harter, and their colleagues. Antibodies to p200, α -adaptin, ERGIC-53, and ARFs were provided by J. Stow, M. Robinson, H.-P. Hauri, and R. Kahn.

Cytosol Preparation and Binding Experiments

Unlabeled CHO cytosol was made as described previously (Podbilewicz and Mellman, 1990). To make 35 S-labeled cytosol, four 10 cm dishes of CHO cells were labeled overnight with 1 mCi of 35 S-ProMix (Amersham, Arlington Heights, IL) per dish. Labeled cells were washed, harvested, and combined with 1×10^9 unlabeled spinner CHO cells. For binding experiments, FFE-isolated membranes (1–5 μ g of protein) were incubated with 3 mg/ml CHO cytosol for 15 min at 37°C in a buffer consisting of 1 mM MgCl₂, 50 mM KCl, 200 mM sucrose, 25 mM HEPES-KOH (pH 7.0), and an ATP-regenerating system con-

taining the indicated additions. Samples were cooled to 4°C and centrifuged for 30 min at 17,000 rpm onto a 1 M sucrose cushion in a TLS-55 rotor. The cushions were diluted to 250 mM sucrose in binding buffer and spun for 30 min at 40,000 rpm through a 0.75 M sucrose cushion. For Western blot experiments, membranes were incubated with 3 mg/ml CHO cytosol as above. Samples were cooled and spun for 10–15 min at 14,000 g in a TLA100.2 rotor. Pellets were resuspended in sample buffer, run on gradient 10%–20% polyacrylamide minigels (Daiichi Pure Chemicals, Tokyo), and transferred to nitrocellulose.

Cryoimmunolectron Microscopy

Fresh membranes were fixed by the addition of 1 vol of 8% paraformaldehyde in 200 mM HEPES (pH 7.0) to the binding reaction. After fixation for 30 min on ice, samples were collected by 30 min spin at 4°C in a microcentrifuge. Pellets were processed for immunoelectron microscopy as described (Amigorena et al., 1994).

Preambedding Labeling of Streptolysin-Permeabilized Cells

CHO cells expressing the human transferrin receptor were labeled with 10 µg/ml Tf_n-HRP for 30 min. Cells were cooled and washed once in αMEM with BSA and once with PBS. Cells were then resuspended in 4 U/ml SL-O (Wellcome Diagnostics) and incubated on ice for 10 min (Sodeik et al., 1994). After excess SL-O had been washed off, cells were washed once in IBS buffer (ICT with 1% BSA and 200 mM sucrose; ICT contains 78 mM KCl, 4 mM MgCl₂, 8.37 mM CaCl₂, 10 mM EGTA, 50 mM HEPES-KOH [pH 7.0]) (Podbilewicz and Mellman, 1990). Cells were resuspended in IBS, 10 µg/ml Tf_n-HRP, an ATP regenerating system, 4 mg/ml cytosol, with or without 25 µM GTPγS. After 20 min at 37°C, samples were placed on ice for an additional 30 min. An equal volume of 8% paraformaldehyde in 200 mM HEPES (pH 7.0) was added, and cells were fixed on ice for 5 min. After washing with IBS, the cells were resuspended in IBS with 20 mM glycine for 10 min on ice and then incubated in primary antibody (crude or affinity purified) at 4°C for 4–6 hr with gentle rotation. Pellets were washed three times for 20 min each with IBS and incubated overnight at 4°C with protein A–gold in IBS. Excess protein A–gold was removed with four 1 hr washes in IBS, and cells were fixed for 30 min with 1% glutaraldehyde in 100 mM Na cacodylate. DAB chemistry was performed as described previously (Hunziker et al., 1991).

Microinjection and Virus Infection of Cells

Vero cells were microinjected with specific antibodies (0.8–1.6 mg/ml) as described (Pepperkok et al., 1993); about 100 cells were usually injected and analyzed per experiment. Cells were infected 2 hr after injection with ts-O45 VSV and incubated at nonpermissive temperature (39.5°C) for 2.5 hr as described (Kreis, 1986). Cells were then fixed and extracted with methanol at –20°C during 4 min and subsequently labeled with specific antibodies. In some experiments, detergent-treated VSV particles were coinjected with antibodies. Purified wild-type VSV (1.6 mg/ml) was diluted with an equal volume of PBS containing 0.1% Triton X-100 and incubated at 37°C for 10 min. An equal volume of antibodies (anti-EAGE or IgG) was then added and this solution microinjected into cells. Cells were further incubated at 37°C for 4 hr in the presence of 20 mM NH₄Cl to block endocytosis and virus infection.

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