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Uptake by COPI-coated vesicles of both anterograde and retrograde cargo is inhibited by GTPγS in vitro

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SUMMARY

On the basis of the cell surface protein CD8 we have constructed reporter molecules for both anterograde and retrograde transport from the Golgi complex. The cytoplasmic tail of CD8 was exchanged by a construct comprising a hemagglutinin (HA) epitope, the C-terminal sequence of the viral protein E19 (containing a KKXX retrieval signal) followed by a myc epitope (CD8-LT). Due to this masking of the KKXX retrieval signal CD8-LT is transported to the cell surface. Since the KKXX motif is joined to the myc epitope via a thrombin cleavage site, CD8-LT in isolated Golgi membranes can be proteolytically converted into an unmasked reporter molecule for retrograde transport (CD8-ST) in vitro. A CHO cell line stably expressing CD8-LT was generated and used for the isolation of Golgi membranes. These membranes were shown to contain CD8-LT en route to the cell surface. By addition of thrombin, CD8-LT could be efficiently converted into CD8-ST, and this allows us to study the sorting into coat protein COPI-coated vesicles of these different kinds of cargo on a comparative basis. COPI-coated vesicles were generated in vitro from Golgi membranes containing either CD8-LT or CD8-ST. When the incubation was performed in the presence of GTP, both CD8-LT and CD8-ST were packaged into COPI-coated vesicles. However, COPI-coated vesicles generated in the presence of the slowly hydrolyzable analogue of GTP, GTPγS contained strikingly lower amounts of CD8-LT and CD8-ST. While COPI-coated vesicles accumulated about 12-fold in the presence of GTPγS these vesicles together contained only one fifth of cargo compared to the few vesicles generated in the absence of GTPγS. These data indicate that cargo packaging into COPI-coated vesicles requires hydrolysis of GTP.

Key words: COP-coated vesicle, Vesicular transport, Protein sorting, Anterograde and retrograde cargo, Biosynthetic protein transport

INTRODUCTION

Biosynthetic protein transport along the secretory pathway involves the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment (IC), the Golgi complex and the *trans*-Golgi network (TGN) (Palade, 1975; Kreis et al., 1995; Bannykh and Balch, 1997). The various organelles of this pathway are functionally connected by transport vesicles that bud from a donor compartment concomitant with uptake of cargo molecules (Rothman, 1994; Rothman and Wieland, 1996). Subsequently, transport vesicles fuse with the appropriate target organelle. The formation of transport vesicles is mediated by the recruitment of cytosolic coat proteins that may act as a mechanical device to shape the membrane into a spherical vesicle (Rothman, 1994; Bednarek et al., 1996; Schekman and Orci, 1997).

Proteins destined for secretion exit the ER in COPII-coated vesicles (Barlowe et al., 1994; Aridor et al., 1995) and are then transported to the IC. Most likely, COPI-coated vesicles are responsible for further transport to the Golgi (Pepperkok et al., 1993; Griffiths et al., 1995; Scheel et al., 1997) as well as for transport through the Golgi stack (Orci et al., 1986; Melancon et al., 1987; Orci et al., 1989). More recently, COPI-coated vesicles have also been implicated to function in the retrieval of ER residents from the Golgi back to the ER in yeast (Cosson and Letourneur, 1994; Letourneur et al., 1994) and mammals (Sönnichsen et al., 1996). Moreover, results from yeast genetic studies were taken as evidence that COPI-coated vesicles might be involved exclusively in retrograde transport (Lewis and Pelham, 1996; Gaynor and Emr, 1997). However, biochemical as well as morphological studies using both yeast and mammalian systems strongly

indicate that COPI-coated vesicles operate in anterograde transport through the early secretory pathway as well (Orci et al., 1986; Melancon et al., 1987; Malhotra et al., 1989; Orci et al., 1989; Serafini et al., 1991; Hosobuchi et al., 1992; Pepperkok et al., 1993; Peter et al., 1993; Duden et al., 1994; Guo et al., 1994; Aridor et al., 1995; Bednarek et al., 1995; Griffiths et al., 1995; Rowe et al., 1996; Scheel et al., 1997; Lowe et al., 1997) Most importantly, two distinct populations of Golgi-associated COPI-coated vesicles have been identified by cryoimmuno-electron microscopy of whole cell sections that carry either anterograde or retrograde cargo (Orci et al., 1997). Bivalent antibodies directed against the cytoplasmic domain of VSV-G protein, an anterograde transport marker, inhibit the formation of COPI-coated vesicles in vitro to about 50%. The remaining vesicles are devoid of VSV G-protein but contain almost the same amount of KDEL receptor, used as a retrograde marker, when compared to control conditions (Orci et al., 1997). These experiments indicate that two independent populations of COPI-coated vesicles can form from Golgi membranes in vitro which are supposed to transport cargo in opposite directions. However, the KDEL receptor is not exclusively present in retrograde vesicles but also in forward moving vesicles, as this protein cycles within the early secretory pathway. The KDEL receptor is a component of machinery rather than a typical cargo molecule (as it sorts retrograde cargo, i.e. ER-escaped KDEL proteins, mainly in the *cis*-Golgi area into transport vesicles that move back to the ER) and it does not contain a typical KKXX retrieval signal. Because of the very low concentrations of escaped ER residents in the Golgi it has been difficult to use a classical retrograde cargo molecule to study uptake of such molecules by COPI-coated vesicles.

In the present study, we have established an in vitro system that allows measurement of the uptake by COPI vesicles of both an anterograde and a retrograde marker. We have constructed an anterograde cargo molecule based on the cell surface protein CD8 that can be proteolytically converted in vitro into a retrograde cargo molecule bearing a KKXX retrieval signal. Following the isolation of Golgi membranes from CHO cells stably expressing the anterograde version of the marker, both the uptake of this molecule and, after proteolysis, the uptake of the converted (retrograde) marker can be measured. It is of note that both markers are present in this membrane fraction in moderate amounts. Thus, it appears unlikely that nonphysiological missorting due to overexpression occurs. We observed no striking difference in the uptake of anterograde and retrograde cargo, respectively. These data are consistent with the idea that COPI-coated vesicles simultaneously operate in bidirectional transport from the Golgi complex (Orci et al., 1997). Most strikingly, the uptake by COPI-coated vesicles of anterograde as well as retrograde transport markers is dramatically reduced when these transport vesicles are generated in the presence of GTPγS, a condition that is generally used to accumulate larger amounts of these vesicles. Thus, GTPγS appears not only to abolish coat dissassembly by inhibiting ADP ribosylation factor (ARF)-catalyzed GTP hydrolysis (Tanigawa et al., 1993), but also to affect the process of cargo packaging into COPI-coated vesicles. Possible molecular mechanisms underlying this effect are discussed.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies directed against the c-myc epitope and the lumenal domain of CD8 were purified from the hybridoma cell lines 9E10 and Okt8, respectively. Polyclonal antibodies were used to detect the COPI vesicle membrane proteins p23 (Sohn et al., 1996) and p24 (K. Sohn et al., unpublished) as well as ARF (Palmer et al., 1993). Monoclonal antibodies directed to the HA epitope, β-COP (M3A5) and polyclonal antibodies against the KDEL receptor were generous gifts from Dr Tommy Nilsson (EMBL, Heidelberg), Dr Thomas Kreis (University of Geneva) and Dr Hans-Dieter Söling (University of Göttingen), respectively. Polyclonal antibodies to mannosidase II were obtained from Dr Kelley Moremen (University of Georgia), and those directed against the transferrin receptor were purchased from Zymed (San Francisco, CA). All secondary antibodies used for immunfluorescence studies were from Dianova (Hamburg, Germany) whereas secondary antibodies used for analysis of western blots came from Bio-Rad (München, Germany). All other reagents and chemicals were of analytical grade.

Construction of CD8 fusion proteins

All cloning procedures were performed according to standard procedures. The eukaryotic expression vector pCMUIV containing the CD8 cDNA was used for all constructs (Ponnambalam et al., 1994). The DNA sequence encoding the cytoplasmic tail of CD8 was replaced by synthetic oligonucleotides encoding the amino acid sequence RPYPYDVPDYAPKYKSKKSFIDEKKPRGSEQKLISEEDL (*Sal*I-*Bam*HI fragment) corresponding to an HA epitope, the adenoviral sequence E19, a thrombin cleavage site as well as a myc epitope (CD8-LT). Arginine residues present in the E19 sequence were substituted by lysines to create a unique thrombin recognition site between the E19 sequence and the myc epitope. A second construct was engineered lacking the myc epitope (CD8-ST). This construct ends on the C-terminal sequence KKPR. The predicted nucleotide sequence of all constructs was confirmed by sequencing using the dideoxynucleotide-method (Sanger et al., 1977).

Cell culture, transfection procedures and indirect immunofluorescence

CHO and COS cells were grown according to standard conditions. For transient expression of cDNAs cells were grown on glass coverslips. DNA transfer was performed using the calcium phosphate precipitation method according to Nilsson et al. (1989). Further processing was performed 24 hours posttransfection. Cells were then prepared for indirect immunofluorescence according to standard protocols applying paraformaldehyde fixation and TX-100 permeabilization. After incubation of primary and secondary antibodies cells were washed with PBS and embedded in Fluoromount G (Biozol, Germany). Samples were analyzed using a Zeiss Axiovert 35 microscope equipped with the appropriate filters for FITC- and TRITC-derived fluorescence.

Metabolic labeling of CD8 fusion proteins

Metabolic labeling was performed according to the method of Jackson et al. (1993). Briefly, COS cells were grown on culture dishes and transfected with the various CD8 constructs using the calcium phosphate precipitation method. 24 hours posttransfection, cells were labeled with 150 μCi/ml [^{35}S]methionine/cysteine (Amersham) for 3 hours. Subsequently, cells were kept on ice and lysed in buffer containing 1% Triton X-100 (TX-100). After removal of unsoluble material, CD8 fusion proteins were immunoprecipitated using monoclonal antibodies directed against the lumenal domain of CD8 (Okt8). After separation on 12% SDS polyacrylamide gels (12 cm × 15 cm) precipitates were analyzed by autoradiography using β-max hyperfilms (Amersham).

Generation of a CHO cell line stably expressing CD8-LT

CHO cells were grown on culture dishes and transfected with a mixture of pCMUIV (containing the CD8-LT cDNA) and $pSV2_{neo}$ (containing the cDNA encoding the neomycin resistance gene product; Southern and Berg, 1982) in a molar ratio of 10:1. Selection of CHO clones was performed in medium containing 500 μg/ml G418 (Gibco BRL). After isolating CHO clones resistent to G418, expression of CD8-LT was analyzed applying indirect immunofluorescence. Several clones (referred to as $CHO_{CD8\text{-}LT}$) were isolated that expressed CD8-LT after more than 8 weeks of cell culture without observing any negative cell.

Isolation of CHO Golgi membranes from $CHO_{CD8\text{-}LT}$ and biochemical characterization of CD8-LT

Golgi membranes were isolated as described by Serafini and Rothman (1992). To proteolytically convert CD8-LT into CD8-ST, a given aliquot of Golgi membranes isolated from $CHO_{CD8\text{-}LT}$ (typically 0.2-0.3 mg protein/ml) was mixed with an equal volume of 0.6 mg/ml thrombin, 20 mM Tris-HCl (pH 8.5), 200 mM sucrose and 100 mM KCl. Where indicated, Tris buffer was adjusted to pH 8.0 and pH 7.5, respectively. Samples were incubated for 30 minutes at 25°C. The reaction was stopped by the addition of PMSF (final concentration 1 mM). Under control conditions thrombin was inactivated with PMSF prior to incubation with Golgi membranes. Control incubations using buffer instead of protease are indicated as 'mock' incubation. SDS-PAGE, western blotting (semidry procedure) and ECL detection (Amersham) of antigens was performed according to standard protocols.

Generation of COPI-coated vesicles in vitro

COPI-coated vesicles were generated and purified to near homogeneity exactly as described by Serafini and Rothman (1992) with the exception that Golgi membranes used as the donor membrane were isolated from the cell line $CHO_{CD8\text{-}LT}$ rather than from CHO wild-type cells.

Electron microscopy

COPI-coated vesicles were purified as described in the previous section. Fractions 7 and 8 (corresponding to 42 and 40% sucrose, respectively) of the final isopycnic sucrose gradient were combined and diluted with an equal volume of PBS. Vesicles were then pelleted onto a 50% sucrose cushion by ultracentrifugation at 150,000 ***g*** for 30 minutes at 4°C. Subsequently, vesicles were collected in a final volume of 20 μl. Negative staining was performed according to standard procedures. Briefly, 1.5 μl of vesicle suspension was incubated on a grid for 2 minutes at room temperature. Grids were washed with 1.5% uranyl acetate and then dried for 30 minutes at room temperature. Samples were analyzed using a Zeiss EM10 transmission electron microscope.

RESULTS

We have constructed an anterograde transport marker that is based on the cell surface protein CD8 (Pascale et al., 1992). In order to establish an in vitro system that allows measurement of the uptake of anterograde and retrograde cargo we have modified the cytoplasmic domain of CD8 (Fig. 1). The complete CD8 tail was exchanged by elongating the lumenal and transmembrane domain of CD8 with an HA epitope plus the cytoplasmic tail of the adenoviral protein E19, containing a typical KKXX retrieval signal (Nilsson et al., 1989) and a myc epitope at the extreme C terminus (referred to as CD8 Long Tail = CD8-LT). Since the KKXX signal is masked the protein was expected to behave like the CD8 wild-type

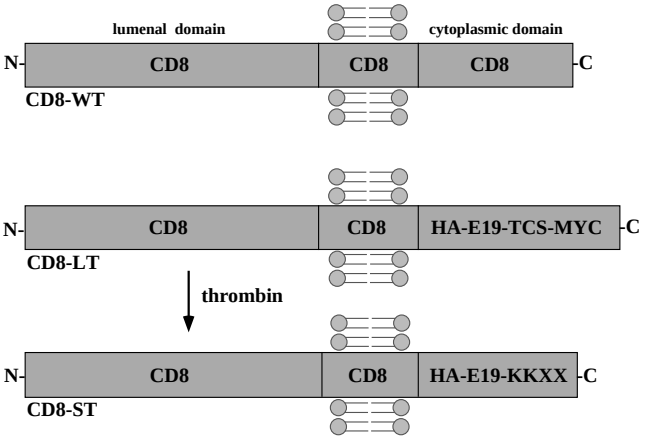


Fig. 1. Schematic description of the structure and topology of the various CD8 constructs used in this study.

molecule (CD8-WT), moving in an anterograde fashion from the ER to the cell surface. To obtain an appropriate retrograde marker as well, we introduced into CD8-LT a consensus sequence between the E19 sequence and the myc epitope that is recognized by the plasma protease thrombin. The essential arginine of this sequence was positioned in such a way that proteolytical cleavage with thrombin results in a product carrying the C-terminal sequence KKPR (refered to as CD8 Short Tail = CD8-ST; Fig. 1). This allows the study of the sorting into COPI-coated vesicles of anterograde and retrograde cargo that is present in roughly the same amounts in the donor membrane.

Transient expression of CD8 fusion proteins in CHO cells

Various cDNAs encoding CD8-WT, CD8-LT as well as a cDNA corresponding to the amino acid sequence of the CD8-LT-protease cleavage product CD8-ST were transiently expressed in both CHO and COS cells. Processing for immunofluorescence detection of the various gene products was performed either with or without permeabilization of the cells in order to discriminate between cell surface staining and intracellular staining of organelles. As reported previously (Pascale et al., 1992), CD8-WT could easily be detected at the cell surface when the cells were processed without adding detergent (Fig. 2). As expected, the same result was obtained with CD8-LT. In contrast, cell surface appearance was not at all detectable with CD8-ST indicating that its KKXX retrieval signal is functional. When the intracellular distribution of the various proteins was studied we found that both CD8-WT and CD8-LT were mainly concentrated in a perinuclear area. This localization is thought to represent at least in part Golgi (see colocalization with the Golgi marker mannosidase II) as newly synthesized material moves towards the cell surface. However, it may in addition represent endosomal compartments as CD8 might be internalized from the cell surface. While this perinuclear signal seems to be strong it might well represent only a small portion of the total population of CD8 molecules because of the compact structure of the Golgi apparatus compared to the plasma membrane. In contrast, CD8-ST showed a typical ER pattern (see colocalization with the ER

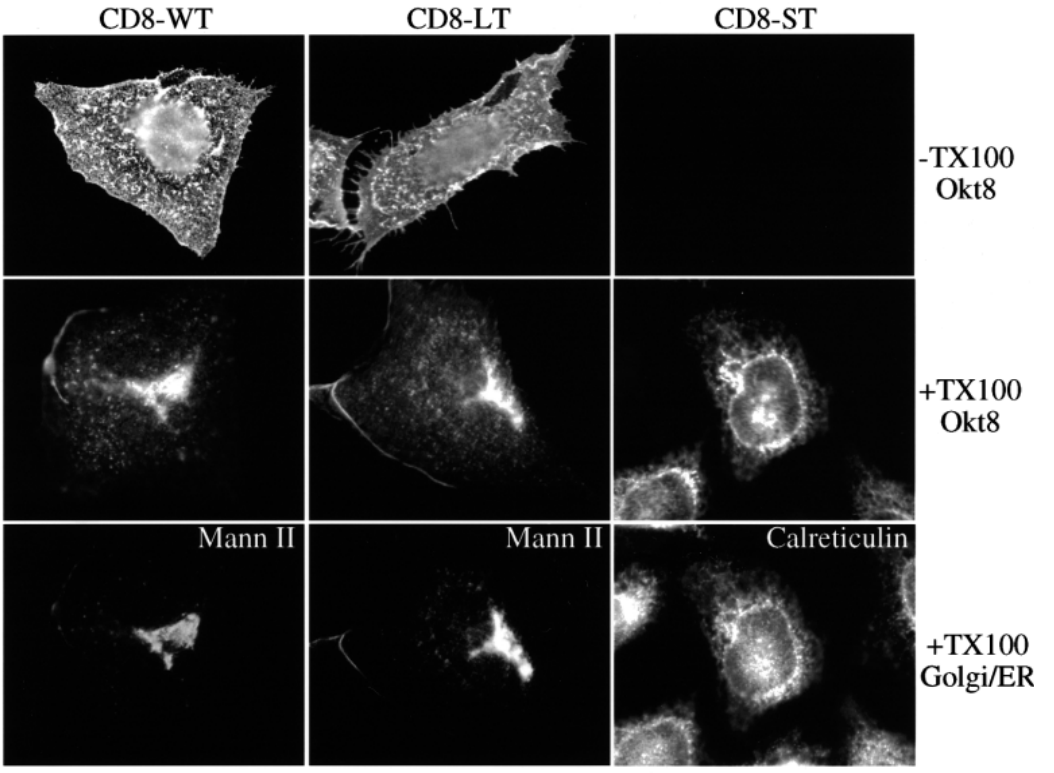


Fig. 2. Characterization of the steady-state localizations of CD8-LT and CD8-ST compared to the CD8 wild-type molecule. CHO cells were transfected with eukaryotic expression plasmids containing the cDNAs indicated. 24 hours posttransfection cells were fixed applying 3% paraformaldehyde. Cells were then either permeabilized with TX-100 or left untreated in order to visualize cell surface staining. CD8 constructs were stained with the monoclonal antibody Okt8 directed against the lumenal domian of CD8. In order to colocalize intracellular populations of CD8 constructs, calreticulin and mannosidase II were used as ER and Golgi marker, respectively.

marker calreticulin) composed of a strong staining of the nuclear envelope and reticular structures scattered throughout the cytoplasm (Fig. 2). For all constructs and conditions similar results were obtained when expressed in COS cells instead of CHO cells (not shown). These data demonstrate that the KKXX retrieval signal is effectively masked in CD8-LT, but confers ER residence to CD8-ST in which the KKXX motif is exposed at the C terminus.

Metabolic labeling of CD8-WT, CD8-LT and CD8-ST

In addition to the characterization of the steady-state localization of the various fusion proteins we studied posttranslational modifications of all constructs by metabolic labeling. Transport through the secretory pathway of proteins carrying the lumenal domain of CD8 can be followed due to the addition of O-linked sugars (Pascale et al., 1992). Three major forms corresponding to the ER (CD8-p), the intermediate compartment/*cis*-Golgi (CD8-i) and the medial/*trans*-Golgi (CD8-m) can be distinguished. As shown in Fig. 3, both CD8-wt and CD8-LT fully mature to the medial/*trans*-Golgi form whereas CD8-ST mainly appears as the intermediate form. It appears, therefore, that CD8-ST continuously exits the ER but is efficiently retrieved from the IC/*cis*-Golgi area resulting in a steady-state localization in the ER (Fig. 2). In contrast, CD8-LT exhibits a behaviour similar to CD8-WT, as it is efficiently converted into the mature form, consistent with its steady-state localization at the cell surface (Fig. 2).

Generation of a CHO cell line stably expressing CD8-LT

To establish the in vitro system we generated a cell line stably expressing CD8-LT. CHO cells were transfected with a mixture of eukaryotic expression plasmids containing the CD8-LT cDNA as well as a cDNA encoding the neomycin resistance gene product. Following isolation of CHO clones that were viable in medium containing G418 these cells were analyzed for expression of CD8-LT (Fig. 4). Localization of CD8-LT in

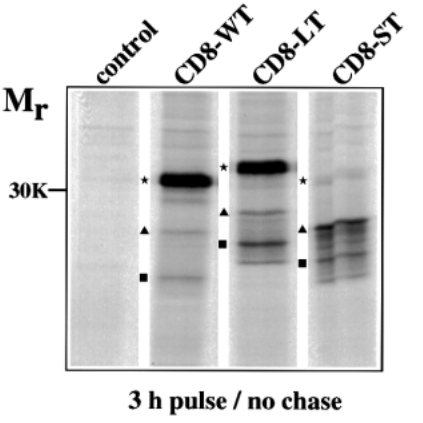


Fig. 3. Metabolic labelling of CD8 fusion proteins. COS cells were transfected with eukaryotic expression plasmids containing the cDNAs indicated. As a control, non-transfected cells were processed as well. 24 hours posttransfection cells were labeled with 150 μCi [^{35}S]methionine/cysteine per ml culture medium for 3 hours without subsequent chase. Cell lysates were prepared using a buffer containing 1% TX-100, and CD8 fusion proteins immunoprecipitated using Okt8 antibodies. Immunprecipitates were analyzed by SDS-PAGE and subsequent autoradiography. Symbol key: ■ = CD8-p (ER form of CD8); ▲ = CD8-i (intermediate form of CD8); ⁕ = CD8-m (mature form of CD8). For details see text.

CHO cells (referred to as $CHO_{CD8\text{-}LT}$,) stably expressing this protein was identical to that in cells expressing the construct transiently (Fig. 2). This was true for both cell surface staining (not shown) and intracellular staining (Fig. 4, lower panels). The perinuclear staining of CD8-LT was at least in part colocalized with the Golgi marker mannosidase II. Western blot analysis of both a total membrane fraction and a Golgi fraction isolated from $CHO_{CD8\text{-}LT}$ demonstrated that CD8-LT migrates at a position consistent with the expected molecular mass of about 35 kDa (Fig. 4, upper panel) corresponding to the mature form of the molecule (compare to Fig. 3). This analysis revealed that the mature form of CD8-LT is enriched in the Golgi fraction compared to total membranes, although the overall signal of the Golgi fraction may in part be due to a contamination with plasma membranes.

Thrombin-catalyzed cleavage of CD8-LT in vitro

A Golgi enriched fraction isolated from $CHO_{CD8\text{-}LT}$ was used to characterize cleavage of CD8-LT by thrombin in vitro. Western blot analysis was performed using an anti-HA antibody that recognizes both CD8-LT and CD8-ST. As shown in Fig. 5A, thrombin treatment of Golgi membranes isolated from $CHO_{CD8\text{-}LT}$ resulted in the appearance of only one additional band migrating faster than the original band. This result is consistent with the unique thrombin cleavage site in CD8-LT (see Materials and Methods). The migration shift corresponded to the expected reduction in size (12 amino acids). Importantly, the cleavage product was formed strictly dependent on the pH value of the incubation buffer (Fig. 5A). No other products were observed. As thrombin treatment was terminated by adding a protease inhibitor, it is unlikely that the C terminus of CD8-LT was unspecifically altered afterwards. Under the conditions used, typically 50 to 80% of CD8-LT could be converted into CD8-ST. In summary, these data confirm that treatment of CD8-LT with thrombin results in the formation in the C-terminal sequence KKPR corresponding to the genetically engineered construct CD8-ST.

We went on to analyze whether thrombin treatment of Golgi membranes damaged other proteins, particularly those which are important for COPI vesicle biogenesis. One such candidate was p23, a major COPI vesicle protein involved in coat assembly (Sohn et al., 1996; Nickel et al., 1997) that contains an arginine residue at the beginning of its cytoplasmic tail close to the membrane. As shown in Fig. 5B, p23 is not cleaved under conditions that promote efficient conversion of CD8-LT into CD8-ST. Moreover, we compared thrombin-treated and untreated Golgi membranes isolated from $CHO_{CD8\text{-}LT}$ by SDS-PAGE and subsequent silver staining, and observed identical protein patterns (not shown). Third, we compared the efficiency of COPI vesicle formation from Golgi membranes either untreated or after exposure to thrombin. No significant reduction in vesicle formation could be observed (not shown). Thus, under the conditions used, thrombin appears to specifically convert CD8-LT into CD8-ST without damaging other proteins and, therefore, without affecting COPI vesicle formation in vitro.

Uptake of cargo into COPI-coated vesicles

Using Golgi membranes isolated from $CHO_{CD8\text{-}LT}$ as a donor membrane, we generated COPI-coated vesicles in vitro. This was performed both in the presence of GTPγS, a condition that allows the generation of larger amounts of COPI-coated vesicles and, in the presence of GTP, using the typical procedure to generate and isolate COPI-coated vesicles (Serafini and Rothman, 1992). With this method, COPI-coated vesicles can be purified to homogeneity applying isopycnic sucrose density centrifugation as the final purification step. Typically, COPI vesicles band at a density corresponding to 40-42% sucrose (fractions 7-8, Fig. 6A). While detectable amounts of COPI-coated vesicles can be generated in the presence of GTP, COPI vesicle formation is increased more than 10-fold in the presence of GTPγS (Fig. 6). Irrespective of the use of GTPγS or GTP, purified COPI-coated vesicles

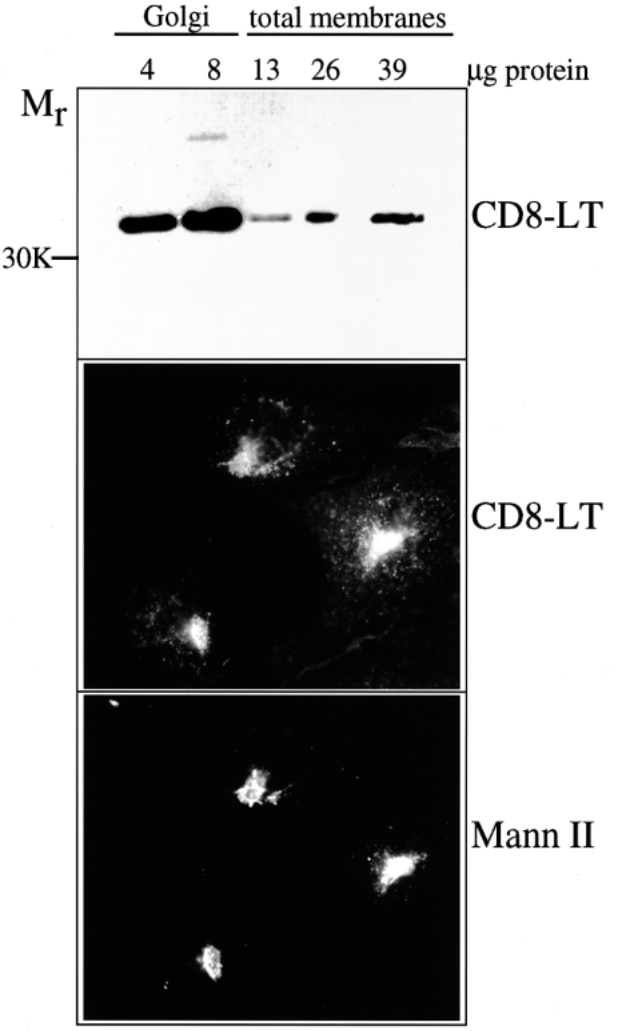


Fig. 4. Generation of a CHO cell line stably expressing CD8-LT. CHO cell clones were selected that were both resistent to G418 and expressed CD8-LT. Upper panel: western blot analysis using anti-myc antibodies directed against the extreme C terminus of CD8-LT. After cell homogenization a 1,000 ***g*** supernatatant was prepared and either used for purification of Golgi membranes (see Materials and Methods) or centrifuged at 100,000 ***g*** to obtain a fraction referred to as 'total membranes'. Protein amounts were loaded as indicated. Lower panels: immunofluorescence analysis of paraformaldehyde/TX100 treated cells using Okt8 antibodies directed against the lumenal domain of CD8. Colocalization was performed using an antibodies directed against mannosidase II.

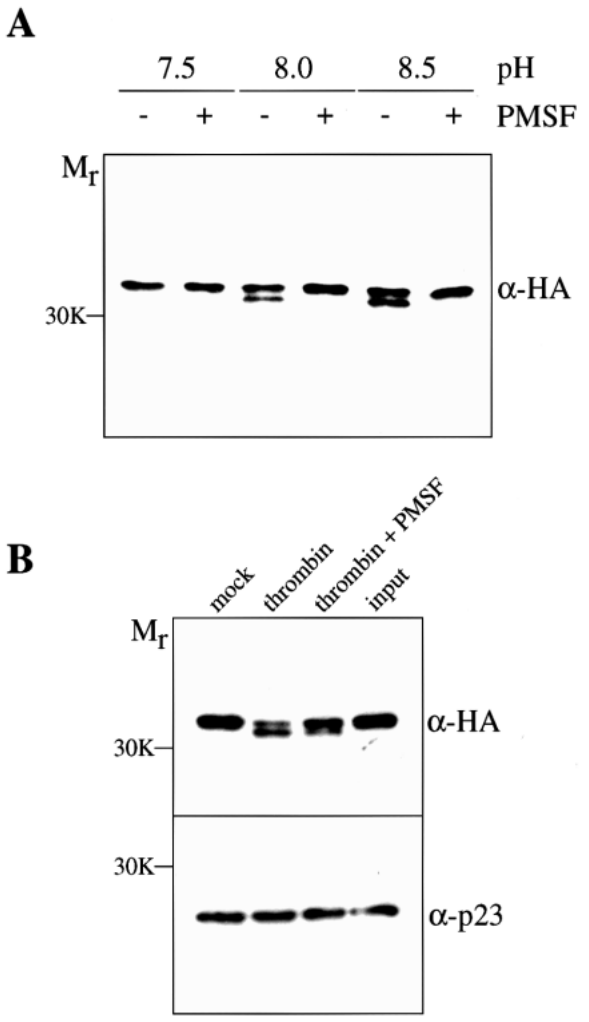


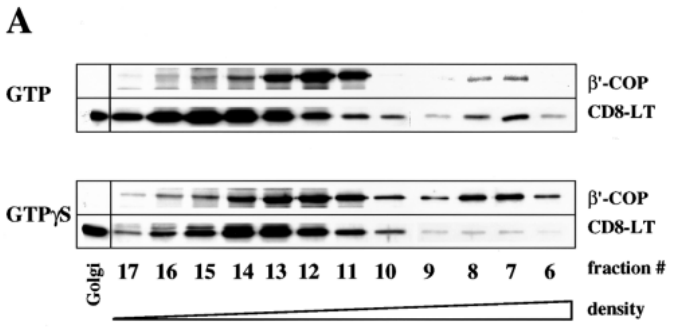
Fig. 5. Cleavage of CD8-LT by thrombin in vitro. Golgi membranes were isolated from $CHO_{CD8\text{-}LT}$ and used as a source for CD8-LT. Incubation with thrombin was performed as described in Materials and Methods. Samples were analyzed by SDS-PAGE and western blotting using anti-HA antibodies followed by ECL detection of antigens. (A) pH dependence of the cleavage reaction. (B) Specificity of thrombin-mediated cleavage of CD8-LT. For details see text.

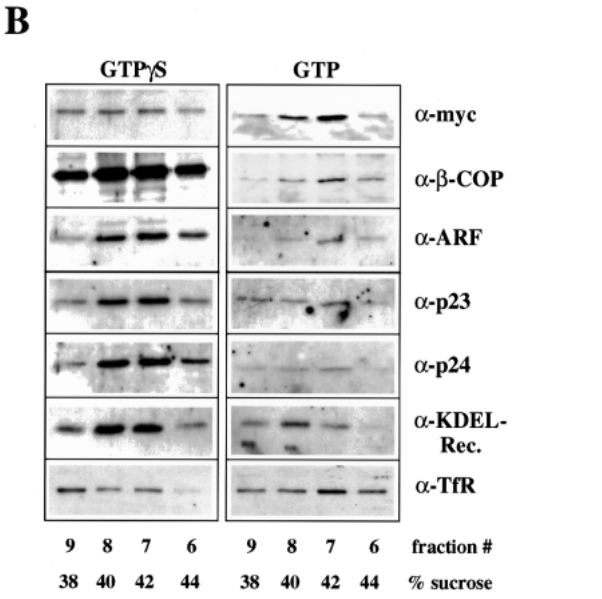
contained all the typical proteins known to be components of this vesicle type at a comparable ratio: β-COP (as a representative for the COPs; (Serafini et al., 1991)), ARF (Serafini et al., 1991), p23 (Sohn et al., 1996), p24 (Stamnes et al., 1995) as well as the KDEL receptor (Sönnichsen et al., 1996). However, striking differences were observed when the uptake of cargo into COPI vesicles was analyzed. Only very small amounts of the anterograde marker CD8-LT could be detected when COPI vesicles were generated in the presence of GTPγS, whereas this cargo was efficiently loaded into vesicles generated in the presence of GTP (Fig. 6A and B, upmost panels). To exclude that this effect is restricted to this particular marker we tested an endogenous anterograde marker as well. As shown in Fig. 6B, the transferrin receptor, present in the Golgi-enriched donor fraction, can easily be detected in COPI vesicles generated in the presence of GTP in amounts exceeding those found in the large amounts of vesicles generated in the presence of GTPγS. In Fig. 6C, the results shown in Fig. 6B have been quantified by scanning of fluorographs using the program NIH Image. Both the ratio of the relative amounts of each protein in 'GTPγS'- and 'GTP' vesicles and the logarithm of this ratio is shown. With GTPγS, COPI vesicles represented by β-COP accumulated roughly 12-fold compared to incubations in the presence of GTP. Similarly, the relative amounts of ARF, p23, p24, and, to a lesser extent, the KDEL receptor were increased with GTPγS (Fig. 6C). Strikingly, quite the opposite was found when the cargo proteins CD8-LT and transferrin receptor were investigated. Given the more than 10-fold excess of COPI vesicles generated in the presence of GTPγS and an excess of CD8-LT of about 5 (3.5 in the case of the transferrin receptor) in vesicles generated in the presence of GTP, about 50-fold more CD8-LT is taken up by 'GTP-COPI' vesicles compared to 'GTPγS-COPI' vesicles. This result identifies p23, p24 and the KDEL receptor as part of the machinery that is needed for COPI vesicle formation rather than as cargo of these carriers. As a consequence, these proteins appear in amounts that represent the amount of vesicles formed under both GTPγS and GTP conditions. The uptake of cargo, however, seems to be sensitive to GTPγS and, thus, requires hydrolysis of GTP.

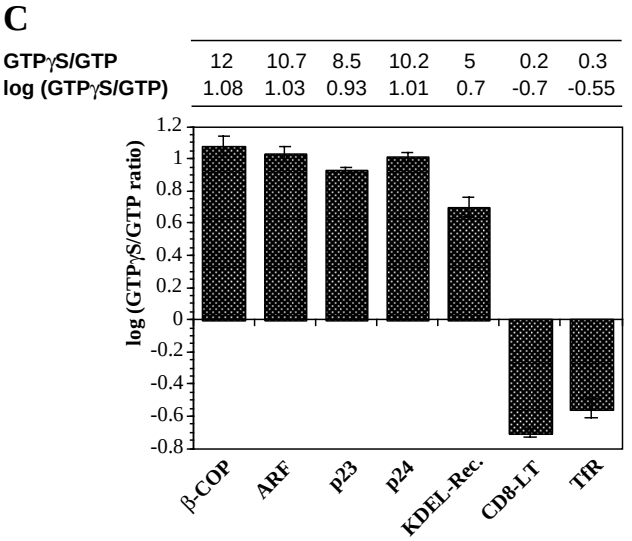
We then analyzed uptake of the retrograde marker CD8-ST. Golgi membranes isolated from $CHO_{CD8\text{-}LT}$ were treated with thrombin and used for COPI vesicle formation in vitro. Again, COPI-coated vesicles could be isolated under both GTPγS- and GTP conditions yielding at least 10fold more vesicles in incubations with GTPγS present (Fig. 6D). Very similar to CD8-LT and the transferrin receptor, CD8-ST was clearly detectable in the small amounts of COPI-coated vesicles generated in the absence of GTPγS. However, the strikingly larger amounts of COPI vesicles generated in the presence of GTPγS did not contain significant amounts of this cargo. As indicated by the Golgi control, about 80% of CD8-LT was converted into CD8-ST. Using anti-myc antibodies, the residual portion of CD8-LT was visualized (Fig. 6D, middle panels), with the same result as observed without thrombin treatment (c.f. Fig. 6A and B). It appears, therefore, that GTPγS in general affects uptake of both anterograde and retrograde cargo molecules by COPI-coated vesicles in vitro.

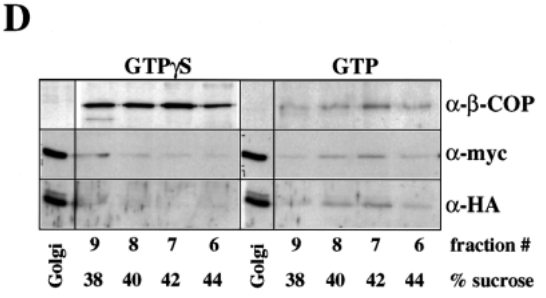
In order to validate the significance of the results described above, formation of COPI-coated vesicles in the presence of GDPβS (instead of GTPγS or GTP) and in the presence of GTP but without cytosol was analyzed. Under both conditions, neither COPI proteins nor CD8-LT cargo could be observed in the fractions that typically contain the majority of purified COPI-coated vesicles (data not shown).

Moreover, the purity of the COPI vesicle fractions was analyzed using electron microscopy. Negative staining was used because GTP-COPI vesicles could not be purified in amounts that would allow processing for cryo-electron microscopy. The combined fractions 7 and 8 of the final isopycnic sucrose gradients were analyzed. Fields at low magnification (Fig. 7A) and representative examples of COPI-coated vesicles at high magnification (Fig. 7B) are shown. Consistent with the biochemical characterization (see Fig. 6), at least 10 times more COPI vesicles could be isolated from incubations in the presence of GTPγS when compared to COPI vesicles purified from incubations in the presence of GTP. Moreover, the purity of both fractions appeared to be similar









without significant contaminants detectable (Fig. 7A). When 'GTP-COPI' vesicles and 'GTPγS-COPI' were visualized at high magnification, no significant morphological differences were observed (Fig. 7B). These data are consistent with the biochemical characterization (see Fig. 6) in that all the typical COPI machinery molecules were found associated with both GTP- and GTPγS-COPI vesicles. Furthermore, the presence of clathrin-coated vesicles at 40-42% sucrose was excluded by visual inspection of electron micrographs, where under the conditions used clathrin-coated vesicles appear clearly distinct from COPI vesicles (data not shown). Moreover, an association of CD8-LT with a contamination of COPII-coated vesicles can be excluded because it is the mature form of CD8-LT that was analyzed. Thus, CD8-LT-containing coated vesicles were formed from a post-ER compartment.

DISCUSSION

From the now long-lasting discussion about the role of COPI-coated vesicles in biosynthetic protein transport through the early secretory pathway two major concepts have emerged: (i) COPI-coated vesicles operate bidirectionally, thereby mediating anterograde IC to Golgi, anterograde intra-Golgi, retrograde intra-Golgi as well as retrograde Golgi to ER transport, as opposed to (ii), where COPI-coated vesicles function exclusively in retrograde transport within the Golgi as well as from the Golgi back to the ER, whereas anterograde transport beyond the IC is mediated by alternative mechanisms (e.g. cisternal progression; for a recent review see Bannykh and Balch, 1997).

With the discovery of COPI-coated vesicles in 1986 (Orci et al., 1986), the carrier mediating protein transport through the Golgi stack appeared to be identified. In this and following reports, striking evidence was provided that COPI-coated vesicles transport VSV-G protein, a classical anterograde

Fig. 6. In vitro generation of COPI-coated vesicles from Golgi membranes isolated from $CHO_{CD8\text{-}LT}$. COPI-coated vesicles were generated as described in Materials and Methods. Incubation of membranes with cytosol was performed either in the presence of GTPγS or in the presence of an equal amount of GTP. Salt-released COPI vesicles were loaded on top of an isopycnic sucrose gradient and centrifuged for 18 hours at 150,000 ***g***. 250 μl fractions were collected from the bottom to the top. Equal volumes of gradient fractions were applied to protein precipitation using the chloroform/methanol procedure and were analyzed by SDS-PAGE and western blotting using the antibodies indicated. Typically, COPI vesicles band in fractions 7-8 corresponding to 40-42% sucrose. (A) Distribution of β-COP and CD8-LT across the isopycnic sucrose gradient after vesicle formation in the presence of GTPγS and GTP, respectively. (B) Comparison of various markers detected in the purified COPI vesicle fraction. (C) Comparitive quantitative analysis of proteins in purified COPI vesicles either generated in the presence of GTPγS or in the presence of GTP. The relative amount of each marker (combined fractions 7 and 8) were quantified by image scanning using the program NIH Image (NIH, Research Services Branch) and are expressed as ratio of the amount of a protein found in vesicles prepared in the presence of GTPγS divided by the amount of the protein found in vesicles prepared in the presence of GTP (upper line) as well as the logarithm of these ratios (bottom line and bar graph). Error bars (s.d.) are calculated from three independent experiments. For further details see text. (D) Comparison of uptake by COPI-coated vesicles of CD8-LT and CD8-ST.

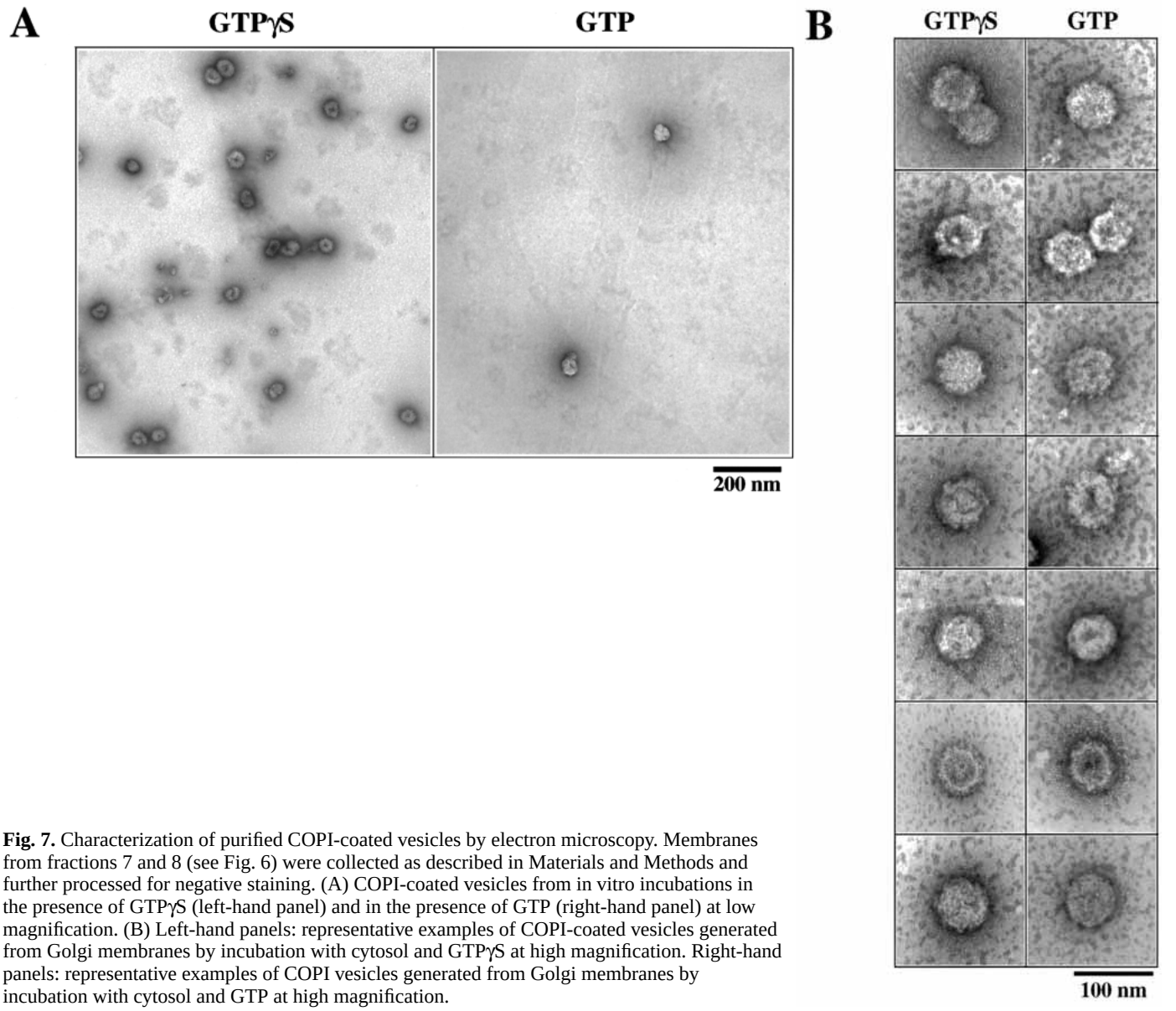


Fig. 7. Characterization of purified COPI-coated vesicles by electron microscopy. Membranes from fractions 7 and 8 (see Fig. 6) were collected as described in Materials and Methods and further processed for negative staining. (A) COPI-coated vesicles from in vitro incubations in the presence of GTPγS (left-hand panel) and in the presence of GTP (right-hand panel) at low magnification. (B) Left-hand panels: representative examples of COPI-coated vesicles generated from Golgi membranes by incubation with cytosol and GTPγS at high magnification. Right-hand panels: representative examples of COPI vesicles generated from Golgi membranes by incubation with cytosol and GTP at high magnification.

transport marker, from one Golgi cisternae to another in vitro (Orci et al., 1986, 1989; Melancon et al., 1987). This kind of protein transport was shown to be inhibited by GTPγS, a drug that causes the accumulation of fusion-incompetent COPI-coated vesicles by abolishing coat disassembly (Tanigawa et al., 1993). More recently, COPI-coated vesicles were shown to act earlier in the secretory pathway as well (Pepperkok et al., 1993; Peter et al., 1993; Griffiths et al., 1995), mediating IC to Golgi transport. In addition, coatomer, the cytosolic precursor of the COPI coat, was shown to bind to the KKXX motif (Cosson and Letourneur, 1994) which is found in many ER resident type I membrane proteins at their extreme C termini (Teasdale and Jackson, 1996). This motif can act as a retrieval signal mediating the back transport of ER residents that have escaped from their intracellular location (Jackson et al., 1993). Thus, COPI-coated vesicles were also implicated in retrograde transport, underlined by the demonstration that retrograde transport is disturbed in yeast cells bearing COPI mutants (Letourneur et al., 1994). Thus, an alternative concept postulates that COPI vesicles exclusively mediate retrograde transport (Pelham, 1994). Support of this idea is based on the analysis of yeast mutants where anterograde transport does not seem to be influenced by several Sec21 (γ-COP) mutants while retrograde transport is clearly inhibited (Gaynor and Emr, 1997). However, anterograde transport should also be inhibited after breakdown of retrograde transport (Pelham, 1994), making these results difficult to interprete. Moreover, recent work demonstrates the existence of two distinct COPI vesicle populations involved in anterograde and retrograde transport, respectively (Orci et al., 1997). While most of the available data favor a role in bidirectional transport, the exact role of COP-coated vesicles is still under debate (Schekman and Mellman, 1997).

This situation has prompted us to develop an in vitro assay that allows comparison of the uptake of anterograde or retrograde cargo. In particular, it has been a major problem to study the sorting of retrograde cargo, because at steady state

ER-resident proteins that have escaped their residence are present in the Golgi in concentrations impeding their biochemical analysis. This situation has forced researchers to use recycling proteins like the KDEL recepter or ERGIC 53 as markers for retrograde Golgi to ER transport (Sönnichsen et al., 1996; Orci et al., 1997). However, these molecules are not only present in retrograde vesicles but also in anterograde vesicles as they shuttle between the ER and the Golgi. Moreover, these proteins are rather machinery than cargo that is being transported from one location to another.

Here we employed a classical anterograde marker (the CD8 cell surface antigen) that, following genetic engineering, can be proteolytically converted into a retrograde marker. Isolation of Golgi membranes from cells stably expressing the anterograde marker led to a membrane fraction with a moderate amount of cargo. Thus, anterograde cargo (CD8-LT) or comparable amounts of anterograde plus retrograde cargo (CD8-ST) were present in the donor membranes used for in vitro formation of COPI-coated vesicles. This enabled us to study the uptake of both markers by COPI-coated vesicles in vitro. Both CD8-LT and CD8-ST were loaded into COPI vesicles as long as these vesicles were generated in the absence of GTPγS. However, COPI vesicles generated in the presence of GTPγS showed a strikingly decreased content of both markers, although at least 10 times more vesicles were formed under this condition. The ratio of cargo to β-COP varied by a factor of about 50 when non-GTPγS conditions were compared to GTPγS conditions. The proportion of machinery molecules like ARF (Serafini et al., 1991) or members of the p24 family (Stamnes et al., 1995; Sohn et al., 1996) to β-COP was roughly constant under both conditions. In addition to the constructed cargo molecules CD8-LT and CD8-ST, we analyzed the uptake of an endogenous anterograde marker, the transferrin receptor. Again, in COPI vesicles generated in the presence of GTPγS, a striking decrease of cargo was observed. Since the appearance of both CD8-LT cargo and COPI proteins in the purified vesicle fraction strictly depended on the presence of cytosol, we conclude that CD8-LT in this fraction is present in COPI-coated vesicles formed in vitro during the incubation. Furthermore, substitution of GTP by GDPβS in the presence of cytosol resulted in the disappearance of both CD8-LT cargo and β-COP from the fractions at 40 to 42% sucrose. These data show that CD8-LT is not present in pre-existing membranes that would contaminate the purified COPI vesicles, but rather that CD8-LT is present in COPI-coated vesicles that had been formed in vitro in a GTP-dependent manner.

We take our findings to indicate that COPI-coated vesicles formed in vitro in the presence of GTPγS might not faithfully represent physiologically loaded carriers. This is of particular interest because COPI vesicles generated in the presence of GTPγS have been used to study their content with respect to cargo and resident proteins of the Golgi (Sönnichsen et al., 1996) in order to gain insight into the function of these transport vesicles. From these experiments, it has been speculated that COPI vesicles might have a major function in the retrograde transport of occupied KDEL receptor because this protein was found to be enriched, whereas anterograde cargo was not found to be enriched in COPI vesicles. From our results, it can be concluded that both anterograde and retrograde cargo have similar access to COPI-coated vesicles, compatible with a role of these carriers in bidirectional transport within the early secretory pathway. This view is consistent with the morphological demonstration of two independent COPI vesicle populations (Orci et al., 1997).

What might be the molecular mechanism underlying the effect observed with GTPγS? It seems that GTP hydrolysis is a prerequisite for proper packaging of cargo into COPI-coated vesicles. A difference might exist between soluble cargo and membrane protein cargo. Uptake by COPI vesicles of bovine serum albumin (BSA) as soluble cargo was compared in the presence and absence of GTPγS, and the amount of BSA was found to increase with the amounts of purified COPI vesicles (Melancon et al., 1987). These data indicate that Golgi-localized soluble cargo is taken up by a bulk mechanism and is not affected by GTPγS. If Golgi-localized membrane protein cargo is also packaged into transport vesicles by a bulk mechanism, the effect of GTPγS described in this study might be due to a kinetic effect. It appears possible that GTPγS-induced enhanced binding of coat proteins to the Golgi results in a non-physiological concentration of p24 proteins that are proposed to form a scaffold to promote COPI vesicle biogenesis (Sohn et al., 1996; Nickel and Wieland, 1997). In this case, membrane protein cargo might be prevented from entering budding zones due to a limited ability of lateral diffusion, as compared to the GTPγS-increased assembly of machinery membrane proteins.

Alternatively, machinery might exist involved in the active packaging of membrane protein cargo into COPI vesicles. In this case the molecular mechanisms of cargo packaging would differ for soluble and membrane protein cargo. It seems possible that GTPγS does not only act on ARF resulting in inhibition of coat disassembly, and thus on vesicle fusion, but also on other GTP-binding proteins involved in packaging into COPI-coated vesicles of both anterograde and retrograde cargo molecules. For example, trimeric G proteins such as $G_{\alpha i3}$, which has been implicated in intra-Golgi transport (Stow et al., 1991), would be candidates for components of machinery that may mediate efficient cargo uptake. When trapped by GTPγS, this machinery might be inhibited resulting in the formation of COPI vesicles that contain all their typical structural components but are devoid of cargo molecules. The in vitro system described in this study should enable us to identify the GTP-binding protein(s) involved, and may eventually allow the isolation of transport vesicles that are enriched in one type of cargo, anterograde or retrograde.

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