

ζ -COP, a Subunit of Coatomer, Is Required for COP-coated Vesicle Assembly

Osamu Kuge,* Sayuri Hara-Kuge,* Lelio Orci,† Mariella Ravazzola,‡ Mylene Amherdt,‡ Gary Tanigawa,* Felix T. Wieland,§ and James E. Rothman*

*Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York 10021; †Department of Morphology, Institute of Histology and Embryology, University of Geneva Medical School, 1211 Geneva 4, Switzerland; and ‡Institut für Biochemie I der Universität Heidelberg, D6900 Heidelberg, Germany

Abstract. cDNA encoding the 20-kD subunit of coatomer, ζ -COP, predicts a protein of 177-amino acid residues, similar in sequence to AP17 and AP19, subunits of the clathrin adaptor complexes. Polyclonal antibody directed to ζ -COP blocks the binding of

coatomer to Golgi membranes and prevents the assembly of COP-coated vesicles on Golgi cisternae. Unlike other coatomer subunits (β -, β' -, γ -, and ϵ -COP), ζ -COP exists in both coatomer bound and free pools.

PROTEIN transport between membrane-bound compartments of eukaryotic cells is carried out by the budding and fusion of transport vesicles (Palade, 1975). Intra-Golgi transport appears to be mediated by COP-coated vesicles that have been isolated and shown to contain a set of coat proteins of apparent molecular mass 160 (α -COP), 110 (β -COP), 110 (β' -COP), 98 (γ -COP), and 61 kD (δ -COP), and several smaller subunits (Orci et al., 1986; Malhotra et al., 1989; Serafini et al., 1991; Stenbeck et al., 1993). Purification of cytosolic β -COP from bovine brain has revealed that it exists as a 650–700-kD complex named coatomer, which contains five peptides with molecular mass identical to α -, β -, β' -, γ -, and δ -COP, and three other peptides with molecular mass 36, 35, and 20 kD, respectively (Waters et al., 1991; Stenbeck et al., 1993). Peptide sequence comparisons have shown that at least three polypeptides (β -, β' -, and γ -COP) are components of both COP-coated vesicles and coatomer (Duden et al., 1991; Serafini et al., 1991; Stenbeck et al., 1992, 1993), and recent studies (Hara-Kuge, S., O. Kuge, L. Orci, M. Amherdt, F. T. Wieland, and J. E. Rothman, manuscript submitted for publication) have directly confirmed that coatomer enters in coated vesicles as an intact unit.

Recently, we have shown that coated vesicle formation and vesicular transport in a cell-free system are dependent on coatomer (Elazar, Z., L. Orci, J. Ostermann, M. Amherdt, G. Tanigawa, and J. E. Rothman, manuscript submitted for publication; Orci et al., 1993). In addition, antibody to β -COP microinjected into cells inhibits transport to the *trans*-Golgi network (Pepperkok et al., 1993), and γ -COP is related to Sec21p, a protein encoded by a yeast gene re-

quired for ER to Golgi transport (Hosobuchi et al., 1992; Stenbeck et al., 1992). Thus, coatomer is required both for intra-Golgi transport and for transport from ER to Golgi, both in vivo and in vitro.

In this study, we define a new coat protein ζ -COP, previously known as p20, the 20-kD subunit of coatomer. We show that this protein is present in coated vesicles, and is required for coatomer binding to the Golgi membranes and for coated-vesicle assembly. While anti- ζ -COP antibody completely inhibits coated vesicle formation, it only partially inhibits the assay signal of intra-Golgi transport reconstituted in a cell-free system. This evidence confirms the dual role of coat assembly in budding and in coupling fusion to budding (Elazar, Z., L. Orci, J. Ostermann, M. Amherdt, G. Tanigawa, and J. E. Rothman, manuscript submitted for publication).

Materials and Methods

Materials

[γ - 32 P]ATP (6,000 Ci/mmol) was purchased from ICN Radiomedicals (Costa Mesa, CA). [α - 32 P]dCTP (3,000 Ci/mmol) and [α - 35 S]thio-dATP (1,500 Ci/mmol) were purchased from DuPont NEN (Wilmington, DE). Peroxidase-conjugated secondary antibodies were purchased from Bio-Rad Labs. (Hercules, CA). A monoclonal antibody to β -COP (M3A5) was kindly provided by Dr. Thomas E. Kreis (Basel, Switzerland). A Golgi-enriched membrane fraction was obtained from CHO cells as described (Balch et al., 1984). Bovine brain cytosol was prepared as described (Malhotra et al., 1989).

DNA Manipulations

DNA manipulations, including restriction enzyme digestion, ligation, plasmid isolation, subcloning, *Escherichia coli* transformation, 32 P-labeling of DNA, and oligonucleotide probes for filter hybridization, were carried out by the standard methods, unless otherwise stated. DNA nucleotide se-

Address all correspondence to James E. Rothman, Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

quences were determined by the dideoxy chain termination methods with Sequenase (United States Biochemical Corp., Cleveland, OH), using walking primers.

Purification of ζ -COP

Coatmer was prepared from bovine liver as described (Waters et al., 1991) and the subunits of coatmer, COPs, were separated by preparative 15% SDS-PAGE. Gels were stained with Coomassie blue and the ζ -COP band was excised. Then, ζ -COP was electroeluted with Bio-Rad model 422 electroeluter, according to the manufacturer's protocol.

cDNA Cloning of ζ -COP

Approximately 100 pmol of ζ -COP was run on a 15% SDS-PAGE, transferred to nitrocellulose filter, stained with Ponceau S, and digested in situ by trypsin (1 μ g sequencing grade trypsin; Boehringer-Mannheim Corp., Indianapolis, IN). The resulting peptides were fractionated by reverse phase HPLC (Tempst et al., 1990) and subjected to amino acid sequence analysis (Tempst and Riviere, 1989). Sequences of two of these peptides were used to design primers for a two stage PCR with a bovine liver λ ZAP II cDNA library (Stratagene, La Jolla, CA) as a template. The primers used for the first round of amplification were AC(ATGC)GA(TC)AG(TC)GA(AG)AT(ATC)GC (codons Thr⁵⁵-Ala⁶⁰, sense) and A(AG) (AG) AA (ATGC) A (AG) (AGTC) CC (TC) TCCAT (codons Met¹¹⁸-Leu¹²³, antisense). The same sense primer and A (AG) (ATGC) CC (TC) TCCAT (AG) TT (TC) TC (codons Glu¹¹⁶-Leu¹²¹, antisense) were used for the second round of amplification. All nucleotides in parentheses were included at that position. The amplification reactions were performed for five cycles with a denaturing temperature of 94°C for 1 min, annealing at 42°C for 1 min, and elongation at 72°C for 2 min, followed by an additional 30 cycles with a denaturing temperature of 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min. The products of the first round amplification were analyzed by Southern blot with an internal probe, AC(AGTC) AC (AGTC) GTIA (AG) (ATGC) CC (TC) TC (codons Glu⁶³-Val⁶⁸, antisense). For Southern blot analysis, DNA fragments were electrophoresed through a 2% agarose gel, transferred to nylon filters, and hybridized with the ³²P-labeled probe for 48 h at 48°C in 3 M tetramethylammonium chloride (Wood et al., 1985), 1 mM EDTA, 5 \times Denhardt's solution, 0.6% SDS, 100 μ g/ml herring sperm DNA, and 0.1 M sodium phosphate (pH 6.8). A final wash was performed in 3 M tetramethylammonium chloride, 0.2% SDS, and 50 mM Tris (pH 8.0) at 42°C for 30 min. The 0.2-kb products of the first round of amplification, which hybridize with the internal probe, were isolated and used as a template for the second round of amplification. The 0.2-kb product of the second round of amplification was subcloned into a plasmid, pBluescript II (Stratagene), sequenced, and used as a probe for screening a bovine liver λ ZAP II cDNA library (Stratagene). Hybridizations were done for 24 h at 65°C in 5 \times SSPE (1 \times SSPE = 0.15 M NaCl, 1 mM EDTA, and 10 mM NaH₂PO₄, pH 7.4), 5 \times Denhardt's solution, 0.5% SDS, and 100 μ g/ml herring sperm DNA. A final wash was performed in 1 \times SSPE, 0.1% SDS at 65°C for 1 h.

The cDNA end corresponding to the 5'-portion of ζ -COP mRNA was amplified by the RACE procedure (Frohman et al., 1988), using bovine liver poly (A)⁺ RNA (Clontech, Palo Alto, CA) as a template. First strand cDNA was synthesized by using a primer, GAGCATCAGCTCATTTTC (codons Glu⁸⁵-Leu⁹⁰, antisense). The gene-specific primers used for cDNA amplification were CACTGTTCAGGCCTTCCAA (codons Leu⁶²-Val⁶⁷, antisense) and GAGGGCAATTCACGTGC (codons Asp³⁶-Leu⁶¹, antisense).

Construction and Purification of ζ -COP Containing a Six-Histidine Extension at Its NH₂ Terminus, His₆- ζ -COP

The coding region of ζ -COP cDNA (clone number 2015) was engineered by PCR to add a BamHI site immediately upstream of the first ATG and a HindIII site immediately downstream of the termination codon. The PCR product was digested with BamHI and HindIII, cloned into the pQE9 vector, and introduced into *E. coli* (M15 harboring plasmid pREP4; Qiagen, Chatsworth, CA). The transformant was grown to a density of A₆₀₀ = 0.9 in Super medium (25 g bacto-tryptone, 15 g yeast extract, and 5 g NaCl per liter with 100 μ g/ml ampicillin and 50 μ g/ml kanamycin at 37°C) and further cultivated for 2 h in the presence of 2 mM isopropyl-thio- β -D-galactoside. The ζ -COP-expressing cells were collected, suspended in Sonication buffer (50 mM Na-phosphate, pH 8.0, and 0.3 M NaCl) containing 1 mg/ml lysozyme, incubated on ice for 10 min, and then disrupted by sonication. The lysate was clarified by centrifugation at 10,000 g for 30 min, and the

supernatant was loaded on a Ni-NTA-agarose (Qiagen) column (8-ml bed volume) previously equilibrated with sonication buffer. The resin was washed with Sonication buffer containing 20 mM imidazole and then His₆- ζ -COP was eluted with a 100 ml imidazole gradient (20–200 mM) in Sonication buffer.

Preparation of Antibody to ζ -COP

A rabbit was injected with 1 mg purified His₆- ζ -COP emulsified in Freund's complete adjuvant. 3 wk later the rabbit was injected with 1 mg purified His₆- ζ -COP emulsified in Freund's incomplete adjuvant, and this booster injection was repeated every 3 wk. Antibody to ζ -COP was affinity purified as described (Harlow and Lane, 1988) by adsorption to His₆- ζ -COP conjugated to AminoLink[®] coupling gel (Pierce Chem. Co., Rockford, IL) according to manufacturer's procedure, precipitated with 60% saturated ammonium sulfate, and dialyzed against 10 mM MOPS-KOH, pH 7.5, and 150 mM KCl. Fab fragments were produced from the affinity-purified anti- ζ -COP antibody using immobilized papain as described by the manufacturer (Pierce Chem. Corp.). The purified Fab fragments (10 μ g) were analyzed by SDS-PAGE and intact antibodies were not detected.

Immunoblot Analysis

Proteins were fractionated by 12% SDS-PAGE under reducing conditions (Laemmli, 1970) and electroblotted onto nitrocellulose in 25 mM Tris, 192 mM glycine, and 20% methanol at 22 V/cm for 1 h. ζ -COP was detected using the affinity-purified anti- ζ -COP antibody (10 ng/ml) and peroxidase-conjugated anti-rabbit IgG (diluted 1:1,000). β -COP was detected using the mouse monoclonal M3A5 (0.4 μ g/ml) and peroxidase-conjugated anti-mouse IgG (diluted 1:1,000). Peroxidase labeling was detected by chemiluminescence using the ECL reagent (Amersham Corp., Arlington Heights, IL) according to manufacturer's procedure.

Immunoelectron Microscopy

Immunoelectron microscopic localization of ζ -COP was performed on CHO Golgi membranes incubated with guanosine 5'-[γ -thio]triphosphate (GTP γ S)¹ to accumulate COP-coated vesicles (Orci et al., 1989), or on COP-coated vesicles purified from rabbit liver as previously described (Malhotra et al., 1989). Both preparations were fixed with glutaraldehyde and embedded in Lowicryl K4M (Armbruster et al., 1982). Thin sections were collected on nickel grids, incubated with anti- ζ -COP antibody, and revealed by the protein A-gold method (Roth et al., 1978).

Preparation of Golgi-derived Coated Vesicles to Be Analyzed by Immunoblot

COP-coated vesicle preparations were performed using CHO Golgi membranes as previously described (Serafini et al., 1991).

Cis- to Medial-Golgi Transport Assay

The preparation of assay components and standard incubation conditions were as described previously (Balch et al., 1984; Malhotra et al., 1989). Reactions (50 μ l) contained 25 mM Hepes (pH 7.0), 2 mM MOPS (from antibody solutions), 30 mM KCl, 2.5 mM magnesium acetate, 5 mM creatine phosphate, 0.25 mM UTP, 0.05 mM ATP, 8 IU/ml creatine kinase, 8 μ M palmitoyl CoA, 0.4 μ M UDP-³H-N-acetylglucosamine (³H]GlcNAc), bovine brain cytosol (5 μ l), donor stacks (5 μ l), and acceptor stacks (5 μ l).

Coatmer Binding Assay

β -COP binding to Golgi membranes was carried out as described (Palmer et al., 1993) except that reactions (50 μ l) contained 25 mM Hepes (pH 7.0), 2 mM MOPS (from antibody solutions), 30 mM KCl, 2.5 mM magnesium acetate, 5 mM creatine phosphate, 0.25 mM UTP, 0.05 mM ATP, 8 IU/ml creatine kinase, 8 μ M palmitoyl CoA, bovine brain cytosol (5 μ l), and CHO Golgi membranes (10 μ l).

1. *Abbreviations used in this paper:* ARF, ADP ribosylation factor; GlcNAc, N-acetylglucosamine; GTP γ S, guanosine 5'-(γ -thio)triphosphate; VSV-G, vesicular stomatitis virus glycoprotein.

Electron Microscopy to Examine the Effect of the Anti- ζ -COP Antibody on Coated Vesicle Assembly

Reactions (500 μ l) contained 25 mM Hepes (pH 7.0), 2 mM MOPS (from antibody solutions), 30 mM KCl, 10 mM NaCl, 1 mM potassium phosphate, 2.5 mM magnesium acetate, 5 mM creatine phosphate, 0.25 mM UTP, 0.05 mM ATP, 10 μ l methanol, 8 IU/ml creatine kinase, 20 μ M palmitoyl CoA, bovine brain cytosol (50 μ l), and acceptor CHO Golgi membranes (50 μ l). Reactions were assembled on ice, incubated at 37°C for 10 min, and incubated for another 10 min after the addition of 1 μ l of 10 mM GTP γ S. Golgi membranes were pelleted for 15 min at 4°C with a microfuge, fixed by glutaraldehyde, and processed for electron microscopy as described previously (Orci et al., 1991). The quantitative evaluation of nonclathrin-coated vesicle and buds was performed as described previously (Orci et al., 1991).

Gel Filtration of ζ -COP in Bovine Brain Cytosol

Bovine brain cytosol (0.2 ml, 33.8 mg/ml of protein) was fractionated using a 24-ml Superose 6 gel filtration column (1-cm ID; Pharmacia Diagnostics Inc., Fairfield, NJ) previously equilibrated with 150 mM KCl, 10% (wt/vol) glycerol, 25 mM Tris-HCl (pH 7.4), and 1 mM dithiothreitol at 4°C. The column was eluted in this buffer at 0.3 ml/min (0.4 ml/fraction). A 10- μ l aliquot of each fraction was subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with the anti- ζ -COP antibody and a monoclonal antibody to β -COP (M3A5) as described above. Quantitation of β -COP was performed as described previously (Waters et al., 1991) using [¹²⁵I]protein A (ICN Biomedicals, Inc., Costa Mesa, CA). Quantitation of ζ -COP was carried out as that of β -COP except that incubation of nitrocellulose with rabbit anti-mouse IgG was omitted.

Results

cDNA Cloning of ζ -COP

ζ -COP purified from bovine liver was digested with trypsin and the isolated peptides were subjected to amino acid sequence analysis to design PCR primers for amplification of ζ -COP cDNA. ζ -COP cDNA was amplified in two stage PCR with a bovine liver cDNA library as a template, by using two pairs of nested primers. The nucleotide sequence of the ~200-bp fragment obtained revealed a reading frame which contained the coding sequence of the two tryptic peptides derived from purified ζ -COP. This fragment was then used to screen a bovine liver cDNA library constructed with λ ZAP II vector. Among 1×10^6 clones screened, four positive clones were isolated. Partial DNA sequencing indicated that three of the clones, numbers 204, 206, and 2016, lacked nucleotides encoding the NH₂-terminal portion of ζ -COP. Another clone, number 2015, was found to contain the poly(A) tail at the wrong cDNA end, when compared with clone 204 and 206 (Fig. 1 a), indicating that clone 2015 is a ligation product of ζ -COP cDNA and another unknown cDNA. To determine the NH₂-terminal end of ζ -COP, the cDNA corresponding to the 5'-portion of ζ -COP mRNA was isolated by the RACE procedure (Frohman et al., 1988). A combined nucleotide sequence of the RACE product and clone 206 contained an open reading frame of 534 bp (Fig. 1 b). This open reading frame was found in clone 2015. The first ATG codon in the open reading frame is in an appropriate context for translational initiation (Kozak, 1987), and an A residue occurs at the -3 position (with respect to the first base of the triplet) while G occurs at the +4 and -6 positions. An in-frame termination codon is found upstream of the first ATG. Thus, this ATG is probably used *in vivo* as a translational initiation codon. The open reading frame contained three ζ -COP-derived tryptic peptides.

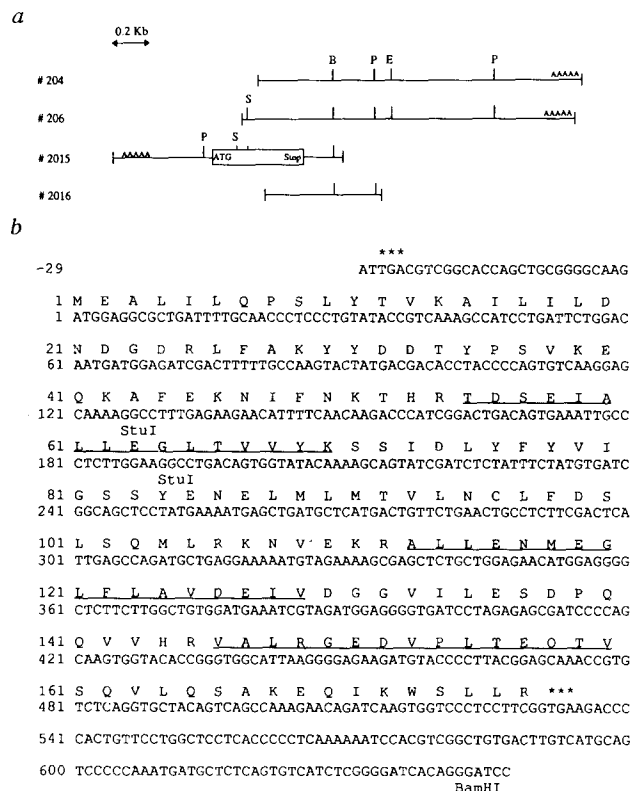


Figure 1. cDNA sequence of ζ -COP. (a) Restriction maps of ζ -COP cDNA clones. The abbreviations for restriction enzymes are B, BamHI; E, EcoRI; P, PstI; and S, StuI. The open box represents an open reading frame of 534 nucleotides. (b) Nucleotide and deduced amino acid sequence of ζ -COP. Underlined regions indicate matches of partial protein sequences of ζ -COP with the cDNA-deduced ζ -COP protein sequence. The asterisks denote stop codons.

The cDNA of ζ -COP predicted a protein with molecular mass of 20,219 D. This is in close agreement with the apparent molecular mass of ζ -COP ($M_r = 20$ kD) determined by SDS-PAGE. A computer search of data libraries revealed that ζ -COP is similar to API7 and API9 (Kirchhausen et al., 1991), the mammalian small chains of the clathrin adaptor complexes. The optimal protein alignment obtained with the BESTFIT program (Smith and Waterman, 1981) indicates that identities of ζ -COP to API7 and API9 are both 24% (Fig. 2).

ζ -COP Is Present in COP-coated Vesicles as Well as in Coatomer

ζ -COP containing a six histidine extension at its NH₂-terminus, His₆- ζ -COP, was expressed in *E. coli*, purified by affinity chromatography on a Ni-NTA column, and used as an antigen to produce polyclonal anti- ζ -COP antibody. An anti- ζ -COP antibody purified by affinity chromatography recognized a 20-kD protein on immunoblots of bovine liver cytosol (Fig. 3, lane I) and purified coatomer (Fig. 3, lane 2). This antibody was used to examine whether ζ -COP is present in COP-coated vesicles as well as in coatomer. We prepared Golgi-derived COP-coated vesicles by isopycnic gradient centrifugation as described (Serafini et al., 1991).

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AP17      .....MIRFILIQNRRAGKTRLAKWYMQ.FDDDEKQKLIIEVHAV
          :: ||| ::::: ::||| ::::: ::||| ::
ζ-COP    1 MEALILQPSLYTVKAILILDNDGDRFLFAKYDDTYPSPVKEQKAFKFNIFN 50
          :: ::::: ::||| ::::: ::||| ::::: ::||| ::
AP19      .....MMRFMLLSRQGLRLQKWLAT..SDKERKKMVRLELMQ

AP17      VT.VRDAKHTNFVEFRNFKIIRRYAGLYFCICVDVNDNNLAYLEAIHNF
          | ::::: ::||| ::::: ::||| ::::: ::||| ::
ζ-COP    51 KT.HRTDSEIALLE..GLTVVYKSSIDLTYFVIGSSYENELMLMTVLNCL 97
          :: ::::: ::||| ::::: ::||| ::::: ::||| ::
AP19      VVLARKPKMCSFLEWRDLKVVYKRYASLYFCCATEGQDNELITLLELTHRY

AP17      VEVLNEYFH.NVCELDLVEFNFKYVTVVDEMFLAGEIRETSQTKVLKQLL
          :: |::: ::||| ::::: ::||| ::::: ::||| ::
ζ-COP    98 FDSLSQLRKNVEKRALLENMGLFLAVDEIVDGGVILESDPQQVVRVA 147
          :: |::: ::||| ::::: ::||| ::::: ::||| ::
AP19      VELLDKYFGSVCELDIIF.NFEKAYFILDEFMGG.....DVQDTSKKSIV

AP17      M.....LQSLK.....
          :      |||
ζ-COP    148 LRGEDVPLTEQTVSQVLSAQEKIKWSLLR 177
          |::: ::||| ::::: ::||| ::::: ::||| ::
AP19      LKAIEQADLLQEEDESPRSVLEEMGLA...
  
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Figure 2. Sequence alignment of ζ-COP with AP17 and AP19. Identities (vertical bars) and conservative replacements (colons and dots) are indicated.

A portion of each isopycnic gradient fraction was subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the anti-ζ-COP antibody and a monoclonal antibody M3A5 (Allan and Kreis, 1986) to β-COP which had been shown to be present in COP-coated vesicles (Duden et al., 1991; Serafini et al., 1991; Waters et al., 1991). The anti-ζ-COP antibody recognized a 20-kD protein, the two peaks of which coincided with those of β-COP on the gradient (Fig. 4). One peak was found at a density of 41% sucrose (Fig. 4), the density expected for the COP-coated vesicles. This result indicates that ζ-COP is present in COP-coated vesicles as well as in coatomer. Immunoelectron microscopy with the anti-ζ-COP antibody confirmed that ζ-COP is localized to COP-coated vesicles; Golgi-derived coated vesicles were able to stain with anti-ζ-COP antibody, as shown in Fig. 5.

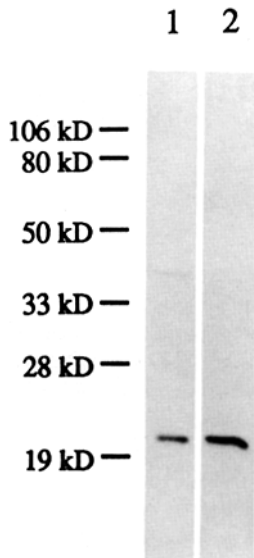


Figure 3. Immunoblotting analysis using the affinity-purified anti-ζ-COP antibody. 15 μg of bovine liver cytosol protein prepared as described (Waters et al., 1991) (lane 1) and 0.1 μg of purified coatomer proteins (lane 2) were separated by SDS-PAGE and probed on Western blots with the affinity-purified anti-ζ-COP antibody as described in Materials and Methods.

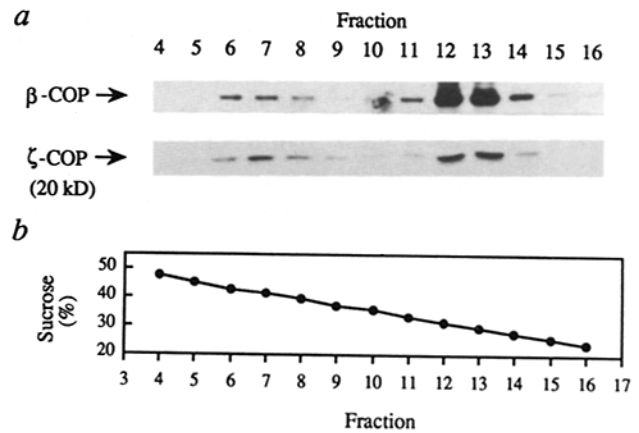


Figure 4. Presence of ζ-COP in COP-coated vesicles. (a) COP-coated vesicles were prepared from CHO Golgi membranes as described (Serafini et al., 1991) and proteins in fractions 4–16 of the isopycnic gradient were subjected to SDS-PAGE in 12.5% acrylamide gels and high and low molecular mass proteins were probed on Western blots either with the monoclonal antibody, M3A5, for β-COP or the affinity-purified anti-ζ-COP antibody, respectively. (b) Percent sucrose (wt/wt) composition of the fractions in a as determined by refractometry.

Effect of the Anti-ζ-COP Antibody on Intra-Golgi Transport

We tested the effect of the anti-ζ-COP antibody on intra-Golgi transport, using a cell-free system (Balch et al., 1984). The cell-free system reconstitutes the transport of the VSV (vesicular stomatitis virus) -G protein between the *cis*- and *medial*-Golgi compartments. Addition of the anti-ζ-COP antibody (up to 10 μg) partially inhibited (up to about 40%) the assay signal as measured by the incorporation of [³H]GlcNAc into VSV-G protein, while the same amount of preimmune IgG had no effect (Fig. 6). The inhibition by the anti-ζ-COP antibody was overcome by the addition of purified His₆-ζ-COP as competitor (Fig. 6 b). As shown in Fig. 6 a, partial inhibition was also observed in reactions containing Fab fragments prepared from the anti-ζ-COP antibody, indicating that partial inhibition was not due to aggregation of proteins or membranes in the presence of divalent antibodies.

The Anti-ζ-COP Antibody Prevents the Assembly of Coated Vesicles on the Golgi Membranes

The association of coatomer (detected with a monoclonal antibody to β-COP) with Golgi-enriched membranes has been studied in a cell-free system (Donaldson et al., 1991, 1992; Palmer et al., 1993). Binding of β-COP is enhanced in the presence of the nonhydrolyzable GTP analogue, GTP[γS]. We tested the effect of anti-ζ-COP Fab fragments on β-COP binding to Golgi-enriched membranes. In the absence of the anti-ζ-COP Fab fragments, addition of GTP[γS] caused a marked increase in β-COP binding (Fig. 7), in agreement with previous data. This GTP[γS]-dependent β-COP binding was completely inhibited by addition of 8 μg of the anti-ζ-COP Fab fragments, whereas the same amount of control Fab fragments prepared from normal rabbit IgG had no effect (Fig. 7).

The blockade of specific β-COP binding by the anti-ζ-

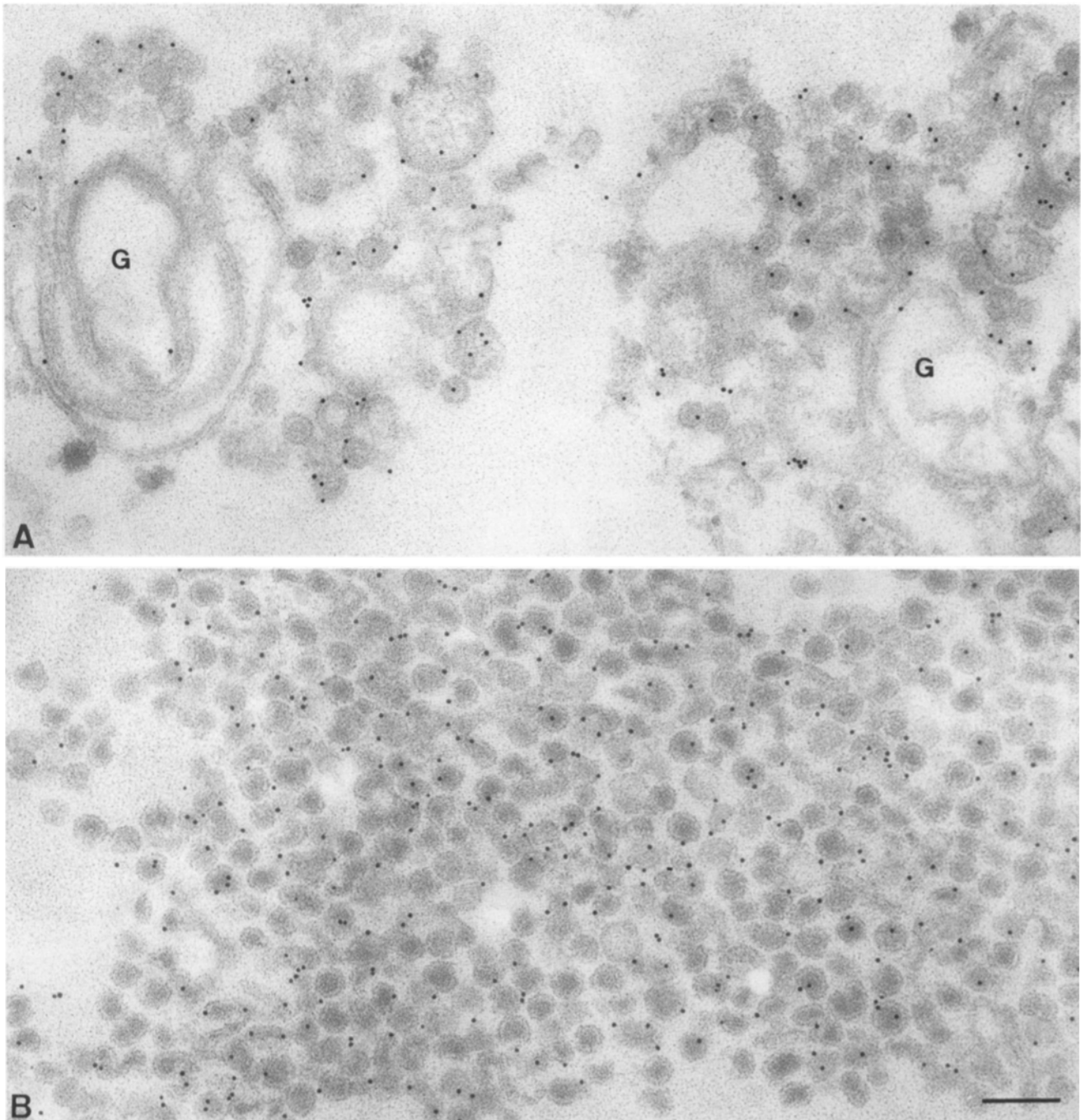


Figure 5. Immunoelectron microscopic localization of ζ -COP in Golgi vesicles. (A) Thin section of a CHO-cell Golgi fraction incubated in the presence of GTP γ S as described (Orci et al., 1989). (B) Thin section of a pellet of Golgi-derived coated vesicles purified from rabbit liver as described (Malhotra et al., 1989). Bar, 200 nm.

COP antibody predicted that the antibody should also prevent the assembly of coated vesicles on the Golgi membranes. Indeed, electron microscopy revealed that when the anti- ζ -COP antibody was added to a reaction containing GTP[γ S], the density of coated vesicles and coated buds were reduced about 25- and 4-fold, respectively (Table I). These results imply that ζ -COP is required for COP-coated vesicle assembly.

Dual Localization of ζ -COP to Coatamer and a Free Cytosolic Pool

Gel filtration analysis of ζ -COP in crude cytosol showed that ζ -COP eluted in two peaks: one (fraction 19) corresponded to the peak of β -COP in coatamer complex and the other (fraction 35) was at a position for much lower molecular size proteins (Fig. 8). This result indicates that ζ -COP exists in

Table I. Effect of the Anti- ζ -COP Antibody on Formation of Golgi-coated Vesicles and Buds

Condition	Density of coated vesicles (number per μm^2)	Density of coated buds (number per μm^2)
Control	5.70 \pm 1.48	8.73 \pm 1.68
+ Anti- ζ -COP Ab	0.21 \pm 0.21	2.39 \pm 0.73

CHO Golgi fractions were incubated in a volume of 0.5 ml in the absence (*control*) or in the presence of 150 μg of affinity-purified anti- ζ -COP antibody (+ *anti- ζ -COP Ab*), as described in Materials and Methods. For electron microscopy, membranes were collected by centrifugation, fixed, and processed as described (Orci et al., 1991). The density of coated vesicles and buds in 10 separate Golgi areas was determined. Numbers are mean values \pm SEM.

cytosol both free from and bound to the coatomer, although it remains to be elucidated whether the free pool of ζ -COP is physiologically significant, or simply reflects dissociation of ζ -COP from coatomer during analysis.

Discussion

In this study, we isolated cDNA of ζ -COP, the coatomer 20-kD subunit. The sequence of the cDNA predicted a novel protein that is similar to AP17 and AP19, the coat proteins of clathrin-coated vesicles (Kirchhausen et al., 1991). The coat of clathrin-coated vesicles consists of clathrin and its adaptor complexes (HAs/APs) (for a review see Keen, 1990). Two main types of HAs/APs, HA-1/AP-1 and HA-2/AP-2, are found in clathrin-coated structures. AP17 and AP19 are the smallest polypeptide components of HA-2/AP-2 and HA-1/AP-1, respectively. The similarity of ζ -COP to AP19 and AP17 are consistent with the idea that a common budding mechanism may underlie the formation of clathrin and COP-coated vesicles. This is also supported by the following: (a) The coat of Golgi-derived COP-coated vesicles is composed of a set of proteins similar in molecular masses to those of clathrin-coated vesicles (Serafini et al., 1991). (b) β -COP is related to β -adaptin, the 115-kD subunit of HA-2/

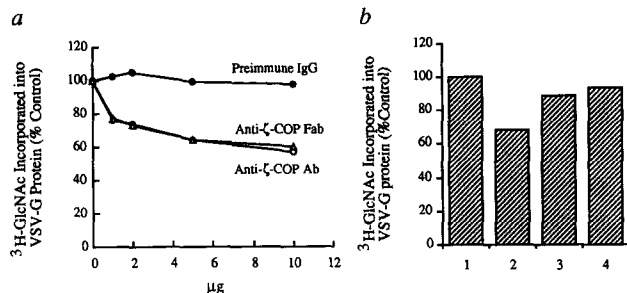


Figure 6. The anti- ζ -COP antibody partially inhibits the assay signal of Golgi cell-free incubation. (a) Enriched Golgi fractions were assayed for transport of VSV-G protein between the *cis*- and *medial*-Golgi compartments as described (Balch et al., 1984). The indicated amounts of preimmune IgG (\bullet), affinity-purified anti- ζ -COP antibody (\circ), or Fab fragments prepared from anti- ζ -COP antibody (Δ) were added to a 50 μl reaction. After incubation on ice for 10 min to promote antibody-antigen binding, the reaction mix was incubated at 37°C for assay. (b) As indicated in a, except these assays contained 10 μg of the affinity-purified anti- ζ -COP antibody (lane 2), 4 μg purified His₆- ζ -COP (lane 3) or 10 μg of the affinity-purified anti- ζ -COP antibody plus 4 μg His₆- ζ -COP (lane 4).

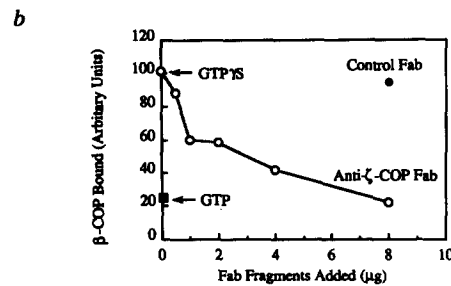
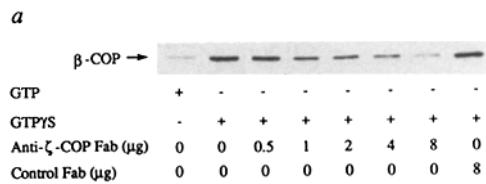


Figure 7. The anti- ζ -COP antibody inhibits coatomer binding to Golgi membranes. (a) CHO Golgi membranes were incubated for 20 min at 37°C with bovine brain cytosol in a reaction mixture containing the indicated Fab fragments in the presence of GTP (10 μM) or GTP γ S (10 μM), as described in Materials and Methods. After incubation, the amount of β -COP bound to the membranes was assayed by SDS-PAGE and immunoblotting with a monoclonal antibody to β -COP, M3A5, as described in Materials and Methods. (b) Shown is a quantitation of the immunoblot in a by densitometry.

AP-2 (Duden et al., 1991; Serafini et al., 1991). (c) The binding of both coatomer and HA-1/AP-1 to Golgi membranes requires ADP-ribosylation factor (ARF), a small GTP-binding protein (Stamnes and Rothman, 1993).

β -, β' -, γ -, and ϵ -COP have previously been shown to be components of both COP-coated vesicles and coatomer (Duden et al., 1991; Serafini et al., 1991; Waters et al., 1991; Stenbeck et al., 1992, 1993; Hara-Kuge, S., O. Kuge, L. Orci, M. Amherdt, F. T. Wieland, and J. E. Rothman, manuscript submitted for publication). ζ -COP is now shown to be present in Golgi-derived coated vesicles as well as in coatomer. This confirms the idea that the entire coatomer assembles as an intact unit into coats (Waters et al., 1991). This idea has been recently established directly (Hara-Kuge, S.,

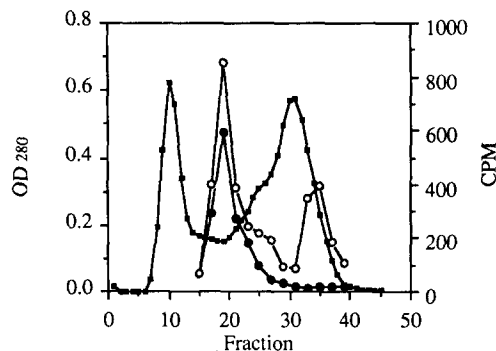


Figure 8. Localization of ζ -COP in cytosol. Bovine brain cytosol was fractionated by gel-filtration column and samples were analyzed by immunoblotting with a monoclonal antibody to β -COP (\bullet) and the anti- ζ -COP antibody (\circ), using [¹²⁵I] protein A. (\blacksquare), optical density at 280 nm.

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Coatmer binding (detected with a monoclonal antibody to β -COP) to Golgi membranes requires myristoylated ARF, and membrane-bound ARF confers to Golgi membranes all of the requirements for specific and saturable coatmer binding (Donaldson et al., 1992; Palmer et al., 1993). Because the anti- ζ -COP antibody inhibits coatmer binding to Golgi membranes, it is possible that ζ -COP is the part of coatmer recognized by ARF and/or the other membrane-bound proteins involved in coatmer binding. To test this, we added purified His₆- ζ -COP to a coatmer binding reaction as a potential competitor but failed to observe competition (data not shown). Thus, ζ -COP appears not to interact by itself with the coatmer binding site of Golgi membranes.

The anti- ζ -COP antibody inhibits partially (40%) the assay signal of cell-free intra-Golgi transport yet completely blocks the formation of Golgi-coated vesicles in a cell-free system. How can this be? Elsewhere, we report (Elazar, Z., L. Orci, J. Ostermann, M. Amherdt, G. Tanigawa, and J. E. Rothman, manuscript submitted for publication) that when coatmer or ARF are left out of transport reactions, vesicular transport stops but an "uncoupled" fusion reaction similar to that which occurs when brefeldin A is added (Orci et al., 1991) is triggered. This involves the direct fusion of donor with acceptor Golgi, without an intervening COP-coated transport vesicle. It is our hypothesis that the coat normally sequesters fusogenic membrane proteins needed in transport vesicles after uncoating (following budding). Thus, these fusion proteins would only be revealed when budding is completed, thereby coupling fusion to the successful completion of budding. When coats cannot assemble, fusion would be immediate and direct. With the glycosylation of VSV-G protein with [³H]GlcNAc as the only measure of transport, this signal will largely persist whether due to vesicular transport (in the presence of coat proteins) or uncoupled fusion (in the absence of coat proteins, or in the presence of brefeldin A). Since anti- ζ -COP antibody blocks coatmer binding, this treatment would be predicted to lead to uncoupled fusion, the hallmark of which is partial inhibition of the assay signal with complete inhibition of coat assembly.

It is striking that ζ -COP exists in cytosol both free and bound to the coatmer, unlike other defined coatmer subunits (β -, β' -, γ -, and ϵ -COP). This explains why p20 (ζ -COP) is present in less than one copy per coatmer (Waters et al., 1991). Together with the fact ζ -COP is required for coatmer function (shown here), this raises the possibility that association-dissociation of ζ -COP with coatmer may represent a means to regulate coat assembly and thus the rate of biosynthetic protein transport. When ζ -COP is bound, coatmer would be switched on, when dissociated, coatmer function would be turned off.

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