En Bloc Incorporation of Coatomer Subunits during the Assembly of COP-coated Vesicles

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Abstract. The cDNA encoding ε-COP, the 36-kD subunit of coatomer, was cloned from a bovine liver cDNA library and sequenced. Immunoblotting with an anti-ε-COP antibody showed that ε-COP exists in COP-coated vesicles as well as in the cytosolic coatomer. Using the cloned cDNA, recombinant His_6-tagged ε-COP was overexpressed in cultured Chinese hamster ovary (CHO) cells, from which metabolically radiolabeled coatomer was purified by taking advantage of the His_6 tag. Radiolabeled coatomer was employed to establish that all the subunits of the coatomer enter coated vesicles as an intact unit.

INTRACELLULAR traffic between the membrane compartments of eukaryotic cells relies on the movement of vesicular carriers (Jamieson and Palade, 1967; Palade, 1975; Rothman et al., 1984*a,b*). Golgi-derived (non-clathrin or COP) coated vesicles can be produced in a cell-free system that reconstitutes intercisternal protein transport (Balch et al., 1983, 1984; Balch and Rothman, 1985; Orci et al., 1986, 1989) and purified (Malhotra et al., 1989; Serafini et al., 1991). This led to the identification of four of the subunits of the coat, or COPs, termed α-COP (160 kD), β-COP (110 kD), γ-COP (98 kD), and δ-COP (61 kD).

Independently, a cytosolic complex that acts as the coat protomer (termed 'coatomer') containing the same four coat proteins was purified (Waters et al., 1991). The coatomer also contains polypeptides of M_r 35–36 kD (a doublet) and 20 kD (ζ-COP: Kuge et al., 1993). The coatomer is required to form Golgi-derived coated vesicles (Orci et al., 1993) and these contain at least the β-COP subunit. But there has not been direct proof that the entire coatomer is incorporated *en bloc* into the coat.

We cloned the cDNA encoding the 36-kD subunit of coatomer (which we now term ε-COP) and used it to express 36-kD protein containing six histidine residues in mammalian cells. These transfectants enabled us to develop a simple method to purify radiolabeled coatomer using the Ni-NTA affinity resin. Using this radiolabeled coatomer, we could follow coat assembly on Golgi membranes, and also determine the stoichiometry of each subunit at every stage.

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Materials and Methods

Materials

EXPRE^{35}S^{35}S protein labeling mix, [α-^{32}P]dCTP and [α-^{35}S]thio-dATP were from DuPont New England Nuclear (Boston, MA). [γ-^{32}P]ATP and [^{125}I]protein A were purchased from ICN Biomedical Inc. (Costa Mesa, CA). FBS was from GIBCO BRL (Gaithersburg, MD). Peroxidase-conjugated secondary antibodies and affinity purified rabbit anti-mouse IgG were purchased from Bio-Rad Labs. (Hercules, CA). A monoclonal antibody to β-COP (M3A5) was kindly donated by Dr. T. E. Kreis. A monoclonal antibody CM1A10 (Palmer et al., 1993) was provided by Dr. D. J. Palmer. A polyclonal anti-ζ-COP antibody was prepared as described (Kuge et al., 1993). Recombinant myristoylated ADP ribosylation factor (ARF1)[1] was provided by Drs. J. B. Helms and G. Tanigawa (Helms et al., 1993).

DNA Manipulations

DNA manipulations, including restriction enzyme digestion, ligation, plasmid isolation, subcloning, *E. 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 sequences were determined by the dideoxy chain termination methods with Sequenase, using walking primers.

Purification of ε-COP

Coatomer was prepared from bovine liver as described (Waters et al., 1991, 1992) and the subunits of coatomer, COPs, were separated by preparative 15% polyacrylamide SDS-PAGE. Gels were stained with Coomassie blue

1. *Abbreviations used in this paper*: ARF, ADP ribosylation factor; GTPγS, guanosine 5;-*O*-(3-thiotriphosphate); PVDF, polyvinylidene difluoride.

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and the doublet band of p35 and p36 was excised and divided into three parts. Then, the upper and the lower parts as p36 and p35 samples, respectively, were electroeluted with a BioRad Model 422 electroeluter, according to the manufacturer's protocol.

cDNA Cloning of ε-COP

Approximately 100 pmols of p35 and p36 were run on a 15% polyacrylamide SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) or nitrocellulose filter and stained with Ponceau S. The proteins on PVDF membrane were subjected to NH_2-terminal amino acid sequence analysis. The proteins on nitrocellulose membrane were digested in situ by trypsin and the resulting peptides were fractionated by reverse phase HPLC (Tempst et al., 1990) and subjected to amino acid sequence analysis (Tempst and Riviere, 1989). The analyzed sequences were used to design primers for a two stage polymerase chain reaction (PCR). The primers used for amplification were TT(TC)GA(TC)GT(AGTC)AA(AG)AA(TC)GC (codons Phe^{20}-Ala^{25}, sense) and GG(AG)TT(TC)TG(AG)TC(AG)TA(AG)AA (codons Phe^{128}-Pro^{133}, antisense). The bovine liver λZapII cDNA library (Stratagene, LaJolla, CA) was used as the template for the first round of PCR. 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 amplification were analyzed by Southern blot with an internal probe, AA(AG)AA(TC)GC-(AGTC)AA(AG)AA (codons Lys^{23}-Ile^{28}, sense) and 0.3-kb product was found to hybridize with the probe. For Southern blot analysis, DNA fragments were electrophoresed through a 2% agarose gel, transferred to nylon filters, and hybridized with the ^{32}P-labeled probe for 48 h at 48°C in 3 M tetramethylammonium chloride (Wood et al., 1985), 1 mM EDTA, 5 × Denhardt's solution, 0.6% SDS, 100 μg/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, 50 mM Tris, pH 8.0 at 42°C for 30 min. The first round reaction was diluted 1:1,000 and used as a template for the second round amplification under the same reaction condition as the first round. The 0.3-kb product on second round amplification was subcloned into a plasmid, pBluescript II (Stratagene), sequenced, and used as a probe for screening a bovine liver λZapII cDNA library (Stratagene). Hybridizations were done for 24 h at 65°C in 5 × SSPE (1 × SSPE = 0.15 M NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.4), 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml herring sperm DNA. A final wash was performed in 1 × SSPE, 0.1% SDS at 50°C for 1 h.

Preparation of Cytosol and Golgi Membranes

Bovine brain cytosol and Golgi-enriched membranes of rabbit livers (Pel-Freez Biologicals) were prepared as described by Malhotra et al. (1989). To obtain the coatomer-depleted cytosol, 1.8 mg each of monoclonal antibody CM1A10, polyclonal anti-ε-COP and anti-ζ-COP antibodies were coupled to 1 ml of protein-G agarose (Boehringer Mannheim Corp., Indianapolis, IN) and packed as described (Harlow and Lane, 1988). The cytosol was passed three times over the coupled-agarose column and eluted cytosol was checked for coatomer depletion by immunoblot. Protein concentration was determined using the Bio-Rad protein assay using BSA as a standard. About 90% of β-COP was recovered by this procedure.

Preparation of Recombinant His_6-ε-COP

The coding region of ε-COP cDNA was engineered by PCR to add a BamHI site upstream of the first ATG and a HindIII site downstream of the termination codon. The PCR product was digested with BamHI and HindIII and cloned into a pQE10 vector (Qiagen), which allows the production of recombinant protein containing an NH_2-terminal affinity tag consisting of six adjacent histidine residues. The resulting plasmid was introduced into *E. coli* (M15 harboring plasmid pREP4, Qiagen). The transformant was grown to a density of A_{600} = 0.9 in Super medium (25 g Bacto-tryptone, 15 g yeast extract and 5 g NaCl per liter) with 100 μg/ml ampicillin, 50 μg/ml kanamycin at 37°C, and further cultivated for 2 h in the presence of 2 mM isopropyl-thio-β-*D*-galactoside. The ε-COP expressing cells were collected, suspended in Sonication buffer (50 mM Na-phosphate pH 8.0, 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 column (8 ml bed volume) previously equilibrated with Sonication buffer. The resin was washed with Sonication buffer, and then His_6-ε-COP was eluted with a 100-ml imidazole gradient (20–500 mM) in Sonication buffer.

Preparation of Affinity Purified Antibodies against ε-COP

A rabbit was injected with 100 μg purified His_6-ε-COP emulsified in Freund's complete adjuvant. Three weeks later the rabbit was injected with 100 μg purified His_6-ε-COP emulsified in Freund's incomplete adjuvant and this booster injection was repeated every 3 wk. After the antiserum was precipitated with 60% saturated ammonium sulfate and dialyzed against PBS, the anti-ε-COP antibody was purified by adsorption to His_6-ε-COP conjugated to AminoLink® coupling gel (Pierce, Rockford, IL) as described (Harlow and Lane, 1988) and dialyzed against 10 mM MOPS/KOH, pH 7.5 and 150 mM KCl.

Western Blot Analysis

Proteins were fractionated by SDS-PAGE under reducing conditions (Laemmli, 1970) and electroblotted on to nitrocellulose in 25 mM Tris, 192 mM glycine and 20% methanol and 0.05% SDS at 22 V/cm for 1 h. β-COP was detected using the mouse monoclonal M3A5 (0.4 mg/ml) and peroxidase-conjugated anti-mouse IgG (diluted 1:1,000). ε-COP and ζ-COP were detected using the affinity purified antibodies against ε-COP (50 ng/ml) and ζ-COP (20 ng/ml), respectively, and peroxidase-conjugated anti-rabbit IgG (diluted 1:1,000). Peroxidase labeling was detected by chemiluminescence using ECL reagent (Amersham Corp., Arlington Heights, IL). Analysis of samples for β-COP, ε-COP, and ζ-COP was achieved by fractionation using 12.5% polyacrylamide gels and immunodetection of β-COP, ε-COP, and ζ-COP on the top, middle, and bottom portions of nitrocellulose blots, respectively. For quantitation, the top portion (β-COP) of nitrocellulose blots was incubated in affinity purified rabbit anti-mouse IgG (1:3,000), and then the top and the middle (ε-COP) portions were incubated in 0.2 μCi/ml [^{125}I]protein A, dried, and exposed to Kodak X-AR 5 film at −80°C for 15 h. The blot was quantitated by excision of the 110-kD and 35/36-kD regions from the nitrocellulose and counting in a γ counter.

Gel Filtration of 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 LKB Biotechnology, Piscataway, 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).

Immunoelectron Microscopy

Rabbit liver Golgi membranes were incubated in the presence of coatomer, coatomer-depleted cytosol, guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS), ATP and ATP generation system, according to the cell free transport assay (Balch et al., 1984). Also CHO Golgi membranes were incubated in AlF_4- as described (Orci et al., 1989). The membranes were collected by centrifugation and fixed with glutaraldehyde. The membrane pellet was cryosectioned as described by Tokuyasu (1980). The protein A-gold method (Roth et al., 1978) was used to visualize antibodies. The affinity purified anti-ε-COP antibody (4.11 mg/ml) was diluted 1:100.

Construction of Plasmids Encoding ε-COP Containing Six Histidine Residues to be Expressed in Mammalian Cells

The coding region of ε-COP cDNA was engineered by PCR to add the region encoding six histidine residues and a KpnI site immediately upstream of the first ATG and a XbaI site immediately downstream of the termination codon. The PCR product was digested with KpnI and XbaI, and cloned into the pSVSport·1 vector (GIBCO BRL).

Introduction of Plasmid into CHO Cells

Transfection of DNA into CHO cells was performed by the calcium phosphate coprecipitation method as described (Lewis et al., 1980). One ml of calcium phosphate/DNA precipitate containing 22 μg of pSVSport·1 carrying His_6-tagged ε-COP cDNA and 2.2 μg of pSV2neo was added to a 100-mm diam dish containing 2 × 10^5 cells. After exposure to the DNA for

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                                                         ***
 -27                                     GAA TTC CGC GGG TGA GAG GGC AGC GAT -1

   1 M   A   P   P   A   P   G   P   A   S   G   G   S   G   E   V   D   E   L   F   20
   1 ATG GCT CCT CCG GCT CCT GGT CCG GCT TCT GGC GGC TCC GGG GAG GTG GAC GAG CTG TTC 60

  21 D   V   K   N   A   F   Y   I   G   S   Y   Q   Q   C   I   N   E   A   Q   R   40
  61 GAC GTG AAG AAC GCC TTC TAC ATT GGC AGC TAC CAG CAG TGC ATC AAC GAG GCG CAG CGG 120

  41 V   K   P   S   S   P   E   R   D   V   E   R   D   V   F   L   Y   R   A   Y   60
 121 GTG AAG CCA TCC AGC CCG GAG AGA GAT GTG GAG CGG GAT GTC TTC CTG TAC AGA GCA TAC 180

  61 L   A   Q   R   K   Y   G   V   V   L   D   E   I   K   P   S   S   A   P   E   80
 181 CTG GCC CAG AGG AAG TAC GGC GTG GTG CTG GAC GAG ATC AAG CCC TCC TCC GCC CCG GAG 240

  81 L   Q   A   V   R   M   F   A   E   Y   L   A   S   H   S   R   R   D   A   I   100
 241 CTG CAG GCC GTG CGC ATG TTT GCT GAG TAC CTG GCC AGC CAC AGC CGG CGG GAT GCG ATC 300

 101 V   A   E   L   D   R   E   M   S   R   S   V   D   V   T   N   T   T   F   L   120
 301 GTG GCC GAG CTG GAC CGG GAG ATG AGC CGG AGC GTG GAT GTG ACC AAC ACC ACC TTC CTG 360

 121 L   M   A   A   S   I   Y   F   Y   D   Q   N   P   D   A   A   L   R   T   L   140
 361 CTC ATG GCT GCC TCC ATC TAT TTC TAC GAC CAG AAC CCA GAT GCA GCC CTG CGC ACC CTT 420

 141 H   Q   G   D   S   L   E   C   M   A   M   T   V   Q   I   L   L   K   L   D   160
 421 CAC CAG GGG GAC AGC CTG GAG TGC ATG GCC ATG ACA GTG CAG ATC CTG CTG AAG CTT GAC 480

 161 R   L   D   L   A   R   K   E   L   K   K   M   Q   D   Q   D   E   D   A   T   180
 481 CGC CTG GAC CTT GCC CGG AAG GAG CTG AAG AAG ATG CAG GAC CAG GAC GAG GAC GCT ACG 540

 181 L   T   Q   L   A   T   A   W   V   S   L   A   A   G   G   E   K   L   Q   D   200
 541 CTC ACC CAG CTG GCC ACC GCC TGG GTC AGC CTG GCT GCG GGA GGT GAG AAG CTC CAG GAC 600

 201 A   Y   Y   I   F   Q   E   M   A   D   K   C   S   P   T   L   L   L   L   N   220
 601 GCC TAC TAC ATC TTC CAG GAG ATG GCC GAC AAG TGT TCG CCG ACC CTG CTG CTG CTC AAT 660

 221 G   Q   A   A   C   H   M   A   Q   G   R   W   E   A   A   E   G   V   L   Q   240
 661 GGG CAG GCA GCC TGC CAC ATG GCG CAG GGC CGC TGG GAG GCT GCT GAG GGC GTA CTG CAG 720

 241 E   A   L   D   K   D   S   G   H   P   E   T   L   I   N   L   V   V   L   S   260
 721 GAG GCA CTG GAC AAG GAC AGC GGC CAC CCT GAG ACG CTG ATA AAC CTC GTT GTC CTG TCC 780

 261 Q   H   L   G   K   P   P   E   V   T   N   R   Y   L   S   Q   L   K   D   A   280
 781 CAA CAC CTG GGC AAG CCT CCT GAG GTG ACA AAC CGC TAC CTG TCC CAG CTG AAG GAC GCC 840

 281 H   R   S   H   P   F   I   K   E   Y   R   A   K   E   N   D   F   D   R   L   300
 841 CAC AGG TCG CAC CCC TTC ATC AAG GAG TAC CGG GCC AAG GAG AAC GAC TTT GAC CGG CTG 900

 301 V   L   Q   Y   A   P   S   A   ***                                             308
 901 GTG CTG CAG TAT GCC CCC AGC GCC TGA GGC CTG CCC CAG ATG CAC TTC CGG AGG AGC CGT 960

 961 GGG AAT GCG AGG CCA GGG CTC TGC TGC CCT CTC TGC TGG GCA CTT GCC CTT TGG CTA GAA 1020

1021 GTA CAG GCT GGA AGC CCA TCC CAG CCT GCC GTT TTT CAA TAA ATG TCT CCA TTC CAG GAA 1080

1081 TTC CAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA 1140
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Figure 1. The sequence of the ε-COP gene. The predicted amino acid sequence is shown in single-letter code. All protein sequences determined by Edman degradation are indicated with an underline. Double-underline shows a polyadenylation signal and (***) shows a termination codon. These sequence data are available from EMBL under accession number X76980.

7 h, the cells were treated with dimethyl sulfoxide (10% for 20 min), grown for 24 h on a nonselecting culture medium, trypsinized, replated onto four plates, and then cultured in the medium containing 500 μg/ml of Geneticin (G-418 Sulfate). Five days after, all resistant cells were collected and cloned at 0.3 cell/well in 2.5 ml medium containing 100 μg G-418, using 24-well plates. After one week, 15 independent colonies were purified and further cultured. The confluent cells were harvested, washed with PBS, suspended in 100 μl of 25 mM Tris/HCl, pH 7.4, 250 mM sucrose and 1 mM PMSF, and disrupted by sonication at 4°C. 40 μg lysates of 15 independent G-418 resistant colonies were checked for expression of ε-COP by immunoblotting. The transformant with the highest level of ε-COP expression was selected and used for purification of coatomer.

Purification of Radiolabeled Coatomer from CHO Transformants

The CHO transformants were cultured in 100-mm plates containing MEMα/10% FBS/500 μg/ml G-418 and passed routinely. For purification of labeled coatomer, the CHO transformants from four confluent culture plates were harvested, suspended in 2 l MEMα/10% FBS and cultured at 37°C for 3 d. The 2 l suspension culture (ca. 2 × 10^9 cells) was harvested and washed with 200 ml of complete DME (Sigma Chem. Co.) without methionine, followed by incubation in 100 ml of the same medium containing 10% dialyzed (against PBS) FBS and 10 mCi [^{35}S]methionine (EXPRE^{35}S^{35}S) at 37°C for 9 h. The metabolic radiolabeled cells were harvested, washed with PBS three times, and suspended in 10 ml 25 mM Tris/HCl, pH 8.0, 500 mM KCl, 250 mM sucrose, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5 mM 1,10-phenanthroline, 2 μM pepstatin A, 2 μg/ml aprotinin and 0.5 μg/ml leupeptin. The cells were disrupted in a French press at 500 psi, and the homogenate was centrifuged at 55,000 rpm in a Beckman SW55 rotor (368,000 g) for 1 h at 4°C. The supernatant was diluted with the same volume of 25 mM Tris/HCl, pH 7.4, 1 mM DTT, 10% glycerol, which should approximate the conductivity of 25 mM Tris/HCl, pH 7.4, 150 mM KCl, 1 mM DTT and 10% glycerol. The supernatant was loaded at 2 ml/min onto an 8-ml Mono Q (Pharmacia LKB Biotechnology) column equilibrated in 25 mM Tris/HCl, pH 7.4, 150 mM KCl, 1 mM DTT and 10% glycerol. The protein was eluted with a 2.2 mM/ml KCl gradient in 25 mM Tris/HCl, pH 7.4, 1 mM DTT and 10% glycerol (5 ml/fraction). An aliquot of each fraction was subjected to immunoblot analysis to detect coatomer. The fractions containing coatomer were pooled (15 ml) and loaded onto an ~2.2 ml Ni-NTA column equilibrated in 25 mM Tris/HCl, pH 7.4, 200 mM KCl, 1 mM DTT and 10% glycerol. The column was washed with 100 ml of equilibration buffer, and then 50 ml of equilibration buffer containing 5 mM imidazole. Labeled coatomer was eluted with equilibration buffer containing 50 mM imidazole (1 ml/fraction). After immunoblot analysis of fractions, the fractions containing purified radiolabeled coatomer (3 ml) were collected and diluted with equilibration buffer, and imidazole was removed by spin dialysis using YM100 membrane (Amicon, Beverly, MA).

β-COP Binding Assay

β-COP binding assay was carried out according to Palmer et al. (1993), using 9.1 μg of CHO Golgi membranes, 2 μg of myristoylated ARF and

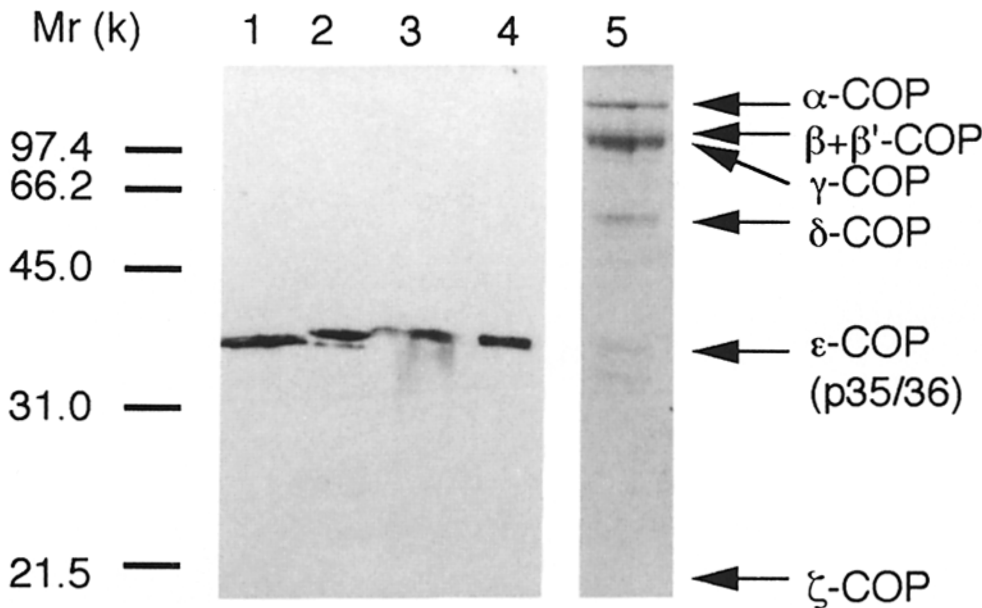


Figure 2. The immunoblot of cytosol and coatomer probed with anti-ε-COP polyclonal antibody. CHO cytosol (60 μg, lane *1*), bovine brain cytosol (60 μg, lane *2*), bovine liver cytosol (60 μg, lane *3*), and purified coatomer from bovine liver cytosol (0.1 μg, lane *4*) were fractionated by 15% polyacrylamide SDS-PAGE and immunoblotted using anti-ε-COP polyclonal antibody as described in Materials and Methods. The Coomassie-staining SDS-PAGE gel (15%) of purified coatomer (5 μg) is shown in lane *5* as a marker.

0.4 μg of labeled coatomer in the presence of 20 μM GTP or GTPγS per 50 μl reaction mixture.

Isolation of Radiolabeled Coated Vesicles

A 2-ml reaction mixture to isolate non-clathrin coated vesicles derived from rabbit liver Golgi membranes was prepared by the same methods as described (Serafini et al., 1991), using 17 μg of labeled coatomer and 10 mg of coatomer-depleted cytosol instead of cytosol in the presence of 20 μM GTP or GTPγS. Also the same 2-ml reaction mixture with 0.5 mg soybean trypsin inhibitor was prepared with or without 20 μg myristoylated ARF in the absence of coatomer-depleted cytosol. The complete reaction mixture was immediately pipetted into two 1.5-ml Eppendorf tubes and incubated for 15 min at 37°C. After this incubation the tubes were chilled for 10 min in an ice/water bath (all subsequent steps were carried out on ice), and the membranes were collected by microcentrifugation for 5 min. The reaction mixture was removed by aspiration, and the membrane pellet was resuspended in 0.6 ml of 50 mM KCl, 25 mM Hepes/KOH, pH 7.2, 2.5 mM magnesium acetate, 0.2 M sucrose and 0.1 mg/ml BSA using a Pipetman P-1000. Resuspended membranes were incubated on ice for 15 min and then collected by microcentrifugation for 5 min. Supernatant was removed by aspiration and discarded, and the membrane pellet was resuspended as before into 0.6 ml of 250 mM KCl, 25 mM Hepes/KOH, pH 7.2, 2.5 mM magnesium acetate, 0.2 M sucrose and 0.1 mg/ml BSA. After incubation for 15 min on ice, the larger contaminating membranes were removed by microcentrifugation as before. The supernatant was recovered, transferred to a new tube, and microcentrifuged for 10 min. 545 μl of recovered supernatant was placed into a new tube, and adjusted to 20% (wt/wt) sucrose by addition of 55% stripping buffer$^+$ (SB$^+$; 250 mM KCl, 25 mM Hepes/KOH, pH 7.2, 2.5 mM magnesium acetate and 0.1 mg/ml BSA, and at the indicated sucrose concentration [wt/wt]. The crude coated vesicles were over layered with 714 μl each of the following in an SW55 tube: 50% SB$^+$, 45% SB$^+$, 40% SB$^+$, 35% SB$^+$, 30% SB$^+$, and 25% SB$^+$. The gra-

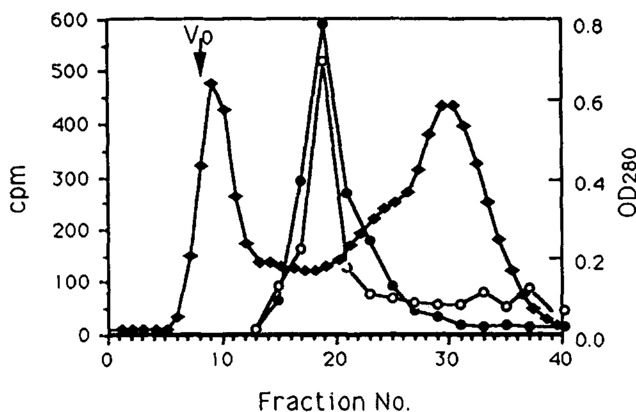


Figure 3. Localization of ε-COP in cytosol. Bovine brain cytosol (2 mg) was fractionated by gel filtration and 10 μl of each fraction (0.4 ml/fraction) was analyzed by immunoblotting with monoclonal antibody M3A5 against β-COP (●) and anti-ε-COP polyclonal antibody (○). OD_{280} (◆) is optical density at 280 nm and V_0 is void volume.

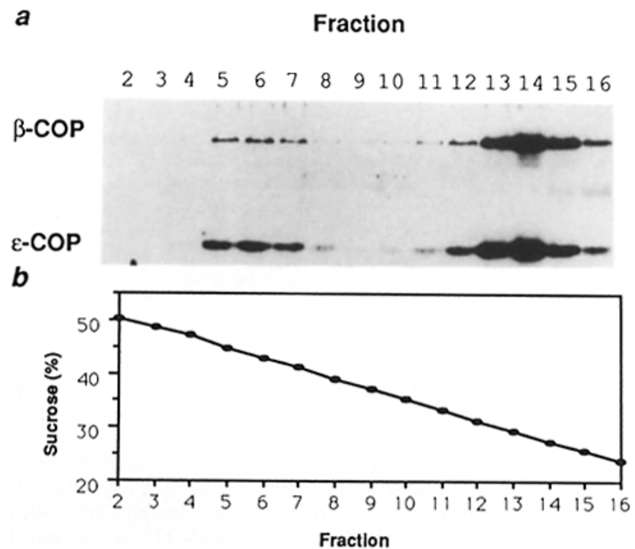
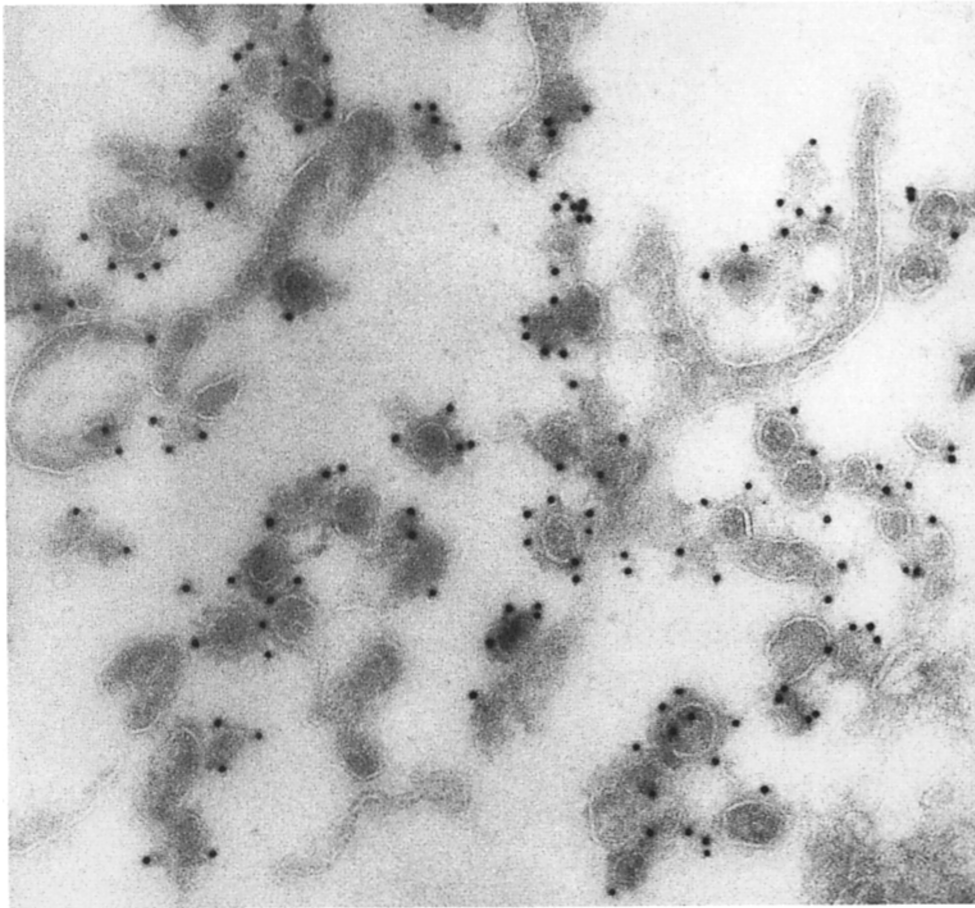


Figure 4. Localization of β-COP and ε-COP in rabbit liver Golgi-derived coated vesicles. (*a*) The sucrose gradient fractions from a coated vesicle preparation using rabbit liver Golgi were immunoblotted using monoclonal antibody M3A5 and anti-ε-COP polyclonal antibody. (*b*) Per cent sucrose (wt/wt) composition of the fractions in *a* as determined by refractometry.

a



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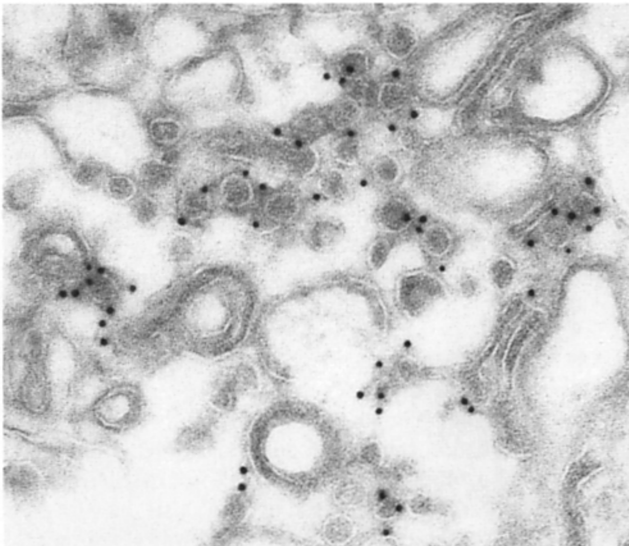

Figure 5. Immunoelectron microscopic localization of ϵ-COP to the coat of non-clathrin-coated vesicles. (*a*) Rabbit liver Golgi membranes were incubated in the presence of coatomer, cytosol depleted of coatomer, GTPγS, ATP and ATP-regenerating system. (*b*) CHO Golgi membranes were incubated in AlF_4^- to accumulate coated vesicles. Immunolabeling was performed on ultrathin cryosections using affinity purified anti-ϵ-COP antibody (dilution 1:100). Protein A-gold was used to localize bound antibodies. Magnifications: (*a*) 102,700×; (*b*) 118,500×.

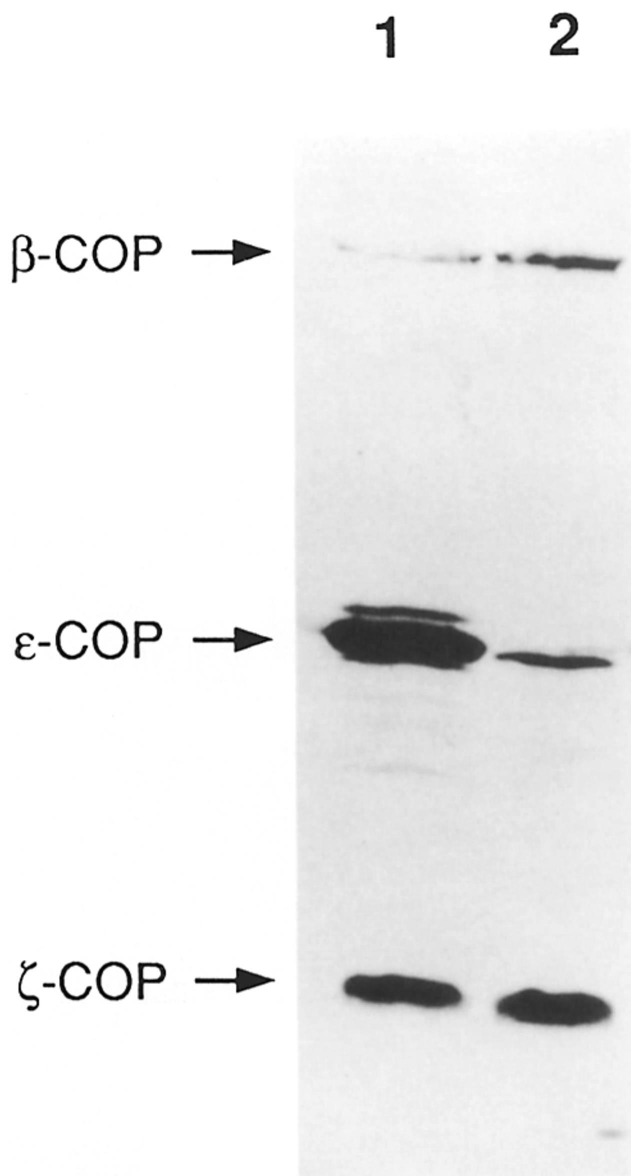

Figure 6. Overexpression of ϵ-COP in CHO cells. (Lane *1*) CHO transfectant (ϵ-COP containing His_6 tag at NH_2-terminus) cytosol (40 μg); and (lane *2*) CHO cytosol (40 μg) were analyzed by immunoblot using M3A5, anti-ϵ-COP polyclonal antibody, and anti-ζ-COP polyclonal antibody.

dient was centrifuged for 16 h at 32,500 rpm in a Beckman SW55 rotor (100,000 *g*) and fractionated from the bottom using a peristaltic pump at the rate of 260 μl/min. Approximately seventeen 260 μl fractions were collected. 200 μl of each sample was mixed with 200 μl of H_2O, transferred into a polycarbonate tube (Beckman Instrs., Fullerton, CA) and centrifuged for 30 min at 100,000 rpm in a Beckman TLA100.2 rotor. Pellets were resuspended in 15 μl of SDS sample buffer and subjected to SDS-PAGE. The gel was dried, immersed in enhancer solution for 20 min (Enlightning, DuPont New England Nuclear), and autoradiographed.

Results

Cloning and Sequencing ϵ-COP

Coatomer was purified from bovine liver (Waters et al., 1991, 1992) and run on an SDS-PAGE gel; Coomassie-staining bands of 35 kD and 36 kD (p35 and p36) were cut out separately. The peptide maps of both proteins after trypsin digestion were similar except for an additional peak in p36 (data not shown). The amino acid sequence of this peak was identical to the NH_2-terminal amino acid sequence of p36 but the NH_2 terminus of p35 lacks the first five amino acids of p36. Therefore the doublet band in coatomer preparations is derived from a single protein: p35 is a proteolytic product of p36, which we call the ϵ-COP subunit of coatomer.

The protein sequences of four additional peptides were determined from the tryptic digest of ϵ-COP (underlined in Fig. 1). Mixed oligonucleotide primers were synthesized according to the first (the NH_2-terminal) and third peptide sequences and used to amplify a 0.3-kb product from a bovine liver cDNA library. The specificity of this band was confirmed by hybridization with another mixed oligonucleotide probe synthesized according to an internal peptide sequence. This PCR product was subcloned, sequenced, and used as a probe against the same cDNA library to select a full-length clone of ϵ-COP. Nineteen positively hybridizing plaques were purified and mapped with restriction endonucleases and eight different cDNAs were identified which share a common restriction map. An open reading frame of 924 bp was identified with a termination codon 12 bp upstream to ATG, a predicted molecular weight of 34.5 kD, a polyadenylation signal (AATAAA; double underlining in Fig. 1), and a poly(A) tail following the 3′ untranslated region (Fig. 1). All protein sequences determined by Edman degradation were contained within this open reading frame (underlined in Fig. 1).

Genomic Southern and Northern blot analyses using the full-length clone of ϵ-COP as a probe also suggested that p35 and p36 are encoded by the same single-copy gene (data not shown).

No protein sequences significantly similar to ϵ-COP were found by the BLASTP program (Altschul et al., 1990).

Localization of ϵ-COP

The full-length cDNA encoding ϵ-COP was cloned into a pQE10 expression vector to generate a plasmid construct which, when transformed into *E. coli*, produced an ϵ-COP recombinant protein containing six histidine residues at its NH_2-terminus. The protein was expressed at a high level and purified by affinity chromatography on Ni-NTA agarose. This protein was injected into rabbits and antiserum was purified by affinity chromatography on AminoLink® Gel coupled to His-ϵ-COP.

Western blot analysis shows that affinity purified anti-ϵ-COP antibody reacts against ϵ-COP specifically in cytosol as well as in purified coatomer preparations (Fig. 2).

In whole cytosol, ϵ-COP is present almost exclusively in a large complex, most likely the coatomer, as is the case for β-COP (Fig. 3). ϵ-COP, as well as β-COP, was also present in COP-coated vesicles (Figs. 4 and 5). Fig. 4 shows a sucrose gradient of crude Golgi-derived COP-coated vesicles formed during an incubation of rabbit liver Golgi membranes, bovine brain cytosol, and GTPγS. Both ϵ-COP and β-COP were found together in fractions 5–7 (Fig. 4 *a*) at ca. 41% (wt/wt) sucrose (1.18 g/ml^{-1}; Fig. 4 *b*), which is the density expected for COP-coated vesicles. Immunoelectron

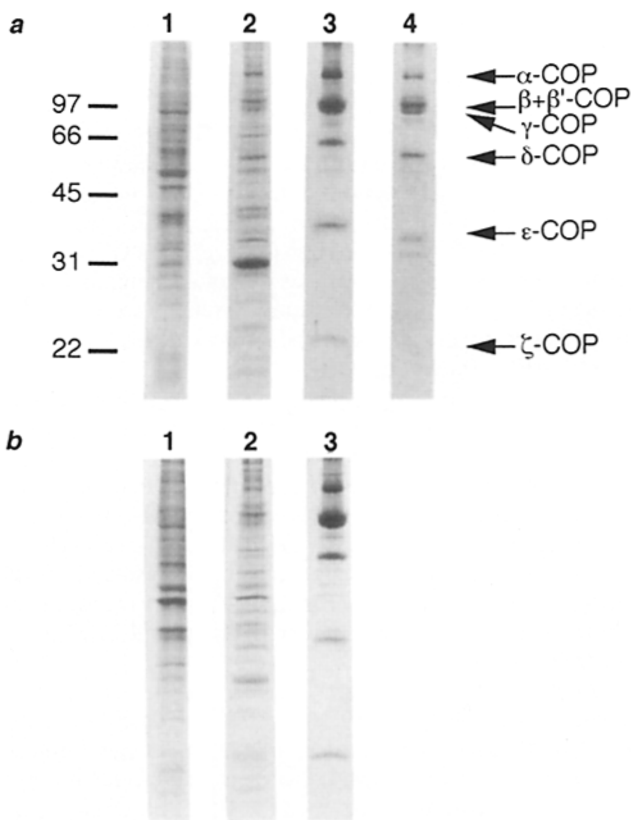

Figure 7. Purification of labeled coatomer. (*a*) Coomassie-stained SDS gel. (12%) (Lane *1*) bovine brain cytosol (18 μg); (lane *2*) mono-Q pool (8 μg) from labeled CHO transfectants; (lane *3*) Ni-NTA pool (6 μg) from same preparation; (lane *4*) coatomer purified from bovine liver (4 μg). (*b*) Autoradiogram of *a*.

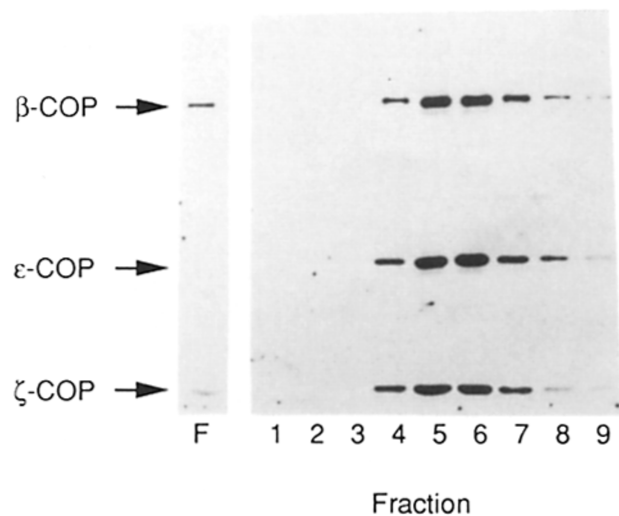

Figure 8. Ni-NTA chromatography of labeled coatomer. (*a*) Fractions of the Ni-NTA column were analyzed by immunoblot using M3A5, anti-ε-COP polyclonal antibody, and anti-ζ-COP polyclonal antibody. (*b*) Radioactivity of 1 μl of each fraction in *a*.

microscopy with anti-ε-COP antibody also shows that ε-COP is associated with the periphery of COP-coated vesicles and buds (Fig. 5). These results show that ε-COP exists in both coatomer and coated vesicles.

Expression of His$_6$-tagged-ε-COP in CHO Cells and Isolation of Radiolabeled Coatomer

cDNAs of ε-COP containing an additional six histidine residues at its amino terminus was cloned into pSVSport·1, a mammalian expression vector. The resulting plasmids were cotransfected with pSV2neo into CHO cells in order to select transfectants in medium containing G418. ε-COP expression in fifteen cloned transfectants was analyzed by Western blot and the cell line with the highest level of expression was used in subsequent studies (Fig. 6).

A suspension culture of CHO transfectants was uniformly labeled with [^{35}S]methionine. Labeled cytosol from the CHO transfectants (Fig. 7, *a* and *b*, lane *1*) was used as starting material and radiolabeled coatomer was purified by the two steps of Mono Q chromatography (Fig. 7, *a* and *b*, lane *2*) and affinity chromatography on Ni-NTA agarose (Fig. 7, *a* and *b*, lane *3*). Unlike *E. coli*, mammalian cells contain many proteins which bind to Ni-NTA agarose. For this reason, one additional step of Mono Q chromatography was needed before Ni-NTA affinity chromatography. The Western blot analysis using a monoclonal antibody (M3A5) to β-COP and the anti-ε-COP antibody showed that the Mono Q flow-through fraction contains small amount of ε-COP but not β-COP (data not shown); the coatomer fraction of course contains both proteins. The ε-COP in the flow-through fraction presumably is due to excess, overexpressed ε-COP not combined into coatomer. Western blot analysis of the Ni-NTA chromatography fractions showed that β-COP and ζ-COP (p20) were co-eluted with ε-COP by 50 mM imidazole (Fig. 8). These data and especially the absence of β-COP and ζ-COP (p20) from the flow-through fraction suggest that the His$_6$-tagged ε-COP is incorporated as a subunit of coatomer in the CHO transfectant, and has replaced virtually all of the native ε-COP in coatomer. Since the CHO transfectant grew as well as wild type, apparently the His$_6$-tagged coatomer is functional.

Analysis of the final material by SDS-PAGE revealed that the major Coomassie-staining bands corresponded to the mobility of the subunits of coatomer purified from bovine liver, except for the bands corresponding to δ-COP and ε-COP (Fig. 7 *a*, lanes *3* and *4*). The slightly higher M_r of ε-COP is pre-

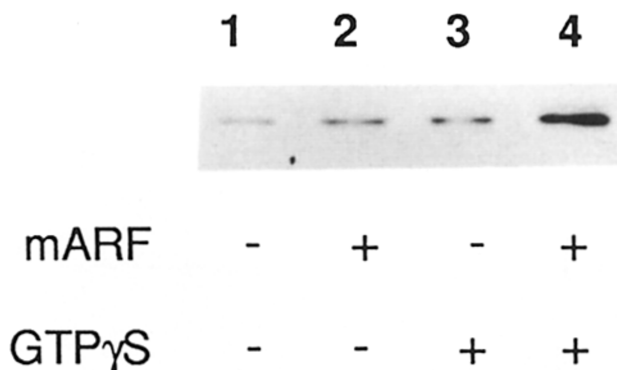

Figure 9. ARF and GTPγS-dependent coatomer binding to Golgi membrane. Golgi membranes were incubated as indicated. After incubation, the membranes were recovered and analyzed by immunoblot using M3A5.

sumably due to the six additional histidine residues; this observation independently confirms the high degree of substitution for native ε-COP. A direct comparison of ε-COP from native CHO coatomer with *E. coli* expressed His_6-ε-COP confirms that this mobility difference is due to His_6. The increased abundance of ζ-COP in CHO coatomer purified by Ni-NTA may be caused by the decreased number of purification steps which might minimize proteolytic degradation or dissociation. The ζ-COP subunit in liver coatomer is in fact substoichiometric (Waters et al., 1991). A typical preparation yields ca. 120 μg (6.0×10^4 cpm/μg) coatomer containing histidine at the NH_2-terminus from a two-liter confluent culture.

Coat Assembly

Purified His_6-tagged coatomer from the CHO transfectant could bind to Golgi membranes in an ARF and GTPγS-dependent fashion (Fig. 9), as is the case for native coatomer (Donaldson et al., 1992; Palmer et al., 1993).

The radiolabeled His_6-coatomer is also assembled into coats on Golgi-derived transport vesicles. Coated vesicles were purified after incubation of Golgi membranes with labeled coatomer and coatomer-immunodepleted bovine brain cytosol (Fig. 10 *a*) in the presence of GTPγS. All of the added radiolabeled coatomer subunits were recovered in an isopycnic gradient in fractions 6–8 (Fig. 10 *b*), which is the density expected for non-clathrin-coated vesicles (ca. 41% (wt/wt) sucrose; Fig. 10 *c*). When GTP replaces GTPγS under this condition, coated vesicles do not accumulate (Malhotra et al., 1989); now the coatomer proteins are not present in the density gradient at the position expected for coated vesicles (Fig. 10 *d*).

We also examined radiolabeled coated vesicles formed with only myristoylated ARF and labeled coatomer as cytosolic factors instead of crude cytosol fractions. Again, all of the subunits of coatomer were recovered in fractions 6–8 (Fig. 11 *c*), as expected for COP-coated vesicles. However, now vesicles can accumulate when GTP replaces GTPγS (Fig. 11 *a*), perhaps because a cytosolic GTPase-activating protein (GAP) is absent. The appearance of coatomer subunits in these fractions required myristoylated ARF1 protein (Fig. 11 *d*).

To determine the ratio of coatomer subunit at various stages, purified labeled coatomer, labeled coatomer bound to Golgi membranes, and coatomer in coated vesicles formed with either coatomer-depleted cytosol or whole cytosol were subjected to SDS-PAGE, and then the [^{35}S]methionine label associated with each coatomer subunit was determined by a phosphorimager. As seen in Table I, the ratios of ^{35}S-counts among subunits is essentially invariant under every condition, suggesting that coatomer exists as a stoichiometric complex containing a distinct complement of proteins as if cycles on and off membranes (The mole ratios in Table I are only approximate, as ^{35}S-counts are corrected for MW, not Met content. This is because the Met content of several COPs is not yet known). However there may be a significant enrichment of ζ-COP as coatomer assembles into coats (Table I),

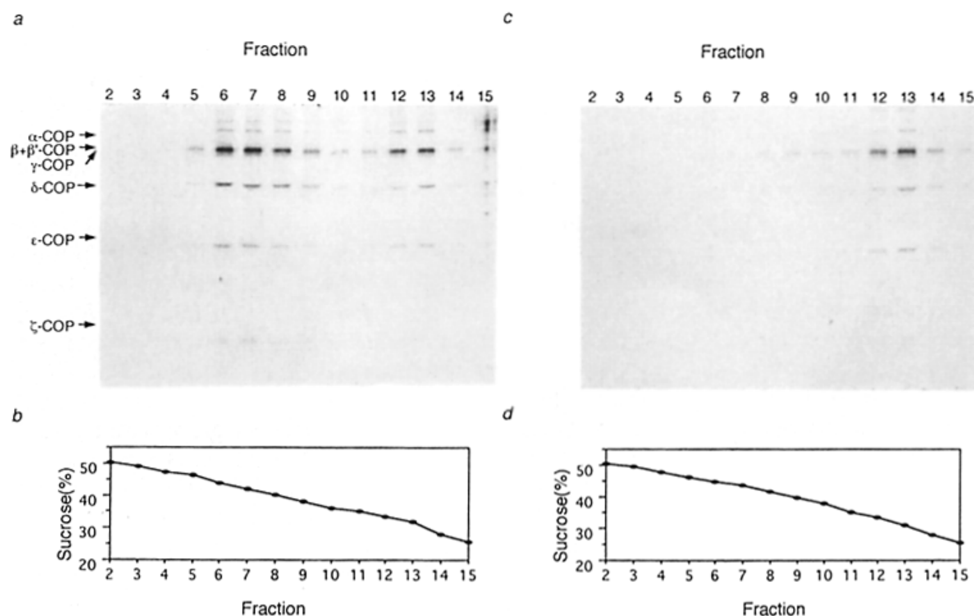

Figure 10. Isolation of labeled coated vesicles. Golgi-derived coated vesicles were prepared by incubation with Golgi membranes, labeled coatomer, coatomer-depleted cytosol and GTPγS (*a*) or GTP (*b*), and proteins from the isopycnic gradients were subjected to SDS-PAGE and autoradiographed. (*c* and *d*) Percent sucrose (wt/wt) composition of the fractions in *a* and *b* as determined by refractometry.

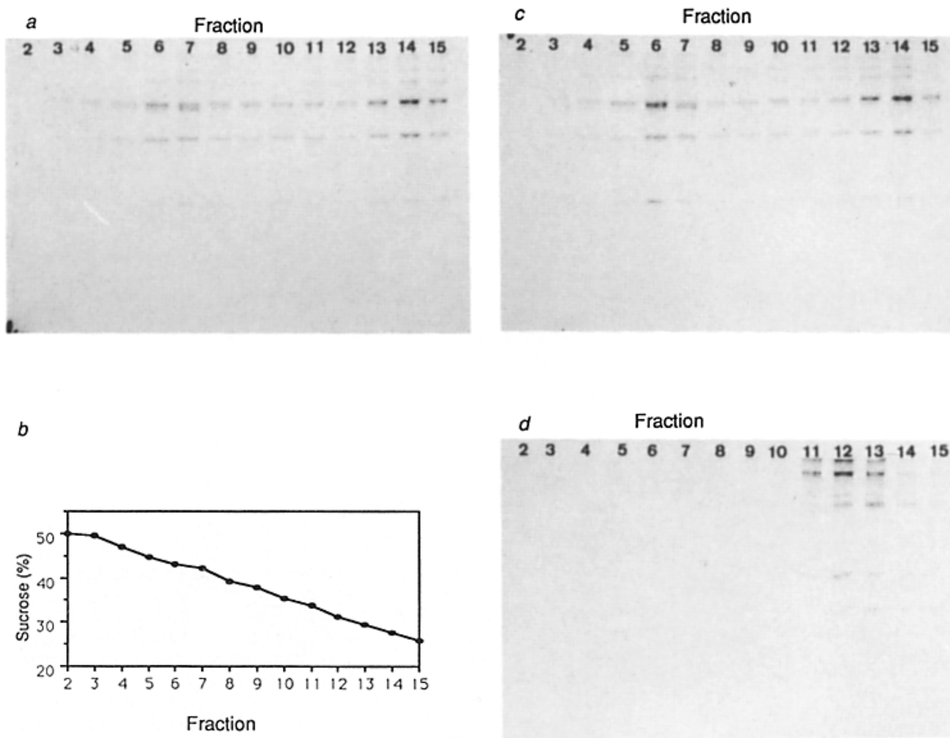

Figure 11. Assembly of coatomer to coated vesicles. Labeled coated vesicles were prepared by incubation of Golgi membranes and labeled coatomer with (*a*) myristoylated ARF1 and GTP, (*c*) myristoylated ARF1 and GTPγS, or (*d*) GTP in the absence of myristoylated ARF1. (*b*) Percent sucrose (wt/wt) composition of the fraction in *a* as determined by refractometry.

raising the possibility that coatomer complexes deficient in this subunit cannot assemble. In fact, antibody directed against ζ-COP inhibits coat assembly (Kuge et al., 1993).

Discussion

The standard coatomer purification procedure requires several steps and is time consuming. This has made it difficult to prepare radiolabeled coatomer in active form, limiting the kinds of experiments that can be done in the cell-free transport system. To circumvent this problem, we prepared a CHO transfectant containing His_6-tagged coatomer. The transfectant enables us to purify native radiolabeled coatomer by a relatively simple method in short order. The isolated His_6-tagged coatomer seems to be functional because the growth of transfectants is identical to that of wild-type cells though the CHO transfectants contain exclusively His_6-tagged coatomer (Fig. 7 *a*). Furthermore, His_6-tagged coatomer shows the same activity as purified coatomer from bovine liver cytosol; for example, coatomer binding to Golgi membranes is dependent on myristoylated ARF and GTPγS (Fig. 9) (Palmer et al., 1993). The His_6-tagged coatomer will be an important tool for further investigations to study the role of coatomer in vesicular transport.

We were also able to prepare functional coatomer with the His_6-tag at the COOH-terminus of ϵ-COP, suggesting that ϵ-COP is functional when it is substituted at either end. The functionality of the NH_2-terminal tagged ϵ-COP confirms that the p35 form (clipped at the NH_2-terminus) is not necessary for coatomer function.

Labeled coatomer could be used to determine the ratio of coatomer subunits at different stages during coated vesicle assembly. The ratio was invariant except for ζ-COP, which is enriched in assembled coats. This is the first demonstration that coatomer complex is incorporated en bloc, as hypothesized (Waters et al., 1991).

Table I. Approximate Molar Ratios of Coatomer Subunits in Coatomer and Coated Vesicles

Stage	α-COP*	β-COP plus β′-COP	γ-COP	δ-COP	ϵ-COP	ζ-COP
1. Purified coatomer	[1]	3.1	1.5	2.5	3.0	2.6
2. Coatomer bound to Golgi membranes	[1]	2.6	1.3	2.4	2.6	2.8
3. Coatomer in coated vesicles (prepared with coatomer-depleted cytosol)	[1]	2.5	1.6	3.5	3.9	5.9
4. Coatomer in coated vesicles (prepared with whole cytosol)	[1]	2.6	1.7	3.3	3.9	5.8

* Approximate molar amounts of each COP (intensity in phosphorimager divided by MW) normalized to α-COP at each stage.

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