Architecture of Coatomer: Molecular Characterization of δ-COP and Protein Interactions within the Complex

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Abstract. Coatomer is a cytosolic protein complex that forms the coat of COP I-coated transport vesicles. In our attempt to analyze the physical and functional interactions between its seven subunits (coat proteins, COPs α-ζ), we engaged in a program to clone and characterize the individual coatomer subunits. We have now cloned, sequenced, and overexpressed bovine α-COP, the 135-kD subunit of coatomer as well as δ-COP, the 57-kD subunit and have identified a yeast homolog of δ-COP by cDNA sequence comparison and by Nδ-terminal peptide sequencing. δ-COP shows homologies to subunits of the clathrin adaptor complexes AP1 and AP2. We show that in Golgi-enriched membrane fractions, the protein is predominantly found in COP I-coated transport vesicles and in the budding regions of the Golgi membranes. A knock-out of the δ-COP gene in yeast is lethal. Immunoprecipitation, as well as analysis exploiting the two-hybrid system in a complete COP screen, showed physical interactions between α- and ε-COPs and between β- and δ-COPs. Moreover, the two-hybrid system indicates interactions between γ- and ζ-COPs as well as between α- and β'-COPs. We propose that these interactions reflect in vivo associations of those subunits and thus play a functional role in the assembly of coatomer and/or serve to maintain the molecular architecture of the complex.

In eukaryotic cells, intracellular traffic between membrane compartments is mediated by vesicular carriers (Palade, 1975; Rothman, 1994). Golgi-derived (COP I) coated vesicles can be produced in a mammalian cell free system that reconstitutes intercisternal protein transport (Balch et al., 1984; Balch and Rothman, 1985; Orci et al., 1986, 1989) and can be purified from such incubations (Malhotra et al., 1989; Serafini et al., 1991a). This led to the identification of their coat components, coatomer and ADP-ribosylation factor (ARF)1 (Serafini et al., 1991b). Coatomer is a cytosolic protein complex that consists of seven subunits (coat proteins, COPs α-ζ) and that is recruited to the Golgi membrane during the budding of a transport vesicle (Waters et al., 1991). A similar complex has been identified in cytosol from yeast (Hosobuchi et al., 1992). All COPs except δ-COP are characterized at a molecular level. α-COP, Mr = 135,000, was defined as a protein in yeast that, when mutated (ret 1-1), leads to a defect in retention/retrieval of ER proteins (Letourneur et al., 1994) and was cloned and sequenced independently to characterize the yeast homolog of mammalian α-COP (Gerich et al., 1995). Recently, the secretory yeast mutant sec 33 exhibiting an ER to Golgi transport defect was shown to bear a mutation in α-COP (Wuestehube et al., 1996). α-COP contains four WD40 repeats in its NH2-terminal third. This motif was originally defined in the 13-subunits of trimeric G-proteins and since was found in subunits of a whole variety of heteromeric protein complexes (Neer et al., 1994). Mammalian α-COP peptide sequences have been characterized from bovine brain and found to be highly conserved (Gerich et al., 1995).

Coatomer contains three subunits with molecular weights around 100 kD: (a) β-COP (Sec26p in yeast) (Duden et al., 1991, 1994; Serafini et al., 1991a), (b) β'-COP (Sec27p in yeast) (Harrison-Lavoie et al., 1993; Harter et al., 1993; Stenbeck et al., 1993; Duden et al., 1994), which, like α-COP, contains WD40 repeats; and (c) γ-COP (Sec21p in yeast) (Hosobuchi et al., 1992; Stenbeck et al., 1992; Harter et al., 1996). Mutations of these COPs in yeast cause an accumulation of ER membranes (Kaiser and Schekman, 1990), and mutated forms of Sec21p and of Sec27p lead further to a defect in retrieval of ER-resident membrane proteins, similar to ret 1-1 (Letourneur et al., 1994). This implication
in ER retrieval has been suggested because coatomer is able to bind to peptides with a sequence motif -KXXXCO; (Cosson and Letourneur, 1994), known as signals for retrieval from the Golgi of escaped ER-resident membrane proteins (Nilsson et al., 1989). Although this binding has been attributed to a ternary complex of α-, β-, and ε-COP in yeast and in mammals (Cosson and Letourneur, 1994; Letourneur et al., 1994; Lowe and Kreis, 1995), photo-cross-linking experiments have pointed to γ-COP as the KXXX-binding subunit of bovine coatomer (Harter et al., 1996). This discrepancy remains unresolved. Furthermore, the binding of γ-COP to a 20-kD protein (likely ζ-COP) has been shown (Lowe and Kreis, 1995). The same authors also suggest an association of β-COP to δ-COP. The δ-COP subunit is an integral part of the COPI coat and is conserved in most eukaryotes and is involved in the budding and fusion of vesicles (Letourneur et al., 1994). In addition, δ-COP has been shown to be implicated in the ER recycling pathway (Lowe and Kreis, 1995) and to be involved in the transport of cargo proteins between the ER and Golgi apparatus (Cosson and Letourneur, 1994).

Comparison of peptide sequences obtained from mammalian COPs isolated from both soluble coatomer and purified transport vesicles revealed that each COP is a constituent of the cytosolic coatomer as well as of the transport vesicle coat (Waters et al., 1991; Stenbeck et al., 1992; Kuge et al., 1993; Hara-Kuge et al., 1994). Both on vesicles and in coatomer, the individual COPs occur in a 1:1 stoichiometry (Waters et al., 1991; Serafini et al., 1991a), and the presence in transport vesicles of some of the COPs has been proven by immunoelectron microscopy (Orici et al., 1993; Stenbeck et al., 1993; Gerich et al., 1995). The coatomer subunits are recruited en bloc during the assembly of COPI coated vesicles (Hara-Kuge et al., 1994).

We wish to understand at a molecular level the mechanisms of the coating and budding reactions during the formation of a transport vesicle. Therefore, it is necessary to elucidate the molecular architecture of the coatomer complex. To this end, we have completed the structural characterization of coatomer subunits by cloning and sequencing the cDNAs for bovine δ-COP and α-COP. This has enabled us to test all seven COPs with each other for possible protein-protein interactions in the two-hybrid system, and we found four interacting pairs of COPs: β/δ-COPs, γ/ε-COPs, α/ε-COPs, and α/β′-COPs. These results are in accordance with the suggested coatomer subcomplexes mentioned above and reveal novel binary COP-COP interactions: α/ε-COPs and possibly α/β′-COPs.

Materials and Methods

Strains, Media, and Microbiological Techniques

General molecular biological methods were as described (Sambrook, 1989). Yeast strain Y190 is MATα, ura3-52, his3-200, ade2-101, lys2-801, can1-100, leu2-3,112, gal4-542, gal80-538, URA3::GAL-LacZ, LYS2::GAL-HIS3, cyh2, and was used for the two-hybrid system. δ-COP was cloned from a bovine liver cDNA library in the ZAP XR vector (Stratagene, La Jolla, CA) and was used as a template to amplify the full-length codon-optimized cDNA for δ-COP using a degenerated primer (GCGCGGATCACCTGGTGCTGTTGGCAGCAGTCTGTA) and a polyA universal primer. The obtained 2,250-bp fragment was sequenced with universal primers and analyzed by Southern blotting: 2 μg of genomic wildtype and mutated DNA was digested with PstI and blotted on nitrocellulose. A random-primed [32p]dCTP-labeled DNA probe obtained from a BglII/ApaI digest of the δ-COP gene was used for hybridization. Transformants were scored on YPA plates (1% Bacto-yeast extract, 2% Bacto-peptone, and 1% potassium acetate) and tetrad analysis on YPD plates was performed to select for mutant and wildtype alleles (Rose et al., 1990).

Disruption of the δ-COP Gene

A DNA fragment containing full-length yeast δ-COP and additional flanking regions was constructed via PCR from genomic DNA of yeast strain RS453. Primers were GTACCTTACTATGGGGATGATTT-GGTGGA and forward and TCTAGCTGGAATCTACTGTTGCA for reverse priming. The obtained 2,250-bp fragment was digested with ApaI and cloned into the Smal/ApaI sites of pBlueScript-SK + (Stratagene) vector. An internal 1396-bp NdeI/BglII fragment containing the DYC gene was inserted into the ApaI site of the vector. The obtained 2,250-bp fragment was sequenced with universal primers and analyzed by Southern blotting: 2 μg of genomic wildtype and mutated DNA was digested with PstI and blotted on nitrocellulose. A random-primed [32p]dCTP-labeled DNA probe obtained from a BglII/ApaI digest of the δ-COP gene was used for hybridization. Transformants were sporulated on YPA plates (1% Bacto-yeast extract, 2% Bacto-peptone, and 1% potassium acetate) and tetrad analysis on YPD plates was performed to select for mutant and wildtype alleles (Rose et al., 1990).

Full-Length cDNA of α-COP

Screening a bovine liver cDNA library in uniZAP XR vector (Stratagene) expressed in E. coli XL1 blue MRF’ (Stratagene) was performed using two degenerated [32p]labeled oligonucleotide coding for the peptides MVFRLERERP (amino acid residues 314–325) and ONGHOIVE (amino acid residues 684–691). From 106 clones, two were detected as double positives in a filter screen. After in vivo excision, the resulting bluescript construct was sequenced with universal primers and several walking primers.

Full-Length cDNA of δ-COP

Screening a cDNA library kg10 (bovine brain) in E. coli C600HI was performed using a degenerated [32p]labeled oligonucleotide coding for the peptide APVGEIDEYG. This screening revealed the COOH-terminal 197-amino acid residues of δ-COP, the nucleotides 941–1536. To recover the whole cDNA sequence, the rapid amplification of cDNA ends (RACE) technique was used (Frohman et al., 1988). mRNA from bovine mammary gland was isolated with the polyATtract mRNA isolation system (Promega Corp., Madison, WI) and reversely transcribed according to the manufacturer’s instructions (GIBCO BRL, Gaithersburg, MD) using primer 1. The resulting cDNA was used as a template for a PCR amplification step with the nested primer 2 for the COOH-terminal and an oligo universal primer for the polyT NH2-terminal end. To obtain a specific product, a subsequent PCR with the universal primer and the nested primer 3 was performed. The obtained sequence did not reveal the NH2 terminus of δ-COP, but an additional cDNA stretch of 155 nucleotides and oligonucleotide 4 was used to rescreen the kg10 library. The newly obtained cDNA sequence (from nucleotide 373 on) was used to design oligonucleotides for two further RACE reactions: first with primer 5 for reverse transcription, primer 6 and a polyA universal primer for the first PCR, and primer 7 and a polyA universal primer for the second PCR. Then with primer 5 for reverse transcription, primers 6 and 7 for the first PCR and primer 60N and 8 for the second PCR. Oligonucleotide 60N codes for the NH2-terminal nine amino acid residues of δ-COP. The obtained clones all revealed the sequence from nucleotide 58 on, but not clearly the NH2 terminus, which was finally found with another RACE reaction with AmpliFlnger Race (Clontech, Palo Alto, CA) using primer 9 for reverse transcription and primer 10 and an anchor primer for PCR, following the manuals suggested by the distributor.

To obtain the full-length cDNA, mRNA from bovine mammary gland was reverse transcribed with the COOH-terminal primer (TCGCGAAATTCATACATTTACATACATTACACTAAG) containing a HindIII site and subsequent PCR with this primer and the NH2-terminal primer (GCGCGGATCCTGGTGCTGTTGGCAGCAGTCTGTA) containing a BamHI site revealed the full-length cDNA of 1533 basepairs, which was subcloned into pQE32 (Qiagen) and sequenced. Primers were (with the position of nucleotides in brackets): 1 (1070–1094), 2 (1053–1070), 3 (1002–1036), 4 (856–885), 5 (840–865), 6 (413–438), 7 (391–415), 8 (368–394), 9 (263–289), and 10 (234–260).

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**Overexpression of β-COP**

The full-length cDNA of β-COP was ligated into pQE32 (Qiagen) with BamHI/HindIII and used to perform overexpression in E. coli M15 [pREP4] (Qiagen) as follows: a 700-ml overnight culture (37°C) of a positive colony (tested previously by Western blotting) in LB medium (Sambrook, 1989) supplemented with kanamycin (25 μg/ml) and ampicillin (100 μg/ml) was used to inoculate 71 LB Kana/Amp and was grown at 37°C to an OD600 of 1.6. This culture was then induced with 15 μM IPTG (isopropyl-β-thiogalactopyranoside, Gerbu Biotechnik GmbH, Gielberg, Germany) and grown for 3 h at 30°C. A control culture was grown under the same conditions without induction (these cultures were used to compare the induced and uninduced cell extracts on a 7.5% SDS-PAGE). Cells were then harvested by centrifugation (8,900 g for 8 min, 4°C) and resuspended in 30 ml breakage buffer (50 mM Hepes, pH 7.0, 100 mM KOAc, 1 M NaCl, 10% glycerol). Further purification was performed using a Ni²⁺-chelating matrix (Qiagen) following a protocol suggested by the distributor, and the obtained protein was analyzed by Western blotting.

**Two-Hybrid System**

Transformations were performed by the lithium acetate method (Gietz and Schiestl, 1991), and selective medium was SD (6.7% nitrogen base without amino acids [Difco Laboratories, Inc., Detroit, MI], 2% glucose) supplemented with Adenine sulfate 20 μg/ml. The template was the 13-COP cDNA inserted in pQE32 (Qiagen), the same vector that was used for subcloning in pGEM4Z (Promega), a gift from Dr. T.E. Kreis (University of Geneva, Switzerland) and X-COP in Bluescript pSK+ (Stratagene) (Hara-Kuge et al., 1994) and the COOH-terminal peptide of bovine α-COP, KDVIGLRISPLQFR, were raised in rabbits. The peptides were coupled to keyhole limpet hemocyanin (216-222), and against the COOH-terminal peptide of bovine α-COP, KDVIGLRISPLQFR, were raised in rabbits. The peptides were coupled to keyhole limpet hemocyanin by glutaraldehyde (Harlow and Lane, 1988) and affinity purification of the β-COP antibody was performed using the peptide coupled to epoxy-activated Sepharose 6B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Antibodies were coupled to protein A-Sepharose beads (CL4B, Pharmacia) as follows: Beads were washed five times with IP buffer (25 mM TrisCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 2 mM benzamidine, and 10% glycerol). After centrifugation, 14,000 g, the supernatant was assayed for protein concentration and 50-100 μg of total protein was assayed for β-galactosidase activity with ortho-nitrophenyl-β-D-galactoside (ONPG) as described (Guarante, 1983). OD600 was measured at least at four time points, and the β-galactosidase activity was determined as follows: 1 U = OD420 × 1000 mg protein × time (min). Standard deviations were in the range of 10-20%.

Quantitative measurement of growth in SD+His + 25 mM 3-AT was performed by growing 50-ml overnight cultures to an OD600 of 0.5-1.0. Fresh medium (100 ml) was inoculated to an OD600 of 0.01, and the OD600 was determined every 4-6 h. Generation times were measured in the range of OD600 0.1-0.8.

**Antibodies**

Antiserum against a synthetic internal peptide of bovine β-COP, RSSPKAL/KEGAKGE (amino acid residues 216-222), and against the COOH-terminal peptide of bovine α-COP, KDVIGLRISPLQFR, were raised in rabbits. The peptides were coupled to keyhole limpet hemocyanin by glutaraldehyde (Harlow and Lane, 1988) and affinity purification of the β-COP antibody was performed using the peptide coupled to epoxy-activated Sepharose 6B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). For immunoprecipitations, antiserum against α- and β-COPs were used as well as anti-β-COP-antiserum (Harter et al., 1993) and anti-α-COP-antiserum (Hara-Kuge et al., 1994).

**Immunoprecipitation**

For electron microscopic immunolocalization, Golgi fractions from rat liver and CHO cells were fixed with 1% glutaraldehyde in phosphate buffer, infiltrated with 2.3 M sucrose, and processed for cryoultramicrotomy according to Tokuyasu (1986). Sections were incubated overnight with affinity-purified anti-β-COP antibody (undiluted). The antibody was revealed with the protein A-gold method (Roth et al., 1978). After immunolabeling, sections were absorption-stained with uranyl acetate (Tokuyasu, 1986) and examined in the electron microscope.

**Plasmid Constructs**

Fusion constructs with the cDNA of the GAL4 DNA-binding domain (plasmid pAS1) and with the GAL4 activation domain (plasmid pACT II) were obtained by in-frame ligation of PCR products of β, β', γ, δ, ε, and ε-β-COP. The primers were designed to give a Ncol/Smal small product for β-COP, a SmaI/BamHI product for β'-COP and Ncol/BamHI products for γ-COP, δ-COP, ε-COP, and ε-γ-COP. Template for the PCR of β-COP, the template was the β-COP cDNA inserted in pGEM4Z (Promega), a gift from Dr. T.E. Kreis (University of Geneva, Switzerland) (Duden et al., 1991). γ-COP cDNA was described (Harter et al., 1996). cDNA of ε-COP in bluescript pKS+ (Stratagene) (Hara-Kuge et al., 1994) and γ-ε-COP in bluescript pSK+ (Stratagene) (Kuge et al., 1993) were generous gifts from Dr. J. Rothman (Memorial Sloan Kettering Cancer Center, New York). The fusion of α-COP with the DNA-binding domain of GAL4 was obtained by ligating an EcoRI/XbaI fragment obtained from the full-length clone after subcloning in pQE30 (Qiagen) and M13mp18 (Promega) into the EcoRI/Xhol sites of the pBD-GAL4 vector (Stratagene). The fusion construct with the activating domain of GAL4 was obtained from the same cDNA fragment by ligating it into the pAD-GAL4 vector (Stratagene), followed by phase isolation and in vivo excision following the manuals of the distributor.

**In Vitro Translation**

mRNAs were in vitro transcribed from the cDNA of β-COP inserted in pGEM4Z (Promega) and of β-COP (Duden et al., 1991) in pGEM4Z (Promega), of α-COP (this work, inserted in bluescript SK), and ε-COP in pBluescriptSK (Hara-Kuge et al., 1994). Transcriptions were performed with T3 RNA polymerase (Promega) or β- and ε-COPs or T7 RNA polymerase (Promega, for α- and ε-COPs) following the protocol suggested by the distributor. The obtained mRNA was chloroform/phenol extracted and precipitated with ethanol/0.9 M ammonium acetate and resuspended in water.

In vitro translation was performed with the Flexi Rabbit Reticulocyte Lysate System (Promega) following a protocol suggested by the distributor. RNase inhibitor (Promega, 1 μg/ml) and dithiothreitol (DTT, final concentration 1 mM) were added to all reactions. 35S-labeled 1-methylmethionine (Amersham Corp., Arlington Heights, IL; 1,000 Ci/mmol) was added for radioactive labeling (final 1-2 μCi/μl). Reaction time was 90 min; longer incubations resulted in no increase of labeled protein, and shorter incubations did not result in less fragmentational translation. The yield of translated protein was detected by TCA precipitation as follows: 1 μl of the sample was dried on a Whatman paper (10 min at room temperature), incubated in 10% TCA (5 min), and then boiled in 10% TCA (5 min). After washing with EtOH and drying (10 min at room temperature), radioactivity was quantified in a liquid scintillation counter.

**Immunocprecipitation**

Antisera were coupled to protein A-Sepharose beads (CL4B, Pharmacia) as follows: Beads were washed five times with IP buffer (25 mM TrisCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 2 mM benzamidine) and resuspended with two vol IP buffer. 300 μl of this suspension (100 μl beads) was incubated with 50 μl antiserum or preimmune serum for 8 h at 4°C. After washing five times with IP buffer, the beads were resuspended in two vol IP buffer and were incubated as follows: all cups were preincubated for 1 h with BSA, 1 mg/ml, and BSA was added to the reactions to a final concentration of 0.1 mg/ml. 10 μl of a 100,000 g supernatant (45 min at 4°C) of the translation reaction was mixed with 30 μl of suspended beads to a final volume of 50 μl IP buffer and incubated for 18 h, 4°C with agitation. After washing the beads five times with IP buffer and twice with IP buffer containing no NP-40, the pellet was resuspended in 20 μl SDS-PAGE loading buffer and 7 μl were separated on a 7.5 or 10% acrylamide gel under reducing conditions.
Results

Primary Structure of δ-COP

The strategy to isolate a cDNA clone of δ-COP was similar to previously described procedures to identify the cDNA of β-COP (Stenbeck et al., 1993). Cytosolic coatomer from bovine brain was isolated and purified as described (Waters et al., 1991). The gel purified subunit of around 61 kD was digested with trypsin and the resulting peptides were microsequenced. One peptide was used to design a corresponding oligonucleotide and a αgt10 cDNA library from bovine brain was screened. A COOH-terminal 594-bp fragment of δ-COP was obtained, and several nested priming reactions of reverse transcribed mRNA from bovine mammary gland cells were used to obtain the NH2 terminus. A full-length cDNA was generated via PCR introducing cloning sites for the overexpression vector and sequencing. This cDNA clone of δ-COP (accession number X94265) encodes a protein of 510-amino acid residues with a calculated molecular weight of 57,140 (Fig. 1). The amino acid sequences of all peptides as determined by Edman degradation can be assigned to the predicted amino acid sequence (Fig. 1, underlined). The sequence data are available from GenBank/EMBL/DDBJ under accession number X94265.

The cloned gene encodes the entire 57-kD subunit of coatomer: δ-COP was synthesized in E. coli using an inducible expression system in which a 6xHis affinity tag is fused to the NH2 terminus. When the promoter was induced, a protein band of an apparent molecular mass of around 61 kD was detected in a corresponding E. coli extract (Fig. 2 A). This protein was neither seen in an extract of cells transformed with the δ-COP expression plasmid but not in an uninduced sample (not shown). Immunological analysis by Western blotting revealed that an affinity purified anti-δ-COP antibody indeed recognizes the overexpressed protein with an apparent molecular mass of around 61 kD, as depicted in Fig. 2 B, but not in an uninduced sample (not shown).

Immunelectron Microscopic Localization of δ-COP

We used the affinity purified anti-δ-COP antibody to identify the subcellular localization of δ-COP. Rat liver Golgi membranes were primed in vitro with ATP, cytosol, and the nonhydrolyzable analog of GTP, GTPγS, to obtain an accumulation of coated transport vesicles (Orci et al., 1989). Protein A gold (Roth et al., 1978) was used to detect the antibody bound to δ-COP on ultrathin cryosections (Tokuyasu, 1986). A distinct labeling was seen on coated vesicular profiles (buds and vesicles) on Golgi membranes (Fig. 3). Quantification of the labeling in 10 Golgi areas revealed that 15% of the gold particles were localized to Golgi cisternae, whereas 85% of the particles were associated with vesicles and buds. No immunogold signal could be identified using preimmune serum to label the same vesicular structures (data not shown). The staining pattern with the anti-δ-COP antibody was similar to those generated by antibodies directed against other COPs (Duden et al., 1991; Serafini et al., 1991a; Kuge et al., 1993; Stenbeck et al., 1993; Gerich et al., 1995).

Knock-out of the δ-COP Gene in Yeast

To test whether δ-COP gene function is essential for yeast cell viability, a gene disruption experiment was performed.
To this end, a 1.4-kb fragment of the coding sequence of yeast δ-COP was replaced by the HIS3 gene. A construct containing the disrupted copy was used to generate a diploid yeast strain RS453 with one wild-type and one mutant copy of the gene. Disruption was confirmed by Southern blot analysis (Fig. 4A). Replacement clones were sporulated, and tetrads of each were analyzed as depicted in Fig. 4B; a 2:2 viable/nonviable pattern was obtained. (No microcolonies were observed to grow out of the nonviable spores.) This result shows that δ-COP is encoded by an essential gene.

**Primary Structure of Bovine α-COP**

With the molecular structure of δ-COP described above, six of the seven COP cDNAs were available from bovine origin; however, α-COP at this time was only characterized in yeast (Letourneur et al., 1994; Gerich et al., 1995). For a complete screen for COP–COP interactions in the yeast two-hybrid system, we had to clone bovine α-COP as well. This was performed using a UniZapXR cDNA library from bovine liver (Stratagene) and degenerated 32P-labeled oligonucleotides. The corresponding amino acid sequence was obtained by microsequencing peptides obtained by trypsin digestion of gel-isolated α-COP. The resulting cDNA derived amino acid sequence of α-COP and an alignment with the yeast homolog is depicted in Fig. 5. As expected from peptide sequence comparisons (Gerich et al., 1995), the derived amino acid sequence of yeast and bovine α-COP show a high degree of conservation (46% identity and 64% similarity). An open reading frame for the human homolog of α-COP (accession number U24105) was identified by a database research with the bovine sequence reported here.

**COP Interactions in the Two-Hybrid System**

The complete set of the seven bovine COP cDNAs could now be analyzed in the yeast two-hybrid system (Fields and Song, 1989; Chien et al., 1991; Durfee et al., 1993) to identify binding partners of each COP within the coatomer complex. Two independent screens were performed: one with the cDNA of a given COP fused to the DNA-binding domain of GAL4 (BD, amino acid residues 1–147), searching for possible binding partners among the individually coexpressed COP proteins fused to the activating domain of GAL4 (AD, amino acid residues 768–881), and a second one with exchanged hybrid vectors (the COP was fused to GAL4[AD]) and the other COPs fused to GAL4[BD]). In each set of experiments, 8–10 independent transformants were assayed for β-galactosidase activity and growth on a minimal medium that lacks histidine. β-galactosidase activities were monitored in a filter lift assay (Breeden and Nasmyth, 1985) and quantified by measurement of β-galactosidase activity units in cell extracts of the corresponding transformants. Response of the second reporter gene, HIS3, was analyzed by growth experiments on a His-selecting medium and also quantified by measurement of the generation time in this medium. As a positive control, a transformant was used that coexpresses SNF1/GAL4(1–147) and SNF4/GAL4(768–881), two proteins known to physically associate in vivo and whose interaction can be detected using the two-hybrid system (Fields and Song, 1989; Durfee et al., 1993).

This complete screen (49 different combinations of COPs) revealed four COP–COP interactions: β/δ-COPs, γ/ξ-COPs, α/ε-COPs, and α/β'-COPs. These results, together with the corresponding controls, are summarized in Table II. Each COP fusion protein alone cannot significantly activate GAL4-dependent transcription by itself in either fusion orientation, except ζ- and β-COP when fused to GAL4[BD]. This transcriptional activation, e.g., in the case of ζ-COP, may result from acidic regions of the polypeptide (since it was not observed when ζ-COP is fused to GAL4[AD]), but still there is a significant stimulation of transcriptional activity resulting from the interaction with γ-COP (62 β-galactosidase units from ζ-COP alone in contrast with 812 units resulting from the interaction with γ-COP, see Table II). Except the α/β'-COP interaction, all binding COP pairs were detected in both fusion orientations. In the experiments with α-COP, either

### Table I. Comparison of Bovine δ-COP with Homologue Proteins

<table>
<thead>
<tr>
<th>Homologue</th>
<th>Species</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
<th>Identity</th>
<th>Similarity</th>
<th>Features</th>
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<td>ARCN1</td>
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<td>57.1</td>
<td>98</td>
<td>99</td>
<td>Presumably δ-COP</td>
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<td>60.6</td>
<td>33</td>
<td>56</td>
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<td>Subunit of the clathrin</td>
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<tr>
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<td>19</td>
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</table>

Sequence homologies of bovine δ-COP with its human and yeast homologs and with two subunits of the clathrin adaptor complexes: AP47 from mouse, AP1, and AP50 from S. pombe, AP2.
in the pBD-GAL4 or pAD-GAL4 plasmid (both plasmids from Stratagene) and ε- or β'-COP in the partner plasmid, the transformants had an elevated β-galactosidase activity; however, these signals were much lower compared to the magnitude of increase of the growth ability in His-selective medium. This may be due to an inhibitory effect on the β-galactosidase activity of α-COP expressed in these transformants. Therefore, those values are not shown. However, these transformants showed highly significant growth in His-selective medium (Table II).

To identify possible interactions between COPs and the small G protein ARF (which is also a component of the coat of COPI-coated vesicles and probably binds coatamer), the cDNAs of ARF and a mutant form of ARF, mARF(Q71L) (Tanigawa et al., 1993), were screened against the individual COPs. The mutant form of ARF has lost the ability to hydrolyze GTP and therefore is fixed in the GTP-bound conformation in which it is known to bind to the membranes. From these experiments, no significant signals were obtained (data not shown), which may be due to the fact that ARF in the GTP-bound (activated) conformation possibly binds to membranes and is therefore not suitable for the two-hybrid assay.

Since the WD40 domains are known to be involved in protein–protein interactions (Neer et al., 1994), we tested this repeat domain of β'-COP with all COPs in both fusion orientations. No significant signals were obtained (data not shown).

**Coimmunoprecipitations of Binary COP Complexes**

To biochemically characterize COP–COP associations as indicated by the two-hybrid experiments, we examined whether in vitro translated COPs could be coimmunoprecipitated. α- and ε-COPs, as well as β- and δ-COPs labeled in vitro by transcription/translation of the corresponding cDNA clones were incubated pairwise with the antibodies as depicted in Fig. 6, A and B. Anti-α-COP antibody precipitated in vitro translated α-COP (Fig. 6 A, lane 5), but not ε-COP (lane 6). However, if α-COP was cotranslationally synthesized together with ε-COP, both COP proteins were coimmunoprecipitated (Fig. 6 A, lane 4). In control reactions with preimmune serum, no labeled proteins were precipitated (Fig. 6 A, lane 7).

When β- and δ-COPs were in vitro cotranslated, a dimer of these coatamer subunits was immunoprecipitated with anti-δ-COP antibodies (Fig. 6 B, lane 4). Interestingly, the binary complex of β- and δ-COP was only precipitated from a cotranslated sample; however if the proteins were in vitro translated separately and then mixed, no coimmunoprecipitation of β-COP was observed with anti-δ-COP antibodies (not shown). In the case of α/ε-COPs, however, it was possible to coimmunoprecipitate the binary complex with anti-α-COP antibodies not only from a cotranslated sample but also from separately translated and subsequently mixed samples (not shown). No dimerization was observed when the pair of β'- and ε-COPs was analyzed, neither with anti–β'-COP nor with anti-ε-COP anti-
Coatomer Subunits Interactions

Discussion

With the cloning and sequencing of the cDNA for δ-COP, the molecular characterization of coatomer, the major cytosolic precursor of the COP I coat, is completed. δ-COP is a stoichiometric component of the coatomer complex, and, like the other COPs, is found in coatomer's soluble state, as well as in the complex when recruited to Golgi membranes, and finally on mature COP I-coated transport vesicles. A complex similar to mammalian coatomer has been identified in yeast (Hosobuchi et al., 1992; Harter et al., 1993; Duden et al., 1994), and direct comparison of peptide structures as obtained by microsequencing and data base searches have revealed a yeast homolog of δ-COP, an open reading frame on chromosome VI that codes for a 60,600-D protein (Murakami et al., 1995). Thus, like the other COPs, δ-COP is strikingly conserved from yeast to mammals. Computer-assisted data base searches have revealed a yeast homolog of g-COP, the molecular characterization of coatomer, the major cytosolic precursor of the COP I coat, is completed. δ-COP is a stoichiometric component of the coatomer complex, and, like the other COPs, is found in coatomer's soluble state, as well as in the complex when recruited to Golgi membranes, and finally on mature COP I-coated transport vesicles. A complex similar to mammalian coatomer has been identified in yeast (Hosobuchi et al., 1992; Harter et al., 1993; Duden et al., 1994), and direct comparison of peptide structures as obtained by microsequencing and data base searches have revealed a yeast homolog of δ-COP, an open reading frame on chromosome VI that codes for a 60,600-D protein (Murakami et al., 1995). Thus, like the other COPs, δ-COP is strikingly conserved from yeast to mammals. Computer-assisted data base search has revealed an open reading frame in the human genome (ARCN1 gene) as a homolog g-COP and two proteins that derive clathrin adaptor complex AP2, from AP1, which is believed to have kinase activity (Nakayama et al., 1991) and AP50, a subunit of the plasma membrane-vacuolar vesicles. A complex similar to mammalian coatomer has been identified in yeast (Hosobuchi et al., 1992; Harter et al., 1993; Duden et al., 1994), and direct comparison of peptide structures as obtained by microsequencing and data base searches have revealed a yeast homolog of δ-COP, an open reading frame on chromosome VI that codes for a 60,600-D protein (Murakami et al., 1995). Thus, like the other COPs, δ-COP is strikingly conserved from yeast to mammals. Computer-assisted data base search has revealed an open reading frame in the human genome (ARCN1 gene) as a homolog g-COP and two proteins that derive clathrin adaptor complex AP2, from AP1, which is believed to have kinase activity (Nakayama et al., 1991) and AP50, a subunit of the plasma membrane-vacuolar vesicles. A complex similar to mammalian coatomer has been identified in yeast (Hosobuchi et al., 1992; Harter et al., 1993; Duden et al., 1994), and direct comparison of peptide structures as obtained by microsequencing and data base searches have revealed a yeast homolog of δ-COP, an open reading frame on chromosome VI that codes for a 60,600-D protein (Murakami et al., 1995).
The interactions of α- with β'-COP (confirming earlier findings; Lowe and Kreis, 1995), of α- with ε-COP, and indicate an association of α- with β'-COP. A trimeric complex of α-, β', and presumably ε-COP has been shown previously (Cosson and Letourneur, 1994; Lowe and Kreis, 1995). Our results suggest that α-COP forms a bridge between β'-COP and ε-COP.

The interactions of α- with ε-COP and β- with δ-COP were confirmed here biochemically by in vitro translation/immunoprecipitation experiments. Remarkably, the binary complex of β- and δ-COP described here was only coimmunoprecipitated in the case β- and δ-COP were synthesized together in the same in vitro translation. If each subunit was synthesized separately and these samples mixed and incubated under several conditions, no β-COP was detectable after precipitation with the anti-δ-COP antibody. Additionally, the anti-δ-COP antibody did not immunoprecipitate a complex of in vitro-translated β-COP and recombinant His6-tagged δ-COP overexpressed in E. coli (data not shown). These observations indicate that the assembly of these coatomer subunits may depend on cotranslational interactions and/or on accessory proteins that interact cotranslationally. On the other hand, a complex of α- and ε-COP was immunoprecipitated by anti-α-COP antibodies if it was either synthesized cotranslationally or formed by mixing the separately translated COPs.

No additional interactions between the individual COPs were revealed by the two-hybrid system, although all COPs were probed, and more than the interactions presented here could be expected. This might be due to the following reasons: while the two-hybrid system is a powerful method to detect protein–protein interactions, its application is limited. One limitation is the fact that it usually reports binary interactions. However, if the binding of a third protein was essential for the association of two proteins in a mammalian cell, this interaction could not be detected with the yeast two-hybrid system. Furthermore, the fact that an interaction is not detectable with the two-hybrid system does not necessarily mean that it does not exist. This may be due to missing postranslational modifications of the mammalian proteins, which may not occur in the yeast cells (Allen et al., 1995), or simply due to an overall structure of one or both hybrid proteins that does not allow interactions for sterical reasons.

Like δ-COP, all deletion mutants of COPs that have been analyzed showed a lethal phenotype (Hosobuchi et al., 1992; Duden et al., 1994; Letourneur et al., 1994; Gerich et al., 1995), a strong indication that it is in fact the complete coatomer complex that is essential in eukaryotic cells. Thus, this quaternary structure is of high functional significance and may turn out to bind various (structurally related) motifs that then might induce functionally different conformations of the protein coat. We propose that the observed interactions of β/δ-COP, γ/ζ-COP, α/ε-COP, and α/β'-COP reflect a function in building up and maintaining the complex and presently develop a biochemical system to reversibly dissociate coatomer into subunits and subcomplexes that should enable us to identify the missing interactions leading to the final picture of its architecture.

Plasmids pASI and pACTIII, as well as pSE1112 and pSE1111, which contain the fusion products GAL4(1-147)-SNF4 and GAL4(768-881)-SNF1, were generous gifts from Dr. Stephen J. Elledge (Dept. of Biochemistry, Baylor College of Medicine, Houston, TX). The β-COP cDNA was kindly pro-
vided by Dr. T. Kreis. The e- and ζ-COP cDNAs, as well as the cDNAs of ARF and mARF(Q71L), were generous gifts from Dr. J. Rothman. We thank Dr. E. Hurt for providing yeast strain RS453 and for help with the gene disruption experiments. We also thank Dr. I. Haas (Institut für Biochemie I, University of Heidelberg, Heidelberg, Germany) and Dr. E. Hurt (Institut für Biochemie I, University of Heidelberg, Heidelberg, Germany) for critically reading the manuscript.

This work was supported by The Deutsche Forschungsgemeinschaft (SFB 352), the Human Frontier Science Program to L. Orci and F. Wieland, and the Swiss National Science Foundation No. 31-43366/95 (to L. Orci).

Received for publication 15 February 1996 and in revised form 8 July 1996.

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Published October 1, 1996

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