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

D. Faulstich,* S. Auerbach,* L. Orci," M. Ravazzola," S. Wegehingel,* F. Lottspeich,§ G. Stenbeck,.§ C. Harter,* F.T. Wieland,* and H. Tschochner*

*Institut für Biochemie I, D-69120 Heidelberg, Germany; †Department of Morphology, University of Geneva Medical School, CH-1211 Geneva 4, Switzerland; ‡Cellular Biochemistry and Biophysics Program, Memorial Sloan Kettering Cancer Center, New York 10021; and §Max-Planck-Institut für Biochemie, Genzentrum, D-82152 Martinsried, Germany

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-ξ), 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 Nα-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 ξ-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-ξ) 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 coatamer is able to bind to peptides with a sequence motif \(-KKXX\) (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 \(\alpha\), \(\beta\)-, and \(\varepsilon\)-COP proteins (Nilsson et al., 1989). The same authors also suggest an association of \(\beta\)-COP to \(\delta\)-COP. Most recently, a new mutant of \textit{Saccharomyces cerevisiae}, RET-2, has been shown to be defective in retrieval as well as in forward transport between ER and Golgi, a mutation attributed to the \(\delta\)-COP gene (Cosson et al., 1996).

Comparison of peptide sequences obtained from mammalian COPs isolated from both soluble coatamer and purified transport vesicles revealed that each COP is a constituent of the cytosolic coatamer 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 (Orci et al., 1993; Stenbeck et al., 1993; Gerich et al., 1995). The coatamer subunits are recruited en bloc during the assembly of COP I 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 \(\delta\)-COP and \(\alpha\)-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: \(\beta\)/\(\delta\)-COPs, \(\gamma\)/\(\varepsilon\)-COPs, \(\alpha\)/\(\varepsilon\)-COPs, and \(\alpha\)/\(\beta\)-COPs. These results are in accordance with the suggested coatomer subcomplexes mentioned above and reveal novel binary COP–COP interactions: \(\alpha\)/\(\varepsilon\)-COPs and possibly \(\alpha\)/\(\beta\)-COPs.

Materials and Methods

Strains, Media, and Microbiological Techniques

General molecular biological methods were as described (Sambrook, 1989). Yeast strain Y190 is MATa, ura3-52, his3-200, ade2-101, lys2-801, trpl-901, leu2-3, 112, GAL4-542, gal80-538, URA3::GAL-LacZ, LYS2::GAL-HIS3, cyh r and was used for the two-hybrid system. RS453 (MATa/his3/his3, leu2/leu2, trpl/trpl, ura3/ura3, ade2/ade2) was used as a control. 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 NH-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 \(\delta\)-COP, but an additional cDNA stretch of 155 nucleotides and oligonucleotide 4 was used to rescreen the \(\alpha\)-COP library. The newly obtained cDNA sequence (from nucleotide 373 on) was used to design oligonucleotides for 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 60 and 7 for the first PCR and primer 60 and 8 for the second PCR. Oligonucleotide 60 codes for the NH2-terminal nine amino acid residues of \(\delta\)-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 AmpliFinder Race (Chontec, Palo Alto, CA) using primer 9 for reverse transcription, 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 (TCGCCAGGCTTCTCAGAAGATTTAATATCGACTTGGAG) containing a HindIII site and subsequent PCR with this primer and the NH-terminal primer (GCCCGGATCTCCTGTGCTTGCGACGAGTCGTGTA) containing a BamHI site revealed the full-length cDNA of 1533 basespairs, which was subcloned into pQE32 (Qiagen) and sequenced. Primers were (with the position of nucleotides in brackets): 1 (1070–1094), 2 (1053–1076), 3 (1012–1036), 4 (856–885), 5 (440–465), 6 (413–438), 7 (391–415), 8 (368–394), 9 (263–289), and 10 (234–260).

Disruption of the \(\delta\)-COP Gene

A DNA fragment containing full-length yeast \(\delta\)-COP and additional flanking regions was constructed via PCR from genomic DNA of yeast strain RS453. Primers were GTACITTCTACTATGTTGGCTACATTTAAGGTTGGCA for forward and TCCAGCTGAATCTACTTGGCCACACAGCGG for reverse priming. The obtained 2250-bp fragment containing the \(\delta\)-COP gene and 224-bp downstream and 387-bp upstream was digested with Apal and subcloned into the Smal/Apal sites of \(\delta\)-COP (Bluestein-SK) (Stagatene) vector. A internal 1396-bp NdeI/BglII fragment of the \(\delta\)-COP gene was removed from the Bluestein-\(\delta\)-COP plasmid and replaced by the HHS gene (1.2 kb) after filling in the restricted ends. 200 bp of the resulting plasmid was digested with BamHI and Apal, and then digested with S. cerevisiae strain RS453 by the lithiumacetate method (Gietz and Schiestl, 1991). The HIS+ transformans were isolated and analyzed by Southern blotting: 2 μg of genomic wildtype and mutated DNA was digested with HindIII and XbaI, separated by agarose gel electrophoresis and blotted onto nitrocellulose. A random-primed [32P]dCTP-labeled DNA probe obtained from a BglII/ Apal 421-bp 5′-end fragment of the \(\delta\)-COP gene was used for hybridization. Transformants were exposed to YPA plates (1% Bacto-yeast extract, 2% Bacto-peptone, and 1% potassium acetate) and tetrad analysis on YPD plates was performed to separate mutant and wildtype alleles (Rose et al., 1990).
Overexpression of δ-COP

The full-length cDNA of δ-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-1-thiogalactopyranoside, Gerbu Biotechnik GmbH, Galberg, 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).

Antibodies

The obtained protein was analyzed by Western blotting. 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, and 2 mM benzamidine). After centrifugation (14,000 g), the supernatant was assayed for 13-galactosidase activity by the filter lift assay (Breeden and Nasmyth, 1985). All positive (blue) transformants were replica plated both on SD+His and SD-His + 25 mM 3-AT. Quantification of 13-galactosidase activity was performed by growing a 100-ml culture in SD+His medium to an OD₆₀₀ of 0.5-1.0. Lysate System (Promega) following a protocol suggested by the distributor. The obtained mRNA was chlorormorphophenol extracted and precipitated with ethanol/0.9 M ammonium acetate and suspended in water.

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 mg/l, L-lysine-HCl 30 mg/ml, and tryptophan (50 μg/ml). After centrifugation (14,000 g), the supernatant was assayed for 13-galactosidase activity by the filter lift assay (Breeden and Nasmyth, 1985). All positive (blue) transformants were growing on the SD-His+3-AT. Quantification of β-galactosidase activity was performed by growing a 100-ml culture in SD+His medium to an OD₆₀₀ of 0.5-1.0. Lysate System (Promega) following a protocol suggested by the distributor. The obtained mRNA was chlorormorphophenol extracted and precipitated with ethanol/0.9 M ammonium acetate and suspended in water.

In Vitro Translation

mRNAs were in vitro transcribed from the cDNA of δ-COP inserted in pGEM7 (Promega) and of β-COP (Duden et al., 1991) in pGEM4Z (Promega), of α-COP (this work, inserted in plueksct SK), and of e-COP in plueksctSK (Hara-Kuge et al., 1994). Transcriptions were performed with T3 RNA polymerase (Promega; for β- and δ-COPs) or T7 RNA polymerase (Promega, for α- and e-COPs) following the protocol suggested by the distributor. The obtained mRNA was chlorormorphophenol extracted and precipitated with ethanol/0.9 M ammonium acetate and suspended in water.

Immunoprecipitation

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 resuspended with 20 μl IP buffer. 35S-labeled 1-methyl-isoleucine (Amersham Corp., Arlington Heights, IL; 1,000 Ci/ml) was added for radioactive labeling (final 1-2 μCi/ml). 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.

Immunolocalization

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 cryoultramicroscopy according to Tokuyasu (1986). Sections were incubated overnight with affinity-purified anti-δ-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, 1988) and examined in the electron microscope.
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 coatamer 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 λgt10 cDNA library from bovine brain was screened. A COOH-termina1 fragment of δ-COP was obtained, and several nested priming reactions of reversely 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 sequenced. 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 of δ-COP (Fig. 1, underlined). The cDNA derived protein consists of 7% positively and 14% negatively charged amino acids with a pI of 6.14. About 25% of the charged amino acid residues are clustered in a region from amino acid residue 129 to 178. Database searching with the full-length clone identified an open reading frame of the ARCN1 gene (Radice et al., 1995) as the human homologue of δ-COP and a hypothetical 60.6-kD protein in the alignment with its yeast homologue.

Two other proteins with weaker homologies to bovine δ-COP could be detected: AP50 from Schizosaccharomyces pombe (accession number Q09718, 20% identity and 44% similarity) and AP47 from mouse ([Nakayama et al., 1991]; 19% identity and 48% similarity) represent subunits of AP2 and AP1, respectively, the two classes of clathrin-associated protein complexes. In these alignments, the strongest homology is found in the NH2-terminal region: for AP50, 24% identity and 51% similarity, and for AP47, 22% identity and 52% similarity were found within the NH2-terminal 120 residues. Table I summarizes the degrees of conservation and possible functions of the homologous proteins.

The cloned gene encodes the entire 57-kD subunit of coatamer: δ-COP was synthesized in E. coli using an inducible expression system in which a 6His 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 without induction nor in an extract of induced cells containing the expression plasmid without insert (data 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).

Immunoelectron 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. 4 A). Replacement clones were sporulated, and tetrads of each were analyzed as depicted in Fig. 4 B: 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 linked 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/ε-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 ε- and β-COP when fused to GAL4(BD). This transcriptional activation, e.g., in the case of ε-COP, may result from acidic regions of the polypeptide (since it was not observed when ε-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 ε-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

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

| Homologue | Species          | M, ×10³ | Identity | Similarity | Features                                      |
|-----------|------------------|---------|----------|------------|-----------------------------------------------|
| ARCN1     | Homo sapiens     | 57.1    | 98       | 99         | Presumably δ-COP                              |
| YFM1      | S. cerevisiae    | 60.6    | 33       | 56         | Coatomer subunit, δ-COP                       |
| AP 50     | S. pombe         | 50.8    | 20       | 44         | Subunit of the clathrin adaptor complex (AP2) |
| AP 47     | Mus musculus     | 48.5    | 19       | 48         | Subunit of the clathrin adaptor complex (AP1); kinase-activity |

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.

Figure 2. Overexpression of (6xHis)-tagged δ-COP. (A) Coomassie staining of equal amounts induced and uninduced lysates of E. coli separated on an SDS gel. (B) Immunostaining of an induced E. coli lysate with anti-δ-COP antibody (sample equivalent to 60 μl of the culture). In the right panel, ~0.3 μg of coatomer isolated from bovine brain was analyzed as a standard for δ-COP.
in the pBD-GAL4 or pAD-GAL4 plasmid (both plasmids from Stratagene) and e- 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 e-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 e-COP (lane 6). However, if α-COP was cotranslationally synthesized together with e-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 δ-COP was observed with anti-β′-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 e-COPs was analyzed, neither with anti-β′-COP nor with anti-ε-COP anti-

Figure 3. Immunoelectron microscopic localization of δ-COP antibody on Golgi-derived buds and transport vesicles generated in presence of GTPγS from rat liver membranes. Affinity-purified anti-δ-COP antibody revealed by the protein A-gold technique. Size of gold particles 10 nm. A comparable labeling was obtained on Golgi fractions isolated from CHO cells. Bar, 100 nm.

Figure 4. Analysis of δ-COP gene disruption. (A) Southern blot analysis of the HindIII/XbaI digested genomic DNA of the wild type (lane 1) and two mutant transformants (lanes 2 and 3). The 2.69-kb fragment is from the wild-type allele, and the 1.86-kb fragment from the mutant allele. (B) Tetrad analysis of the two mutant transformants shown in A. Of the four spores, only the two carrying the wild-type allele are able to grow.
Coatomer Subunits Interactions

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. 

Discussion

Figure 5. cDNA-deduced amino acid sequence of bovine α-COP and alignment with its yeast homolog. Sequences determined by Edman degradation are underlined. These sequence data are available from GenBank/EMBL/DDJB under accession number X96768.

Table II. COP–COP Interactions Detected in the Two-Hybrid System

| Gal4 DNA-binding domain fusion | Gal4 activating domain fusion | β-Gal activity | Generations/day in -His +25 mM AT |
|-------------------------------|-------------------------------|----------------|---------------------------------|
| β-COP | δ-COP | 74 | 4.08 |
| δ-COP | β-COP | 540 | 4.80 |
| ζ-COP | γ-COP | 812 | 4.80 |
| γ-COP | ζ-COP | 50 | 1.32 |
| α-COP | ε-COP | s.t.* | 5.20 |
| ε-COP | α-COP | s.t. | 2.80 |
| β-COP | α-COP | s.t. | 2.11 |
| α-COP | β-COP | < 5 | < 0.1 |
| β-COP | α-COP | 14 | 1.92 |
| δ-COP | β-COP | < 5 | < 0.1 |
| β-COP | δ-COP | 12 | < 0.1 |
| ε-COP | γ-COP | 5 | < 0.1 |
| γ-COP | ε-COP | 62 | 1.68 |
| α-COP | ε-COP | < 5 | < 0.1 |
| ε-COP | α-COP | 15 | < 0.1 |
| SNF1 | ζ-COP | 500 | 4.30 |

Quantitative measurement of transcriptional activation of the reporter genes resulting from COP–COP interactions. One unit β-galactosidase activity (reporter gene LacZ) is defined as 1,000 ΔOΔ405/min mg lysate protein (total). In the right column, the generation times in minimal medium lacking histidine (and containing 25 mM 3-amino-1,2,4-triazole) are given to quantitate the response of the reporter gene HIS3. Except the α/β-COP interaction, all interactions are shown in both fusion orientations.

* s.t., see text.
cession number Q09718). Similar weak homologies were found in two other subunits of mammalian coatomer: β-COP is slightly related to the human β-subunit of AP2 (17% homology over the NH2-terminal 450 residues [Duden et al., 1991], and the smallest subunit ζ-COP is reminiscent of both the small subunits of AP1 and AP2 [Kuge et al., 1993]).

We have probed possible protein–protein interactions the individual COPs might exert with each other in vivo using the two-hybrid system in yeast. Therefore, we tested mammalian α, β, γ, δ, ε, and ζ-COP pair-wise in both possible orientations of fusion proteins. The most striking result of this study was a specific interaction of β-COP with δ-COP, as suggested previously (Lowe and Kreis, 1995). Additionally, these experiments revealed binary interactions of γ- with ζ-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.

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