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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 NH_2-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.

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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., 1991*a*). This led to the identification of their coat components, coatomer and ADP-ribosylation factor (ARF)[1] (Serafini et al., 1991*b*). 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, M_r = 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 NH_2-terminal third. This motif was originally defined in the β-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., 1991*a*), (*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

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1. *Abbreviation used in this paper*: ARF, ADP ribosylation factor.

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in ER retrieval has been suggested because coatomer is able to bind to peptides with a sequence motif -KKXXCO$_2^-$ (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 KKXX-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. Most recently, a new mutant of *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 δ-COP gene (Cosson et al., 1996).

Comparison of peptide sequences obtained from mammalian COPs isolated from both soluble coatomer and purifed 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., 1991*a*), 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 coatomer 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 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/ζ-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 MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, 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/α, his3/his3, leu2/leu2, trpl/trpl, ura3/ura3, ade2/ade2) was used for the δ-COP gene disruption and PCR experiments. Yeast was grown in YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose) at 30°C. For selection of transformants, SD medium was used containing 0.7% nitrogen base, 2% glucose, and the appropriate amino acids.

Bacterial strains were *Escherichia coli* XL1 blue (Stratagene, La Jolla, CA) and *E. coli* M15 [pREP4] (Qiagen, Inc., Chatsworth, CA) for overexpression of δ-COP. Library screenings were performed in *E. coli* C600 Hfl and *E. coli* XL1 blue (Stratagene), subsequent cloning of the isolated DNA in CMK603 and *E. coli* XL1 blue (Stratagene).

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 ^{32}P-labeled antisense strand oligonucleotides coding for the peptides MIVFKLERERPA (amino acid residues 314–325) and QGNHQIVE (amino acid residues 684–691). From 10^6 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 λgt10 (bovine brain) in *E. coli* C600Hfl was performed using a degenerated ^{32}P-labeled oligonucleotide coding for the peptide APVIGEIDGEY. 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 polyATract 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 oligoA-universal primer for the polyT NH_2-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 NH_2 terminus of δ-COP, but an additional cDNA stretch of 155 nucleotides and oligonucleotide 4 was used to rescreen the λgt10 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 60N and 7 for the first PCR and primer 60N and 8 for the second PCR. Oligonucleotide 60N codes for the NH_2-terminal nine amino acid residues of δ-COP. The obtained clones all revealed the sequence from nucleotide 58 on, but not clearly the NH_2 terminus, which was finally found with another RACE reaction with Amplifinder 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 (TCGCGAAGCTTCTACAGAATTTCATACTTATCCACTAGGA) containing a HindIII site and subsequent PCR with this primer and the NH_2-terminal primer (GCGCGGATCCTGGTGCTGTTGGCAGCAGCAGTCTGTA) 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 (1053–1076), 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 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 GTACTTTACTATGTGGGTACGTATTTAAGGTGGCA for forward and TTCCAGGTCGAATACTACTTGCACACAGGCG for reverse priming. The obtained 2,250-bp fragment containing the δ-COP gene and 224-bp downstream and 387-bp upstream was digested with ApaI and subcloned into the SmaI/ApaI sites of pBluescript-SK$^+$ (Statagene) vector. An internal 1396-bp NdeI/BglII fragment of the δ-COP gene was removed from the pBluescript–δ-COP plasmid and replaced by the HIS3 gene (1.2 kb) after filling in the restricted ends. 20 μg of the resulting plasmid was digested with BamHI and ApaI and transformed in *S. cerevisiae* strain RS453 by the lithiumacetate method (Gietz and Schiestl, 1991). The HIS$^+$ transformants 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 on nitrocellulose. A random-primed [^{32}P]dCTP-labeled DNA probe obtained from a BglII/ApaI 421-bp 5′-end fragment 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 separate mutant and wildtype alleles (Rose et al., 1990).

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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 7l LB Kana/Amp and was grown at 37°C to an OD_{600} of 1.6. This culture was then induced with 15 μM IPTG (isopropyl-β-thiogalactopyranoside, Gerbu Biotechnik GmbH, Gaiberg, 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).

Breakage of cells was done in a French press three times. Unsoluble protein was separated by centrifugation of the cell lysates at 150,000 *g* for 30 min at 4°C. The supernatants were dialyzed against 50 mM Hepes, pH 7.2, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM PMSF, and 2 mM benzamidine. Further purification was performed using a Ni^{2+}-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 mg/l, L-lysine-HCl 30 mg/l, and (if selection was not desired) L-histidine-HCl 20 mg/l. After growing for 3–4 d, 8–10 transformants were replica plated both on SD+His and SD−His + 25 mM 3-amino-1,2,4-triazole (3-AT; Sigma Immunochemicals, St. Louis, MO) and monitored for β-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 OD_{600} of about 2. The cells were centrifuged (5 min 500 *g*, 4°C), and the pellet was resuspended in 1 vol of water, resuspended in 500 μl buffer B(400) supplemented with 1 mM PMSF and 2 mM benzamidine, frozen/thawed in liquid nitrogen, and finally broken with glassbeads (size 0.5 mm) in a mini bead beater (three times for 90 s, 4°C). Buffer B(400) is 50 mM Tris-Cl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 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 (Guarente, 1983). The OD_{405} was measured at at least four time points, and the β-galactosidase activity was determined as follows: $1\ U = \Delta OD_{405} \times 1{,}000/\text{mg protein} \times \text{time (min)}$. 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 OD_{600} of 0.5–1.0. Fresh medium (100 ml) was inoculated to an OD_{600} of 0.01, and the OD_{600} was determined every 4–6 h. Generation times were measured in the range of OD_{600} 0.1–0.8.

Antibodies

Antisera against a synthetic internal peptide of bovine δ-COP, RPSGPSKALKLGAKGKE (amino acid residues 216–232), 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 δ-COP antibody was performed using the peptide coupled to epoxy-activated Sepharose 6B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). For immumoprecipitations, antisera against α- and δ-COPs were used, as well as anti-β′-COP-antiserum (Harter et al., 1993) and anti-ε-COP-antiserum (Hara-Kuge et al., 1994).

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 cryoultramicrotomy 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, 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 ζ-COP. The primers were designed to give a NcoI/SmaI product for β-COP, a SmaI/BamHI product for β′-COP and NcoI/BamHI products for γ-COP, δ-COP, ε-COP, and ζ-COP. Template for the PCR of δ-COP was the cDNA inserted in pQE32 (Qiagen), the same vector that was used for sequencing and performing the overexpression of the (6xHis)-tagged protein. 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). The γ-COP cDNA is described (Harter et al., 1996). The cDNA of ε-COP in bluescript pKS+ (Stratagene) (Hara-Kuge et al., 1994) and ζ-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/SalI fragment obtained from the full-length clone after subcloning in pQE30 (Qiagen) and M13mp18 (Promega) into the EcoRI/XhoI 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 phage isolation and in vivo excision following the manuals of the distributor.

In Vitro Translation

mRNAs were in vitro transcribed from the cDNA of δ-COP inserted in pGEMT (Promega) and of β-COP (Duden et al., 1991) in pGEM4Z (Promega), of α-COP (this work, inserted in pbluescript SK), and ε-COP in pBluescriptSK (Hara-Kuge et al., 1994). Transcriptions were performed with T3 RNA polymerase (Promega; for β- and δ-COPs) or with 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 U/μl) and dithiothreitol (DTT, final concentration 1 mM) were added to all reactions. ^{35}S-labeled L-methionine (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.

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 two vol IP buffer. 300 μl of this suspension (100 μl beads) was incubated with 50 μl antiserum or preimmune serum for 8 h, 8°C with agitation. 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 assay 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.

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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 λgt10 cDNA library from bovine brain was screened. A COOH-terminal 594-bp 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 NH_2 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 yeast (Murakami et al., 1995) that shows 33% identity and 56% similarity to bovine δ-COP. NH_2-terminal Edman degradation of a candidate δ-COP homologue isolated from yeast coatomer revealed the same NH_2-terminal amino acid sequence as deduced from the yeast DNA sequence (Fig. 1, *underlined*). Fig. 1 shows the complete cDNA-derived amino acid sequence of bovine δ-COP and the alignment with its yeast homologue.

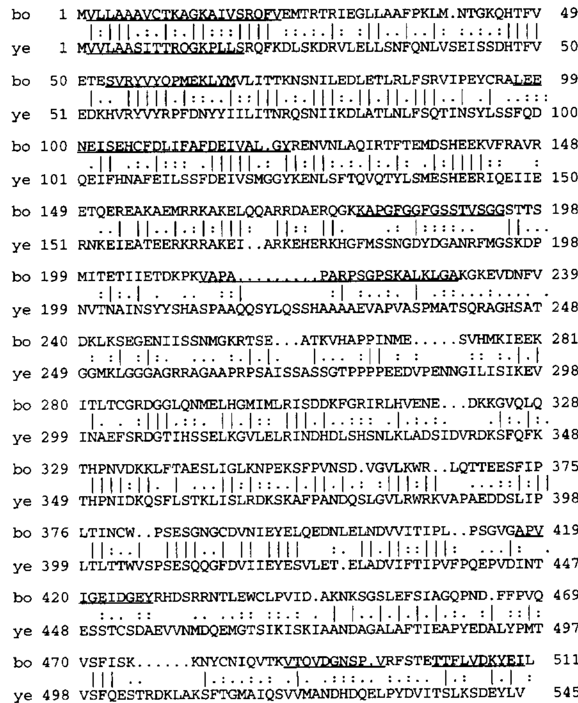

Figure 1. cDNA-deduced amino acid sequence of bovine δ-COP and alignment with a sequence deduced from a homlogous yeast open reading frame. Sequences determined by Edman degradation are underlined. The sequence data are available from GenBank/EMBL/DDBJ under accession number X94265.

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 NH_2-terminal region: for AP50, 24% identity and 51% similarity, and for AP47, 22% identity and 52% similarity were found within the NH_2-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 coatomer: δ-COP was synthesized in *E. coli* using an inducible expression system in which a 6xHis affinity tag is fused to the NH_2 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., 1991*a*; 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.

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

Homologue	Species	M_r	Identity	Similarity	Features
		$\times 10^3$	%	%	
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.

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 ^{32}P-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

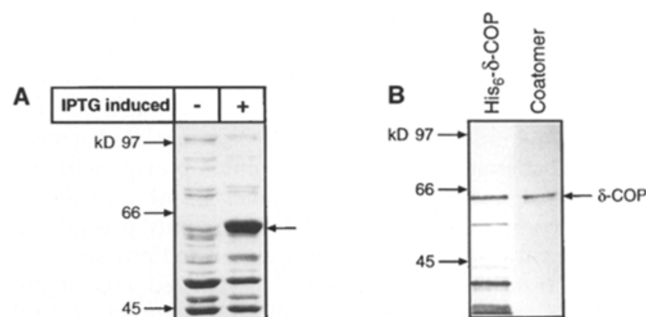

Figure 2. Overexpression of (6xHis)-tagged δ-COP. (*A*) Coomassie staining of equal amounts induced and uninduced lysates of *E. coli* separated on an SDS gel. (*B*) Immunostaining of an induced *E. coli* lysate with anti–δ-COP antibody (sample equivalent to 60 μl of the culture). In the right panel, ~0.3 μg of coatomer isolated from bovine brain was analyzed as a standard for δ-COP.

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/ζ-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

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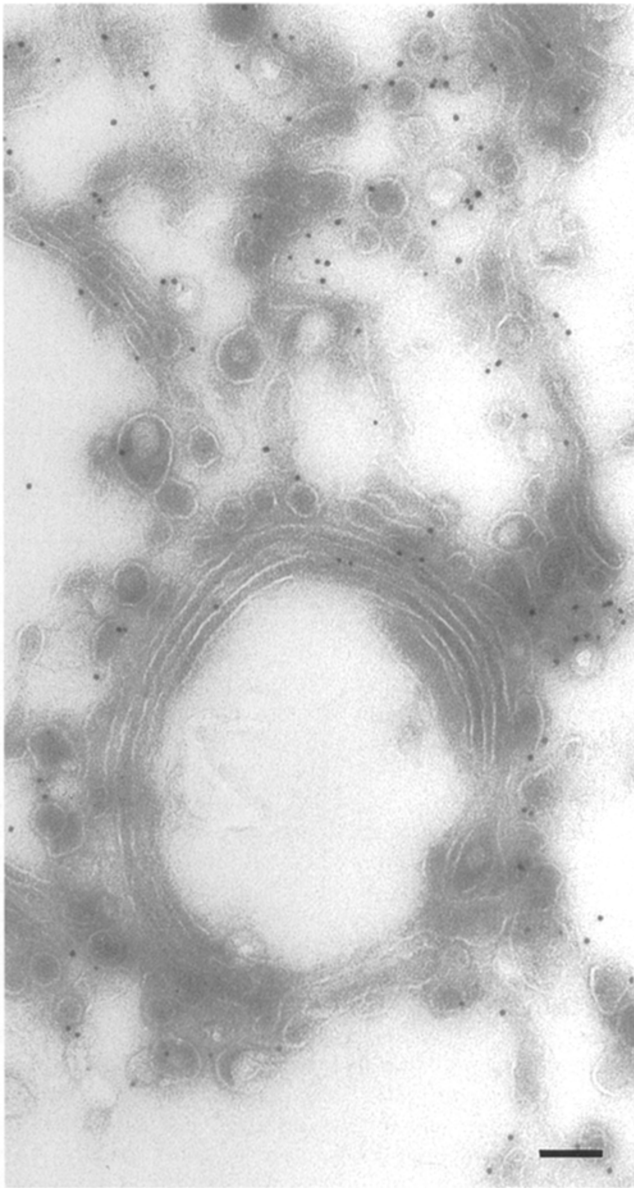

Figure 3. Immunoelectron microscopic localization of δ-COP antibody on Golgi-derived buds and transport vesicles generated in presence of GTPγS from rat liver membranes. Affinity-purified anti–δ-COP antibody revealed by the protein A–gold technique. Size of gold particles 10 nm. A comparable labeling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

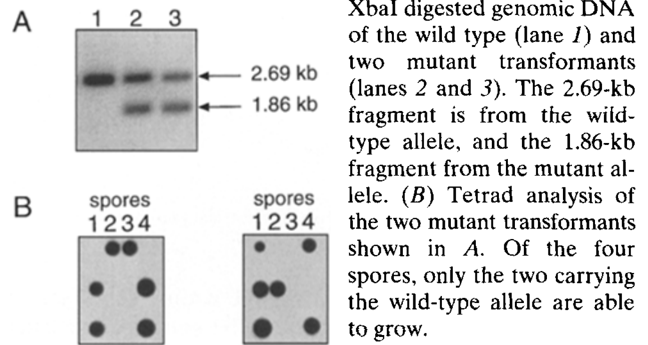

Figure 4. Analysis of δ-COP gene disruption. (*A*) Southern blot analysis of the HindIII/XbaI digested genomic DNA of the wild type (lane *1*) and two mutant transformants (lanes *2* and *3*). The 2.69-kb fragment is from the wild-type allele, and the 1.86-kb fragment from the mutant allele. (*B*) Tetrad 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.

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 coatomer), 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 hydrolize 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 that would indicate any COP interactions with this motif (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 coatomer 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-

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bovine 1  ..MLTKFETKSARVKGLSFHPKRPWILTSLHNGVIQLWDYRMCTLIDKFD 48
yeast  1  MKMLTKFESKSTRAKGIAFHPSRPWVLVALFSSTIQLWDYRMGTLLHRFE 50

bo    49  EHDGPVRGIDFHKQQPLFVSGGDDYKIKVWNYKLRRCLFTLLGHLDYIRT 98
ye    51  DHEGPVRGLDFHPTQPIFVSAGDDYTIKVWSLDTNKCLYTLTGHLDYVRT 100

bo    99  TFFHHEYPWILSASDDQTIRVWNWQSRTCVCVLTGHNHYVMCAQFHPSED 148
ye   101  VFFHRELPWIISASDDQTIRIWNWQNRKEIACLTGHNHFVMCAQFHPTDD 150

bo   149  LVVSASLDQTVRVWDISGLRKKNLSPGAVESDVRGITGVDLFGT..TDAV 196
ye   151  LIVSASLDETIRIWDISGLRKRHSAPGTSSFEEQMSAQQNLLDGSLGDCV 200

bo   197  VKHVLEGHDRGVNWAAFHPTMPLIVSGADDRQVKIWRMNESKAWEVDTCR 246
ye   201  VKFILEGHTRGVNWASFHPTLPLIVSGSDDRQVKLWRMSATKAWEVDTCR 250

bo   247  GHYNNVSCAVFHPRQELILSNSEDKSIRVWDMSKRTGVQTFRRDHDRFWV 296
ye   251  GHTNNVDSVIFHPHQNLIISVGEDKTLRVWDLDKRTPVKQFKRENDRFWL 300

bo   297  LAAHPNLNLFAAGHDGGMIVFKLERERPAYAVHGNMLHYVKDRFLRQLDF 346
ye   301  IAAHPHINLFGAAHDSGIMVFKLDRERPCSFIHQNQLFFVNAEKQIQ.SF 349

bo   347  NSSKDVAVMQLRS....GSKFPVF.SMSYNPAENAVLLCTRASNLENSTY 391
ye   350  NFQKRVASLPYASLKGIGQPWDAFRSISYNPSQHSVLV.....NEANGKF 394

bo   392  DLYTIPKDADTQNPDAPEGKRSSGLTAVWVARNRFAVLDR.MHSLLIKNL 440
ye   395  ALVILPKQP.VGAVEPTSVTQDTGNFATFVGRNRFVVYNKNTESVEVRSL 443

bo   441  KNEITKKVQVPN.CDEIFYAGTGNLLLRDAESITLFDVQQKRTLASVKIS 489
ye   444  ENKVTRNIKVEETVRTIVAAGPGSVLVIHPREVILYDVQQGKKVSQLAVK 493

bo   490  KVKYVIWSADMSHVALLAKHAIVICNRKLEALCNIHENIRVKSGAWDESG 539
ye   494  NVKYVSWSLDGQYVALMSKHTITLATKKLELINSMHETIRIKSAAWDESG 543

bo   540  VFIYTTSNHIKYAVTTGDYGIIRTLDLPIYVTRVKGNNVYCLDRECRPRV 589
ye   544  VLIYSTLNHIRYSLLNGDRGIIKTLEKTLYITKVQGKLVYCLNREGEIEI 593

bo   590  LTIDPTEFKFKLALINRKYDEVLHMVRNAKLVGQSIIAYLQKKGYPEVAL 639
ye   594  LTIDPTEYRFKKALVNKNFPEVLRLIKDSNLVGQNIISYLQKSGYPEIAL 643

bo   640  HFVKDEKTRFSLALECGNIEIALEAAKALDDKNCWEKLGEVALLQGNHQI 689
ye   644  QFVQDPHIRFDLALEYGNLDVALDEAKKLNDSSTWERLIQEALAQGNASL 693

bo   690  VEMCYQRTKNFDKLSFLYLITGNLEKLRKMMKIAEIRKDMSGHYQNALYL 739
ye   694  AEMIYQTQHSFDKLSFLYLVTGDVNKLSKMQNIAQTREDFGSMLLNTFYN 743

bo   740  GDVSERVRILKNCGQKSLAYLTAATHGLDEEAESLKE..TFDPEKETIPD 787
ye   744  NSTKERSSILAEGGSLPLAYAVAKANGDEAAASAFLEQAEVDEQDVTLPD 793

bo   788  IDPNAKLLQPPAPIMPLDTNWPLLTVSKGFFEGSIASKGKGGALAADIDI 837
ye   794  QMDASNFVQRPVISKPLE.KWPLKEAELSYFEKAVL..GQIDDLTIDDET 840

bo   838  DTVGTEGWGEDAELQLDEDGFVEATEGLGDDALGKGQEEGGGWDVEEDLE 887
ye   841  PAVNT...TQEQEEPLGEENFNDEDIGEDEGAWDLGDED...LDVGEELP 884

bo   888  LPPELDIPPGAAGGAEDGFFVPPTKGTSPTQIWCNNSQLPVDHILAGSFE 937
ye   885  EEVEQGEITSPAQEVE.............TAIWTKNSKLPAVLVAAGAFD 921

bo   938  TAMRLLHDQVGVTQFGPYKQLFLQTYARGRTTYQALPC.LPSMYGYPNRN 986
ye   922  AAVQALSKQVGVVKLEPLKKYFTNIYEGCRTYIPSTPCELPAQLGYV.RA 970

bo   987  WKDAGLKNGVPAVGLKLNDLIQRLQLCYQLTTVGKFEEAVEKFRSILLSV 1036
ye   971  YDDTVSEDQILPYVPGLDVVNEKMNEGYKNFKLNKLDIAIECFREAIYRI 1020

bo  1037  PLLVVDNKQEIAEAQQLITICREYIVGLSMETERKKLPKETLEQQKRICE 1086
ye  1021  TLLMVDNTEDEKLAHKILETAREYILGLSIELERRSLKE...GNTVRMLE 1067

bo  1087  MAAYFTHSNLQPVHMILVLRTALNLFFKLKNFRTAAAFARRLLELGPKPE 1136
ye  1068  LAAYFTKAKLSPIHRTNALQVAMSQHFKHKNFLQASYFAGEFLKIISSGP 1117

bo  1137  VAQQTRKILSACEKNPTDAYQLNYDMHNPFDICAASYRPIYRGKPVEKCP 1186
ye  1118  RAEQARKIKNKADSMASDAIPIDFDPYAKFDICAATYKPIYEDTPSVSDP 1167

bo  1187  LSGACYSPEFKGQICKVTTVTEIGKDVIGLRISPLQFR 1224
ye  1168  LTGSKYVITEKDKIDRIAMISKIGAPASGLRIRV.... 1201
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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/DDBJ 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
α-COP	β'-COP	s.t.	2.11
—	—	6	0.12
α-COP	—	< 5	< 0.1
β-COP	—	14	1.92
β'-COP	—	< 5	< 0.1
γ-COP	—	12	< 0.1
δ-COP	—	< 5	< 0.1
ε-COP	—	< 5	< 0.1
ζ-COP	—	62	1.68
—	α-COP	< 5	< 0.1
—	β-COP	10	< 0.1
—	β'-COP	< 5	< 0.1
—	γ-COP	9	0.24
—	δ-COP	6	< 0.1
—	ε-COP	< 5	< 0.1
—	ζ-COP	15	< 0.1
SNF1	SNF4	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 ΔOD405/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.

bodies used for immunoprecipitation. The same holds for the combination of β-COP with γ-COP (not shown).

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 research has revealed an open reading frame in the human genome (ARCN1 gene) as a homolog δ-COP and two proteins that show a faint homology to bovine δ-COP: murine AP 47, a subunit of the Golgi-derived clathrin adaptor complex AP1, which is believed to have kinase activity (Nakayama et al., 1991) and AP50, a subunit of the plasma membrane–derived clathrin adaptor complex AP2, from *S. pombe* (ac-

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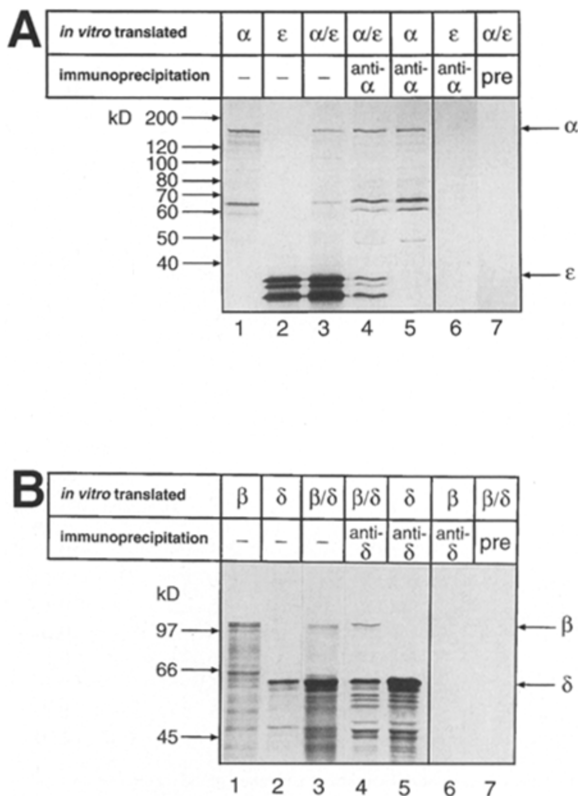

Figure 6. Immunoprecipitation of binary coatomer subcomplexes. (*A*) Immunoprecipitation of in vitro cotranslated α- and ϵ-COPs. ^{35}S-Met–labeled samples were separated by SDS–gel electrophoresis and proteins visualized by autoradiography. The first three lanes show in vitro translated α- (lane *1*) and ϵ-COP (lane *2*) and a cotranslated sample (lane *3*). Lanes *4–6* show immunoprecipitations with an anti–α-COP antibody as indicated in the individual lane headings: precipitation from a cotranslated sample (lane *4*), of in vitro translated α-COP (lane *5*), and, as a control, of in vitro translated ϵ-COP (lane *6*); control precipitation with preimmune serum from a cotranslated sample (lane *7*). (*B*) Immunoprecipitation of in vitro translated β- and δ-COPs. In vitro translated β- (lane *1*) and δ-COP (lane *2*) and a cotranslated sample (lane *3*); precipitation from a cotranslated sample (lane *4*), of in vitro translated δ-COP (lane *5*), and of β-COP (lane *6*); control precipitation with preimmune serum from a cotranslated sample (lane *7*).

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 NH_2-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, 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 His_6-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 synthezised 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 posttranslational 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 pACTII, 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-

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vided by Dr. T. Kreis. The ϵ- 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.

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