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Non-clathrin-coat protein α is a conserved subunit of coatomer and in *Saccharomyces cerevisiae* is essential for growth

(secretory pathway/coat protein-coated vesicles/Golgi membranes/WD-40 repeat)

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ABSTRACT **To complete the molecular characterization of coatomer, the preformed cytosolic complex that is involved in the formation of biosynthetic transport vesicles, we have cloned and characterized the gene for non-clathrin-coat protein α (α-COP) from *Saccharomyces cerevisiae*. The derived protein, molecular weight of 135,500, contains four WD-40 repeated motifs (Trp/Asp-containing motifs of ≈40 amino acids). Disruption of the yeast α-COP gene is lethal. Comparison of the DNA-derived primary structure with peptides from bovine α-COP shows a striking homology. α-COP is localized to coated transport vesicles and coated buds of Golgi membranes derived from CHO cells.**

Transport of newly synthesized proteins from the endoplasmic reticulum (ER) via the Golgi apparatus to the plasma membrane occurs in vesicles (1, 2). In mammalian cells, budding of a transport vesicle involves the recruitment of a protein coat structure from the cytosol consisting of ADP-ribosylation factor (ARF) and coatomer, a preformed complex of seven non-clathrin-coat proteins (COPs; α-, β-, β′-, γ-, δ-, ε-, and ζ-COPs) (3, 4). ARF is a small GTP-binding protein that docks to Golgi membranes upon nucleotide exchange of ARF-bound GDP for GTP. Only after ARF-GTP is bound are these membranes able to recruit coatomer from the cytosol (5, 6). This binding of coatomer is concommitant with the formation of buds within these membranes, strongly suggesting that it is the coatomer coat that shapes a forming vesicle (7, 8). Of the seven COPs that make up the coatomer complex, six are known at a molecular level (refs. 9–14 and S. Auerbach, D. Faulstich, H. T., and F. W., unpublished data).

In yeast, two sets of proteins have been identified that are involved in vesicular transport. One is yeast coatomer with a protein composition similar to its mammalian homologue (15, 16). Three secretion mutants, SEC21, SEC26, and SEC27, of *Saccharomyces cerevisiae* have led to the identification of proteins that represent homologues of the mammalian coatomer subunits γ-COP, β-COP, and β′-COP (16, 17). The second is COP II, another coat structure composed of proteins that are recruited from the cytosol—Sec13p, Sec31p, Sec23p, Sec24p, and Sar1p (18–21). *In vivo* and *in vitro* studies have demonstrated that COP II is a coat structure that covers ER-derived transport vesicles. The secretion mutant homologues of mammalian γ-, β-, and β′-COP show ER phenotypes, implicating that coatomer is also involved in ER budding. However, differences in the function of coatomer vs. COP II coat are unknown at present. To ultimately discriminate between the functions of these two sets of proteins, the knowledge of the molecular structure of each subunit involved is essential. To this end, we have completed the molecular characterization of the COPs and characterize here the DNA-derived primary structure of α-COP from *S. cerevisiae*.§

MATERIALS AND METHODS

Yeast Strains and Media. *S. cerevisiae* strains used in this study were as follows: BJ926 (wild type) for the isolation of coatomer and RS453 (*MAT***a**/α, *his3/his3, leu2/leu2, trp1/trp1, ura3/ura3, ade2/ade2*) for gene disruption and PCR experiments. Yeast was grown in YPD medium [1% Bacto yeast extract/2% (wt/vol) Bacto Peptone/2% (wt/vol) glucose] at 30°C. For selection of *HIS3* transformants, SD medium was used containing 0.7% nitrogen base, 2% glucose, and the appropriate amino acids.

Isolation of Coatomer and COP-Coated Vesicles. Mammalian coatomer was purified from bovine brain by the procedure of Waters *et al.* (4); yeast coatomer was isolated as described by Hosobuchi *et al.* (16). Golgi-derived transport vesicles and tryptic peptides were generated as described (22).

Preparation of Antisera. Anti-α-COP antibodies were produced by immunizing rabbits with a synthetic peptide corresponding to the N-terminal sequence of α-COP from bovine brain (MLTKFETKSL) that was coupled to a branched polylysine core (23). Antisera were affinity-purified by using the antigenic polypeptide coupled to CNBr-activated Sepharose 6B (Pharmacia).

DNA Cloning and Sequence Analysis. DNA isolation from transformed bacteria and recombinant DNA manipulation were performed by standard procedures (24). The N-terminal amino acid sequence information was used to construct two completely degenerated oligonucleotides. The oligonucleotide 5′-ATGAA(GA)ATG(CT)T(GACT)AC(GACT)AA(GA)-TT-3′, corresponding to amino acid residues 1–7, was used as a forward primer, and the oligonucleotide 5′-GG(GA)TG-(GA)AA(GACT)GC(GAT)AT(GACT)CC(CT)TT-3′, corresponding to amino acids 21–15, was used as a reversed primer for a two-stage PCR with 0.5–1.0 μg of genomic DNA from yeast strain RS453. *Taq* DNA polymerase from Perkin–Elmer was used for PCR. A PCR product of the expected size (62 bp) was subcloned and sequenced by using Sequenase (United States Biochemical). A 32-bp oligonucleotide, 5′-ACGAAA-TTTGAATCAAAGTCCACTAGGGCCAA-3′, derived from the PCR product was end-labeled with [γ-^{32}P]ATP (Amersham) and used to screen a λ DASH II library of *S. cerevisiae* genomic sequences. Two positive clones were isolated, subcloned into pUC18 (GIBCO), and sequenced.

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Abbreviations: ARF, ADP-ribosylation factor; ER, endoplasmic reticulum; GTP[γS], guanosine 5′-[γ-thio]triphosphate; COP, non-clathrin-coat protein.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X83754).

***Escherichia coli* Expression of α-COP.** A full-length α-COP gene with introduced *Nco* I and *Bam*HI sites was constructed in pBluescript SK(+) (Stratagene): An internal *Hin*dIII–*Sph* I fragment of the α-COP gene was ligated in a pBluescript SK(+) vector. At the 5′ end, a 725-bp *Nco* I–*Hin*dIII fragment was generated by PCR with primer 1 (5′-TGCCGACCATGGGAATGAAGATGTTAACTAAATTTGAATCA-3′, including the *Nco* I site) and primer 2 (5′-CTATCAACATTGTTAGTGTGACCTC-3′, an internal oligonucleotide corresponding to amino acids 258–250). At the 3′ end, a 406-bp *Sph* I–*Bam*HI fragment was generated with primer 3 (5′-GGCGAAATTACCTCTCCAGCACAGGA-3′, an internal oligonucleotide corresponding to amino acids 891–898) and primer 4 (5′-AGATCTGGATCCTACACGTATTCTTAATCCGGATGCA-3′, including the *Bam*HI site and without termination codon). The two fragments were ligated to the *Hin*dIII–*Sph* I fragment. The resulting α-COP gene was digested with *Nco* I and *Bam*HI, cloned into pQE-60, an expression vector (Diagen, Hilden, Germany), and transformed into *E. coli* (M15 harboring plasmid pREP4). Transformants were grown to an OD_{600} of 1 in selection medium (1% Bacto tryptone/0.5% yeast extract/1% NaCl with ampicillin at 100 μg/ml and kanamycin at 25 μg/ml) at 37°C after induction with 30 μM isopropyl β-D-thio-galactoside for 3 h. α-COP-expressing cells were harvested by centrifugation and directly resuspended in SDS sample buffer. Proteins were separated on a SDS/7.5% polyacrylamide gel under reducing conditions and analyzed for expression of α-COP on an immunoblot.

Disruption of the α-COP Gene. A DNA fragment containing full-length α-COP and additional flanking regions was constructed. A 3521-bp *Hin*dIII–*Sal* I fragment containing the C terminus of the α-COP gene and 682 bp downstream was subcloned in pBluescript SK(+) vector. A 1054 bp *Sac* II–*Hin*dIII fragment derived from a PCR product was ligated to this construct. The *Sac* II restriction site was introduced by the appropriate primer. For the PCR, the following primers were used: primer 5 (5′-TTGGTTCCGCGGATCCGTCTTCGAGACTGTGACGAT-3′) and primer 2 as described above.

An internal 2.8-kb *Xba* I–*Sph* I fragment of the α-COP gene was removed from the pBluescript-α-COP plasmid and replaced by the *HIS3* gene (1.2 kb). The resulting plasmid (20 μg) was digested with *Sac* II and *Sal* I and transformed in *S. cerevisiae* RS453 by the lithium acetate method (25). The *HIS*$^+$ transformants were isolated and analyzed on a Southern blot: 2 μg of genomic wild-type or mutated DNA was digested with *Hin*dIII, separated by agarose gel electrophoresis, and blotted onto nitrocellulose. A random-primed [^{32}P]dCTP-labeled DNA probe obtained from a *Sph* I–*Bam*HI 430-bp 3′-end fragment of the α-COP gene was used for hybridization. Transformants were sporulated on YPA plates (1% Bacto yeast extract/2% Bacto Peptone/1% potassium acetate) and tetrad analysis on YPD plates was performed to separate mutant and wild-type alleles (26).

Immunocytochemistry. Immunoelectron microscopic localization of α-COP was performed on CHO Golgi membranes exposed to bovine brain cytosol in the presence of guanosine 5′-[γ-thio]triphosphate (GTP[γS]) (22). The preparation was fixed with 0.1% glutaraldehyde and cryosectioned as described (27). The protein A-gold method was used to reveal the antibody on sections (28). The affinity-purified anti-α-COP antibody was applied at a concentration of 150 μg/ml for 3 h and then the protein A-gold solution diluted 1:100 was applied. As a control, sections were incubated with preimmune serum (diluted 1:2) under the same conditions as the anti-α-COP antibody.

SDS/PAGE, Immunoblot Analysis, and Protein Sequencing. Proteins were separated on SDS/7.5% polyacrylamide gels with an acrylamide/*N,N′*-methylenebisacrylamide ratio of 100:1 (wt/wt) under reducing conditions. Western blot analysis and protein sequencing were performed as described (15). α-COP was detected by chemiluminescence (ECL; Amersham) by using affinity-purified anti-α-COP antibody at a concentration of 0.5 μg/ml and peroxidase-conjugated anti-rabbit IgG (diluted 1:5000).

RESULTS

DNA-Derived Structure of α-COP from *S. cerevisiae*. By following the procedure for the isolation of mammalian coatomer, a similar complex was isolated from yeast cytosol, essentially as described by Waters *et al.* (4) and Hosobuchi *et al.* (16). The protein of highest apparent molecular mass (≈160 kDa) closely related to the apparent molecular mass of mammalian α-COP was sequenced N-terminally, yielding the following peptide sequence: MKMLTKFESKSTRAKGIAFHPSRP. This information was used to design two completely degenerated oligonucleotides that cover the N-terminal part (amino acids 1–7) and the C-terminal part (amino acids 15–21) of the peptide described above, leaving a central gap of 7 amino acids. These oligonucleotides were used for the PCR with genomic DNA from yeast strain RS453 as a template. A PCR product of the expected size (62 bp) was subcloned and sequenced. The nucleotide sequence between the two primers coded for the correct amino acids ESKSTRA in positions 8–14. A probe corresponding to the codons for amino acids 5–15 of the PCR product was used to screen a genomic yeast library. Four related clones were obtained; the DNA was subcloned and sequenced, revealing an open reading frame of 3603 bp (Fig. 1). The gene for yeast α-COP is bounded at its 5′ end by an open reading frame corresponding to the cytosolic chaperonin TCP1 (data not shown), formerly localized to yeast chromosome 9 (29). It codes for a protein of 1201 amino acids corresponding to a molecular weight of 135,591 and a calculated pI value of 5.93. The complete DNA-derived amino acid sequence is shown in Fig. 2*A*. In Fig. 2*B*, peptide sequences of the N terminus and three internal peptides of bovine brain α-COP are compared with its yeast homologue. The mammalian sequences were obtained from α-COP isolated from the cytosolic coatomer and from purified Golgi-derived transport vesicles. Identity between single peptides (8–21 amino acids long) of yeast and mammalian α-COP is in the range of 80–44%; when conserved amino acids are taken into consideration, similarity ranges from 90 to 67%. The average identity and homology of the peptides compared (50 amino acid residues) are 60 and 84%, respectively. Data-base searches for homologies with the program TFASTA revealed the presence of four WD-40 (Trp/Asp-containing motifs of ≈40 amino acids) repeated motifs that are localized in the N-terminal part of α-COP. The alignment of these motifs found in yeast α-COP

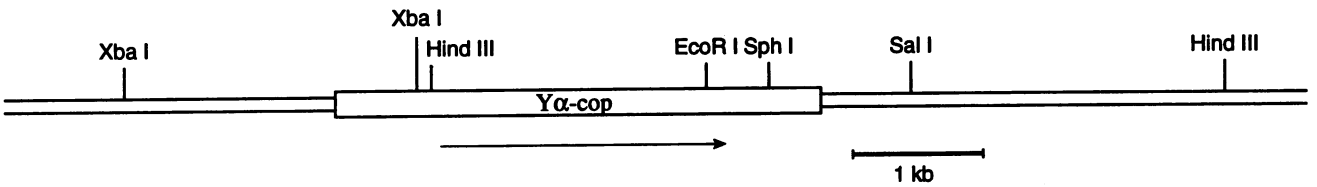

FIG. 1. Restriction enzyme map of the chromosomal region containing the α-COP gene. The arrow shows the direction of transcription.

A

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   1 MKMLTKFESK STRAKGIAFH PSRPWVLVAL FSSTIQLWDY RMGTLLHRFE
  51 DHEGPVRGLD FHPTQPIFVS AGDDYTIKVW SLDTNKCLYT LTGHLDYVRT
 101 VFFHRELPWI ISASDDQTIR IWNWQNRKEI ACLTGHNHFV MCAQFHPTDD
 151 LIVSASLDET IRIWDISGLR KRHSAPGTSS FEEQMSAQQN LLDGSLGDCV
 201 VKFILEGHTR GVNWASFHPT LPLIVSGSDD RQVKLWRMSA TKAWEVDTCR
 251 GHTNNVDSVI FHPHQNLIIS VGEDKTLRVW DLDKRTPVKQ FKRENDRFWL
 301 IAAHPHINLF GAAHDSGIMV FKLDRERPCS FIHQNQLFFV NAEKQIQSFN
 351 FQKRVASLPY ASLKGIGQPW DAFRSISYNP SQHSVLVNEA NGKFALVILP
 401 KQPVGAVEPT SVTQDTGNFA TFVGRNRFVV YNKNTESVEV RSLENKVTRN
 451 IKVEETVRTI VAAGPGSVLV IHPREVILYD VQQGKKVSQL AVKNVKYVSW
 501 SLDGQYVALM SKHTITLATK KLELINSMHE TIRIKSAAWD ETGVLIYSTL
 551 NHIRYSLLNG DRGIIKTLEK TLYITKVQGK LVYCLNREGE IEILTIDPTE
 601 YRFKKALVNK NFPEVLRLIK DSNLVGQNII SYLQKSGYPE IALQFVQDPH
 651 IRFDLALEYG NLDVALDEAK KLNDSSTWER LIQEALAQGN ASLAEMIYQT
 701 QHSFDKLSFL YLVTGDVNKL SKMQNIAQTR EDFGSMLLNT FYNNSTKERS
 751 SILAEGGSLP LAYAVAKANG DEAAASAFLE QAEVDEQDVT LPDQMDASNF
 801 VQRPVISKPL EKWPLKEAEL SYFEKAVLGQ IDDLTIDDET PAVNTTQEQE
 851 EPLGEENFND EDIGEDEGAW DLGDEDLDVG EELPEEVEQG EITSPAQEVE
 901 TAIWTKNSKL PAVLVAAGAF DAAVQALSKQ VGVVKLEPLK KYFTNIYEGC
 951 RTYIPSTPCE LPAQLGYVRA YDDTVSEDQI LPYVPGLDVV NEKMNEGYKN
1001 FKLNKLDIAI ECFREAIYRI TLLMVDNTED EKLAHKILET AREYILGLSI
1051 ELERRSLKEG NTVRMLELAA YFTKAKLSPI HRTNALQVAM SQHFKHKNFL
1101 QASYFAGEFL KIISSGPRAE QARKIKNKAD SMASDAIPID FDPYAKFDIC
1151 AATYKPIYED TPSVSDPLTG SKYVITEKDK IDRIAMISKI GAPASGLRIR
1201 V
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B

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3MLTKFESKST12          S.cerevisiae
 ||||||·||
 MLTKFETKSL            bovine brain

163IWDISGLR170
 :·||||||
 VSDISGLR

300LIAAHPHINLF310
 ::||||::|||
 VLAAHPNLNLF

861EDIGEDEGAWDLGDEDLDVGE881
 :· || :·|: ||    ||||
 DTSGEYQNALYLG····DVGE
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C

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                 A          D    V               V  V R
        V        V          N    I S A S         I  I N
xx GH xxx L xx L x Fx P xx P x x L G G G x D xx L x LWD x

LT GH LDY V RT V F FH R EL P W I I S A S D D QT I R IWN W   (91-124)
LT GH NHF V MC A Q FH P TD D L I V S A S L D ET I R IWD I   (133-166)
LE GH TRG V NW A S FH P TL P L I V S G S D D RQ V K LWR M   (205-238)
CR GH TNN V DS V I FH P HQ N L I I S V G E D KT L R VWD L   (249-282)
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FIG. 2. DNA-derived amino acid sequence of α-COP. (*A*) Full-length sequence of α-COP from *S. cerevisiae*. (*B*) Sequence alignment of peptides derived from yeast and bovine α-COP. Identities are indicated by vertical bars and conservative replacements are indicated by colons and dots. (*C*) Sequence alignment of four repeated motifs of α-COP to a consensus sequence of the WD-40 repeated motif (30). The positions of the motifs within the amino acid sequence of α-COP are given in parentheses.

with the consensus derived from a number of proteins related to the β-subunit of trimeric G proteins (30) is depicted in Fig. 2*C*.

Immunological Characterization of α-COP. The striking homology between the N terminus of yeast and mammalian α-COPs prompted us to probe for a cross reaction of the yeast gene product with an antibody raised against a synthetic N-terminal peptide of α-COP from mammals. Immunological analysis on Western blots revealed that the antibody indeed cross reacts with a protein component of the yeast coatomer (Fig. 3, lane 2) and with the yeast gene product expressed in *E. coli* (Fig. 3, lane 1). The bacterially expressed protein shows an apparent molecular mass of 160 kDa, confirming that the yeast gene characterized contains the full-length information for α-COP.

Disruption of the α-COP Gene in *S. cerevisiae*. To test whether α-COP gene function is essential for cell viability, a gene disruption experiment was performed. To this end, a 2.58-kb fragment of the coding sequence of α-COP was replaced by the *HIS3* gene (Fig. 4*A*). 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*B*). Seven replacement clones were sporulated, and 10 tetrads of each were analyzed. In Fig. 4*C*, a tetrad analysis is depicted from one of these transformants: for all tetrads, a 2:2 viable/nonviable pattern was obtained. This result shows that α-COP is essential for growth.

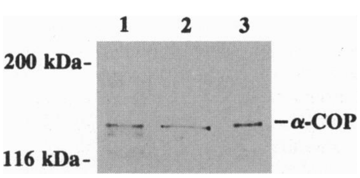

FIG. 3. Immunoblot analysis of α-COP. Lanes: 1, bacterially expressed α-COP; 2, α-COP from yeast coatomer; 3, α-COP from bovine brain coatomer.

Immunolocalization of α-COP in CHO Cells. The antipeptide antibody was used to localize α-COP at the ultrastructural level. For immunoelectron microscopy of Golgi membranes and Golgi-derived transport vesicles, CHO Golgi membranes were "primed" *in vitro* in the presence of the nonhydrolyzable analogue of GTP, GTP[γS], to generate coated transport vesicles as described (22, 31). Immunolocalization was performed on ultrathin cryosections by using affinity-purified antibody and protein A-gold to label bound antibodies. Immunogold particles were predominantly found in association with vesicles and buds (Fig. 5*a*). Quantification of gold particles revealed that 87 ± 2% were associated with vesicles and buds and 13 ± 2% were associated with Golgi cisternae. Incubation of the vesicle preparation with preimmune serum resulted in no labeling of vesicular structures (Fig. 5*b*).

DISCUSSION

Mutations in all genes identified for yeast coatomer subunits (Sec21p/γ-COP, Sec26p/β-COP, and Sec27p/β′-COP) lead to phenotypes defective in ER-to-Golgi transport (16, 17). To our knowledge, no secretion mutant for α-COP has been identified so far. We have isolated a gene coding for the α subunit of yeast

coatomer involved in vesicular transport, based on the following data: (*i*) immunoblot analysis shows that the bacterially expressed gene product corresponds to the 160-kDa subunit of isolated coatomer, and (*ii*) immunoelectron microscopy reveals that α-COP localizes to the coat of Golgi-derived buds and transport vesicles. The finding that disruption of the α-COP gene in yeast is lethal indicates that α-COP, like β-COP (17), serves an essential function within the coatomer complex in secretory processes.

The strong conservation between sequences of yeast and mammalian α-COP, shown here, and the conservation of γ-, β-, and β′-COPs from yeast and mammals (9, 15, 17) point to a strict conservation of the overall function of coatomer. Biochemical approaches in mammalian systems have elucidated a function of coatomer in anterograde vesicular protein transport (32, 33). This pathway can be disrupted by specific antibodies directed against distinct coatomer subunits (14, 34) and by a mutated ε-COP in mammalian cells (35).

Recently, coatomer has been shown to bind to the C-terminal dilysine motif (36) known to act as a signal for the retrieval of ER-resident proteins from the Golgi complex (37, 38). Thus, coatomer might be involved in vesicle formation of both the anterograde and retrograde pathways.

The only striking structural homology of yeast α-COP with known proteins is the presence of four WD-40 repeated motifs. These repeats are found in a large and still expanding family of proteins that are subunits of oligomeric protein complexes (for review, see refs. 30 and 39). Members of this family regulate diverse cellular functions such as transmembrane signaling, cell division, gene transcription, mRNA modification, and protein secretion. In addition to α-COP, two other WD repeat proteins are known to be involved in vesicular biosynthetic protein transport: β′-COP as part of coatomer (10, 12) and Sec13p as part of COP II (40). β′-COP is a subunit

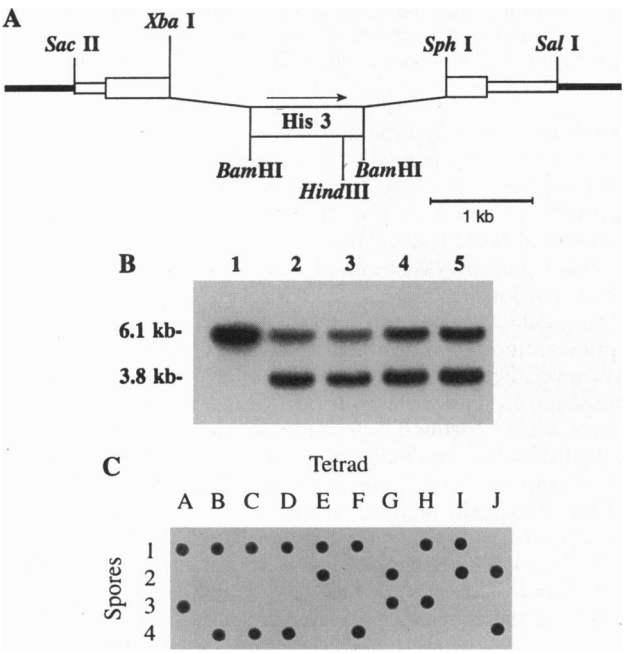

FIG. 4. Analysis of α-COP gene disruption. (*A*) Restriction enzyme map of the construct that was used to replace the α-COP gene. The arrow shows the direction of transcription. (*B*) Southern blot analysis of the *Hin*dIII-digested genomic DNA. Lanes: 1, digestion product of the wild type; 2–5, digestion products of four transformants. The 6.1-kb fragment is from the wild-type allele, and the 3.8-kb fragment is from the mutant allele. (*C*) Tetrad analysis of one transformant. Ten tetrads (columns A–J) were dissected into their four individual spores (rows 1–4). Of the four spores, two carrying the wild-type allele are able to grow into colonies, and two carrying the disrupted α-COP::*HIS3* gene are unable to grow.

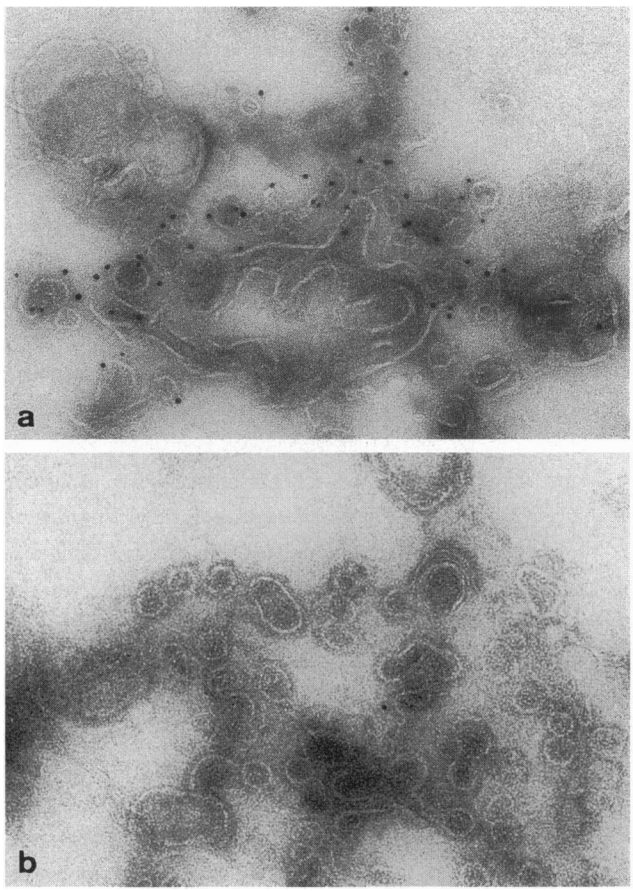

FIG. 5. Immunoelectron microscopic localization of α-COP on Golgi-derived buds and transport vesicles from CHO membranes generated in the presence of GTP[γS]. (*a*) Labeling with the affinity-purified anti-α-COP antibody used at a concentration of 0.15 μg/ml followed by protein A-gold (dilution 1:100). (*b*) Labeling of the same vesicle preparation with preimmune serum (dilution 1:2) and protein A-gold as described in *a*. (×76,000.)

of the cytosolic coatomer complex and is found on transport vesicles derived from the Golgi complex (10, 12). Similarly, functional Sec13p is associated with Sec31p in a large cytosolic complex that is required for the formation of ER-derived transport vesicles (18, 19, 21). Specific functions of the repeated motif have not yet been attributed to any of the individual proteins. WD-40 repeats are mainly found in oligomeric complexes involved in regulatory functions, suggesting that they might serve as motifs that mediate specific protein–protein interactions to build up the quaternary structure of these complexes in a reversible regulated manner. A highly speculative view is that coatomer function in anterograde vs. retrograde transport may be regulated by protein–protein interactions via the various WD-40 repeated motifs present in α- and β′-COPs.

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