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β′-COP, a novel subunit of coatomer

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Several lines of evidence favour the hypothesis that intracellular biosynthetic protein transport in eukaryotes is mediated by non-clathrin-coated vesicles (for a review see Rothman and Orci, 1992). The vesicles have been isolated and a set of their surface proteins has been characterized as coat proteins (COPs). These COPs exist in the cytosol as a preformed complex, the coatomer, which was prior to this study known to contain six subunits: four (α-, β-, γ- and δ-COP) with molecular weights between 160 and 58 kDa, and two additional proteins of ~36 and 20 kDa, ε- and ξ-COP. Here we describe a novel subunit of the coatomer complex, β′-COP. This subunit occurs in amounts stoichiometric to the established COPs both in the coatomer and in non-clathrin-coated vesicles and shows homology to the β-subunits of trimeric G proteins.

Key words: biosynthetic protein transport/COPs/non-clathrin-coated vesicles/trimeric G protein β-subunits

Introduction

Non-clathrin-coated vesicles have been implicated as carriers of biosynthetic protein transport through the Golgi apparatus (Malhotra *et al.*, 1989; Serafini *et al.*, 1991a) (for a review see Rothman and Orci, 1992). These vesicles contain on their surface a set of proteins named COPs (coat proteins). So far, six COPs have been described: α-COP (~170 kDa), β-COP (107 kDa), γ-COP (~100 kDa), δ-COP (~58 kDa), ε-COP (~36 kDa) and ξ-COP (20 kDa). The individual COP polypeptide chains form a soluble complex, the coatomer, in the cytosol (Waters *et al.*, 1991). Two of the COPs have been described at a molecular level: β-COP, which shows homology to the β-adaptin of clathrin-coated vesicles (Duden *et al.*, 1991; Serafini *et al.*, 1991a) and γ-COP, which represents the mammalian homologue of the yeast *SEC21* gene product (Stenbeck *et al.*, 1992). Sec21p is required for the budding of ER to Golgi transport vesicles (Kaiser and Schekman, 1990) and has been shown to exist as a subunit of a yeast cytosolic complex that resembles the mammalian coatomer (Hosobuchi *et al.*, 1992). The phenotype of the *SEC21* mutant strongly suggests that the ER to Golgi transport vesicles in mammalian cells might be formed by recruiting coatomer from a cytosolic pool which they share with the Golgi transport vesicles. Thus, COP-coated vesicles seem to mediate biosynthetic protein transport from the ER via the various stations of the Golgi up to at least the *trans* Golgi network.

Recently the first molecular details have emerged underlying the mechanisms of coat formation and budding: ADP ribosylation factor (ARF), a cytosolic small GTP-binding protein, is a constituent of the coat of non-clathrin-coated vesicles, but it is not a constituent of the cytosolic coatomer itself (Serafini *et al.*, 1991b). Binding of ARF is a prerequisite for the binding of coatomer to Golgi membranes (Donaldson *et al.*, 1991; Orci *et al.*, 1991), a process that is inhibited by the antibiotic brefeldin A (Donaldson *et al.*, 1992a). Cytosolic ARF carries a GDP molecule (Kahn and Gilman, 1986) which has to be exchanged for GTP in order to enable ARF to bind to the Golgi membrane. This exchange is mediated by a Golgi enzyme that is inhibited by brefeldin A (Donaldson *et al.* 1992b; Helms and Rothman, 1992). Recruitment of coatomer to the membrane thus requires GTP. Additional G proteins are likely to be involved in vesicular transport (reviewed by Pfeffer, 1992).

There seems to be one copy of each COP present in a single coatomer complex, except for β-COP, whose broad and more intense Coomassie staining suggested either a 2:1 stoichiometry or the presence of another protein of similar molecular weight. In order to assess the actual composition of the coatomer complex we have separated its subunits in

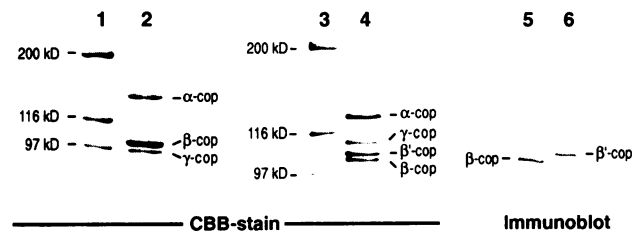

Fig. 1. Analysis of the 100 kDa COP family by SDS–PAGE and immunoblotting. (**Left**) Comparison of the separation of purified coatomer in 6% acrylamide gels either with an acrylamide to *N,N′*-methylene-bisacrylamide ratio of 37.5:1 (lane 2) or with a ratio of monomer to crosslinker of 100:1, and additional 6 M urea in the separating gel (lane 4). In lane 2 the COPs are designated according to Serafini *et al.* (1991a) and Waters *et al.* (1991). The three proteins around 100 kDa in lane 4 were identified by protein sequence analysis (see text) and immunoblotting (see below). (**Right**) Immunological identification of β-COP and β′-COP in coatomer separated on a 6% acrylamide gel as in lanes 3 and 4. Lane 5, immunoblot using the anti-β-COP monoclonal antibody M3A5. Lane 6, immunoblot using the polyclonal antibody C1-PL directed against the N-terminal 12 amino acids of β′-COP. In the urea-containing gel system the two bands with apparent molecular weights of 96 and 105 kDa shown in lane 2 were clearly separated into three bands migrating with apparent molecular weights between 105 and 110 kDa (lane 4).

EDMAN CYCLE:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
a:		P	L	R	L	D	I	K	R	K	L	T	A	X	S	D
b:		-	-	-	L	D	I	K	R	K	L	T	A			

Fig. 2. N-terminal amino acid sequences of β′-COP (Figure 1, lane 4). Line a: protein isolated from coatomer. Line b: protein isolated from non-clathrin-coated vesicles. The first three amino acids released during the sequencing of the vesicle-derived material could not be firmly attributed, probably due to the low quantity of protein sequenced. From step four onwards the improved background allowed the unambiguous identification of the phenylthiohydantoin amino acids until cycle 12.

a modified electrophoretic system. Here we report the detection of an additional stoichiometric coatomer component of 102 kDa. Furthermore we describe the complete cDNA sequence coding for this novel coat protein, named β′-COP, and show that β′-COP is a constituent of both the coatomer and the non-clathrin-coated Golgi transport vesicles. The cDNA-derived N-terminal third of β′-COP is made up of five repeats typical of the β-subunits of trimeric G proteins.

Results

Identification of a novel COP present in both coatomer and transport vesicles

Coatomer from bovine brain cytosol (Waters *et al.*, 1991) as well as that from transport vesicles (Serafini *et al.*, 1991a) is slightly heterogeneous on SDS gels in the region where β-COP migrates (Figure 1, lane 2). In our attempt to improve the resolution of this region, we used a modified gel system containing urea (Ahle and Ungewickell, 1989) and an increased ratio of acrylamide to bisacrylamide (100:1). In this system, a triplet of clearly separated bands appears (Figure 1, lane 4). The lower protein in this triplet represents β-COP, as revealed by its reaction with the monoclonal antibody M3A5 (Duden *et al.*, 1991) in a Western blot (Figure 1, lane 5). After blotting to glass fibre support, the three proteins were subjected to N-terminal microsequencing. The material attributed to β-COP turned out to be blocked N-terminally, as was expected (Duden *et al.*, 1991). Likewise, the protein of the upper band, γ-COP, is blocked. The N-terminal amino acid sequence of the material in the middle band is shown in Figure 2, line a. N-terminal sequencing of the corresponding protein obtained from isolated transport vesicles (Serafini *et al.*, 1991a) did not allow identification of the amino acids released in the first three Edman steps, but from step four onwards, the peptide sequence shown in line b of Figure 2 was obtained; it is identical to the upper sequence from step four to step 12. Thus, the additional protein present in coatomer is a constituent of non-clathrin-coated transport vesicles as well. The apparent molar ratios of the coatomer subunits at ~100 kDa as determined by densitometric scanning of the gels stained with Coomassie blue are 0.5 for γ-COP, 1.0 for the middle protein, which we have named β′-COP, and 0.7 for β-COP. Polyclonal rabbit antibodies directed against a synthetic peptide corresponding to the first 12 amino acids were used after immunoaffinity purification for an immunological analysis of the novel coat protein by Western blotting and immunocytochemistry. As depicted in Figure 1, lane 6, the antibodies react exclusively with the middle protein of the triplet. Electron microscopy of isolated Golgi stacks derived from CHO cells treated with AlF_4^- using the immunogold method (Orci *et al.*, 1989) with the β′-COP peptide antibody (C1-PL) shows a clear-cut

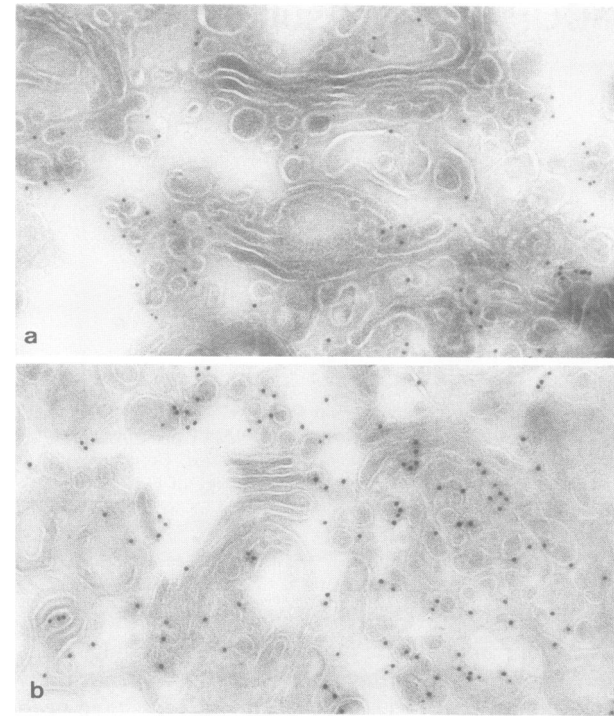

Fig. 3. Immunoelectron-microscopic localization of β-COP (**a**) and β′-COP (**b**) to the coat of non-clathrin-coated vesicles. CHO Golgi membranes were incubated in AlF_4^- to allow coated vesicles to accumulate (Orci *et al.*, 1989). Immunolabelling was performed on ultrathin cryosections using affinity-purified anti-EAGE antibodies (a gift from Thomas Kreis) (panel a) or affinity-purified C1-PL antibodies (panel b). Protein A−gold was used to localize bound antibodies. Numerous immunogold particles are associated with the periphery of non-clathrin-coated vesicles and buds. Magnifications: (a) 49 300×; (b) 46 000×.

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   1 PLRLDIKRKL TRRSDRVKSV DLHPTEPWML RSLVNGSVCV WNHETQTLVK
  51 TFEVCDLPVR RRKFVRRKNW VVTGRDDMQI RVENYNTLER VHMFEAHSDY
 101 IRCIRVHPTQ PFILTSSDDM LIKLWDWDKK WSCSQVFEGH THYVMQIVIN
 151 PKDNNQFASA SLDRTIKVWQ LGSSSPNFTL EGHEKGVNCI DYYSGGDKPY
 201 LISGADDRLV KIWDYQNKTC VQTLEGHAQN VSCRSFHPEL PIIITGSEDG
 251 TVRIWHSSTY RLESTLNYGM ERVWCVASLR GSNNVALGYD EGSIIVKLGR
 301 EEPAMSMDAN GKIIWRKHSE VQQANLKAMG DAEIKDGERL PLAVKDMGSC
 351 EIYPQTIQHN PNGRFVVVCG DGEVIIYTAM ALRNKSFGSA QEFAWRHDSS
 401 EYAIRESNSV VKIFKNFKEK KSFKPDFGRE SIVGGFLLGV RSVNGLAFYD
 451 WENTELIRRI EIQPKHIFWS DSGELVCIAT EESFFILKYL SEKVLRAQET
 501 HEGVTEDGIE DGFEVLGEIQ EIVKTGLWVG DCFIYTSSVN RLNYYVGGEI
 551 VTIAHLDRTM YLLGYIPKDN RLVLGDKELN IVSVSLLVSV LEVQTAVMRR
 601 DFSMADKVLP TIPKEQRTRV AHFLEKQGFK QQALTVSTDP EHRFELALQL
 651 GELKIAVQLA VERESEQKWK QLAELAISKC PFGLAQECLH HAQDYGGLLL
 701 LATASGNASM VNKLAEGAER DGKNNVAFMS YFLQGKLDAC LELLIRTGRL
 751 PEAAFLARTY LPSQVSRVVK LWRENLSKVN QKAAESLADP TEVENLFPGL
 801 KEAFVVEEWV KETHADLWPA KQVPLVTPNE ERNVMEEAKG FQPSRSAAQQ
 851 ELDGKPASPT PVIVTSQTAN KEEKSLLELE VDLDNLEIED IDTTDINLDE
 901 DILDD
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Fig. 4. cDNA-derived amino acid sequence of β′-COP. Peptides as known from microsequence analysis are underlined.

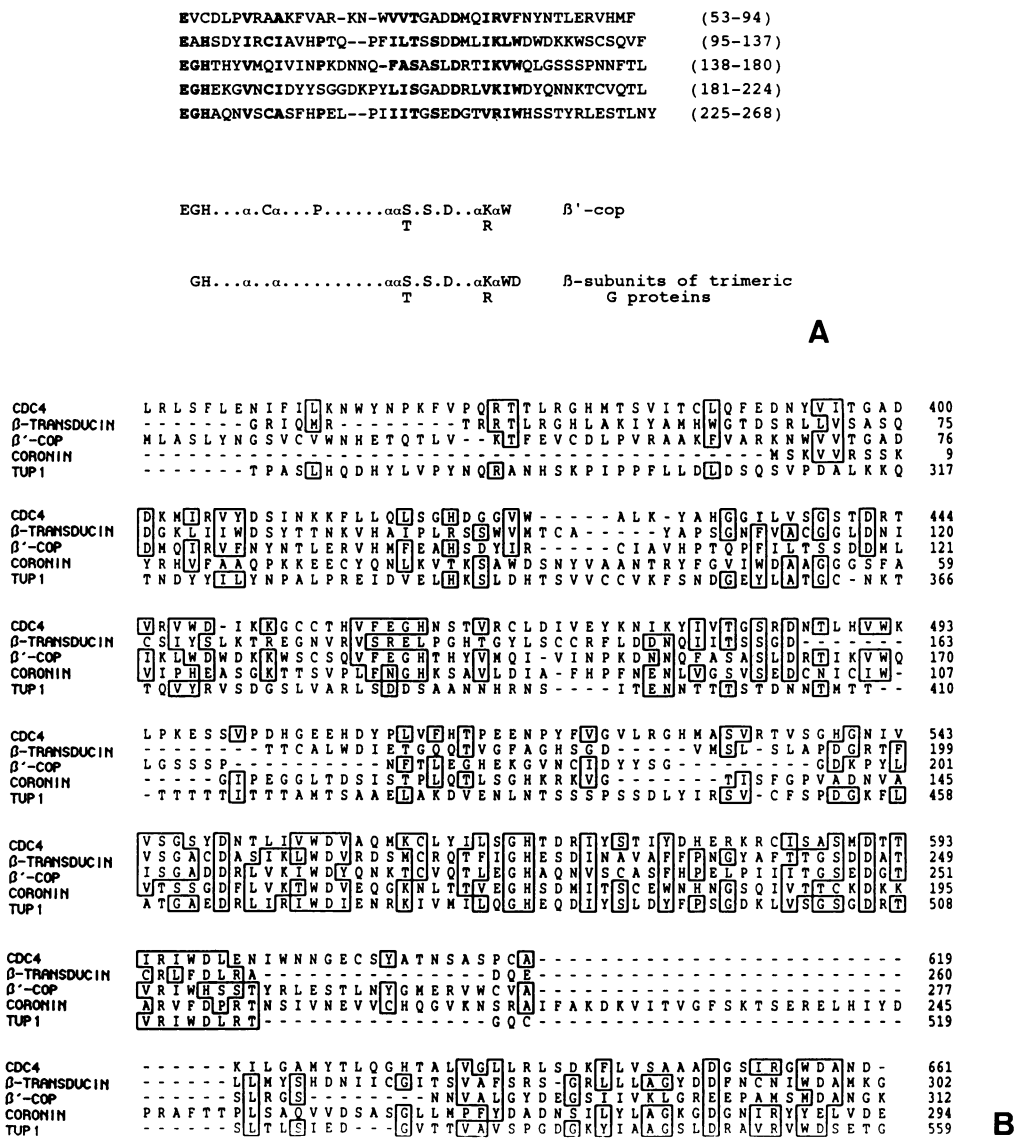

Fig. 5. Homology of β′-COP to the β-subunit motif. (**A**) Five stretches of β′-COP homologous to the β-subunit motif are depicted and aligned with the consensus sequence of this motif in trimeric G proteins. Optimal alignment was achieved by manually introducing a few gaps into four of the five segments. The positions of the segments within the amino acid sequence of β′-COP are given in brackets. The resulting repeated motif of β′-COP is shown below and is aligned with the established motif of the trimeric G protein β-subunits. α stands for hydrophobic and neutral amino acids; the single letter code for amino acids is used. (**B**) Sequence alignment of the N-terminal domain of β′-COP with β-subunit repeats of various proteins. Comparison of the amino acid sequences of CDC4, β-transducin (Fong *et al.*, 1986), coronin (de Hostos *et al.*, 1991) and TUP1 (Williams and Trumbly, 1990). Amino acid residues are boxed if three of the five in a given match column are conserved. Sequence positions for the rightmost residue of each protein are given in the right-hand column.

localization to the non-clathrin-coated vesicles (Figure 3b). The same labelling pattern was obtained with the anti-β-COP peptide antibody (EAGE) demonstrating co-localization of these two COPs (Figure 3a).

cDNA-derived sequence of the novel coatomer subunit

In order to characterize β′-COP better, tryptic peptides were prepared according to Serafini *et al.* (1991a) and sequenced. An oligonucleotide probe designed based on one of these peptides was used to screen a λgt10 cDNA library from bovine brain. The largest insert comprised an open reading frame (ORF) of 2637 bp; this spanned ~97% of the complete ORF (2715 bp). The RACE procedure (Frohman *et al.*, 1988) was used to obtain the missing 3% (60 bp at the 5′ and 20 bp at the 3′ end of the cDNA). The cDNA-derived amino acid sequence is shown in Figure 4. The cDNA contains an ORF predicted to encode a protein of 918 amino acid residues. As microsequencing from a blot had shown that the N-terminal amino acid is a proline residue (Figure 2), the mature polypeptide chain of β′-COP consists of 905 amino acid residues (M_r = 102 041 Da) consistent with its apparent M_r of ~100 kDa on SDS gels. Sequence comparison (Swissprot and PIR databases) revealed striking homology to a variety of regulatory proteins that share motifs of the β-subunits of trimeric G proteins (Figure 5B). This homology is not spread along the complete sequence but is restricted to the N-terminal third of the deduced protein (Figure 5A). β-Subunit motifs are repeated five times in this stretch, with closest similarities to *Saccharomyces cerevisiae* TUP1 (Williams and Trumbly, 1990), *S.cerevisiae* CDC4, and β-subunits of mammalian trimeric G proteins (Fong

et al., 1986) (Figure 5A). In single peptide stretches (~60 amino acids long) up to 40% identical amino acid residues are found. If conserved amino acid changes are considered, similarities of up to 67% are obtained.

Discussion

We have shown that the coatomer from mammalian cytoplasm contains a previously undetected component, β′-COP, in addition to the α-, β-, γ-, δ-, ε- and ξ-COPs. The coatomer complex is formed by association of the seven COP subunits in stoichiometric amounts. The same pattern of COPs, including β′-COP, is also present in non-clathrin-coated vesicles.

The N-terminal third of β′-COP is made up of five repeated motifs typical of the β-subunits of trimeric G proteins (WD-40 repeats, for reviews see van der Voorn and Ploegh, 1992, and Duronio *et al.*, 1992). It is striking that such a domain is found in a protein of the size of β′-COP (~100 kDa). However, this is not without precedent. Structurally related proteins that are much larger than the conventional β-subunits of ~36 kDa are known in several organisms. They contain from three [*Dictyostelium discoideum* coronin, 55 kDa (de Hostos *et al.*, 1991)] to six [yeast CDC4, 86 kDa (Fong *et al.*, 1986)] repeated motifs. The β-subunits of trimeric G proteins themselves show eight such repeats.

The precise functions of these large proteins containing repeated motifs of the β-subunit type are not known. As in β′-COP, the motifs are clustered, although in most cases within the C-terminal half of the protein.

What could be a general function of the repeated motif domains in the β-subunit? Analogous to the trimeric G protein complexes, many of the multidomain proteins that contain such β-repeat motifs have been shown to represent parts of protein complexes that mediate various cellular events. The yeast protein TUP1 (a β-repeat protein) forms a complex with Ssn6 (CYC8) which interacts with an appropriate DNA-bound protein resulting in the repression of transcription (Williams *et al.*, 1991; Keleher *et al.*, 1992). A mammalian protein with the characteristic β-repeat (Takagaki and Manley, 1992) is a 50 kDa subunit of the human cleavage stimulation factor (CstF) required for polyadenylation of pre-mRNA. CstF is a multimeric protein complex of three distinct subunits of 77, 64 and 50 kDa. The 50 kDa subunit may be responsible for the reversible interaction of CstF with other protein factors involved in RNA adenylation (Takagaki and Manley, 1992). The activity of such complexes may be regulated by controlled dissociation/association of their subunits. This reaction is not necessarily dependent on GTP as in the trimeric G protein complexes.

What might be the function of the β-repeat domain in β′-COP? Bearing in mind that proteins with this structural motif can generally form specific complexes, one might speculate that the repeated motifs in β′-COP represent a binding site for the contact with another coatomer subunit, thereby stabilizing the complex, or that they represent the linking elements that bind the coatomer to the Golgi membranes. Recalling that the release of coatomer from the membrane requires the hydrolysis of GTP (Melançon *et al.*, 1987; Orci *et al.*, 1989), one simple explanation would be that the β-repeat domain of β′-COP is involved in this reversible binding of the coatomer to ARF, which of the small G proteins is most similar in sequence to Gα (Sevell and Kahn, 1988). In contrast to Gα, in this case ARF would bind to a β-like domain (in coatomer/β′-COP) in its GTP-bound form, and ARF would dissociate from this site of coatomer when GTP is hydrolysed, helping to control the coating and uncoating processes.

In order to understand fully the process of coating and budding, it will be necessary to examine the complete set of components involved. Previously it has been shown that ARF-GTP and coatomer are the only effectors needed for the generation of coated buds and vesicles in an *in vitro* system consisting of isolated rabbit liver Golgi membranes (Palmer *et al.*, 1993). Therefore, knowledge of the molecular structure of every COP, and specifically a functional characterization of individual domains, such as the repeated motif domain of β′-COP, will help to explain these initiating steps of vesicular transport at a molecular level.

Materials and methods

Isolation of Golgi-derived coated vesicles and coatomer

Non-clathrin-coated vesicles were obtained using rabbit liver Golgi as a membrane source and cytosol from bovine brain as described by Serafini *et al.* (1991a). 'Coatomer', the cytosolic protein complex containing subunits of non-clathrin-coated vesicles, was prepared from bovine brain cytosol by the procedure of Waters *et al.* (1991).

SDS–PAGE, protein sequencing and immunoblotting

Purified proteins from Golgi-derived vesicles and coatomer were separated on 6% SDS–polyacrylamide gels under reducing conditions according to Laemmli (1970) or using a modified separating gel (Ahle and Ungewickell, 1989). The ratio of acrylamide to *N,N′*-bisacrylamide was increased to 100:1 and 6 M urea was added. For protein sequencing ~100 μg of protein was fractionated on a 1.5 mm thick gel and electroblotted onto Glassybond membranes (Biometra) in a Bio-Rad semi-dry apparatus. The Coomassie-stained β′-COP was excised and sequenced using an Applied Biosystems gas phase sequencer with on-line HPLC detection (Eckerskorn *et al.*, 1988). For analytical purposes ~5 μg of purified protein was subjected to SDS–PAGE in a minigel and directly stained with Coomassie blue. Densitometric scanning was performed on a Joyce–Loebl scanning densitometer. Immunostaining was performed after transfer onto PVDF membranes (Millipore) according to Kyhse-Andersen (1984). The membrane was preblocked with 5% (w/v) non-fat milk in PBS and cut into two identical halves. One half was incubated with the monoclonal anti-β-COP antibody M3A5 at a dilution of 1:150. The other half was incubated with the affinity-purified anti-β′-COP peptide antibody C1-PL at a dilution of 1:1000. Peroxidase-conjugated secondary antibodies were used as a detection system.

Antibodies

Antibodies directed against the N-terminal sequence of β′-COP (PLRLDIKRKLTA) were prepared by immunizing rabbits with this peptide coupled to keyhole limpet haemocyanin by glutaraldehyde (Kreis, 1986). The antibodies were affinity-purified on the peptide coupled to epoxy-activated Sepharose 6B (Pharmacia). The mouse monoclonal antibody M3A5 and the anti-EAGE antibody, both directed against β-COP, were kindly provided by Thomas Kreis.

Electron microscopy

CHO Golgi membranes were incubated with AlF_4^- as described by Orci *et al.* (1989). The membranes were fixed for 30 min on ice by overlaying the pellet with 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. Ultrathin sections were prepared from part of the pellets and immunolabelling was performed as described previously (Orci *et al.*, 1986). The anti-β′-COP antibody was used at a dilution of 1:5; the anti-EAGE antibody was diluted 1:40.

Isolation of cDNA clones and sequencing

Tryptic peptides from coatomer β′-subunit, isolated as described above, were prepared and sequenced according to Serafini *et al.* (1991a). The degenerate oligonucleotide gtgatgca(g,a)at(c,a,t)gtnat(c,a,t)aa(c,t)cccaa

corresponding to amino acids 144–152 was used as a probe to screen 1.2×10^6 plaques of a randomly primed λgt10 bovine brain cDNA library (Clontech). Hybridization and washing were performed at 60°C in 6×SSC (Sambrook *et al.*, 1989). Five positive clones were sequenced in the M13mp18 system (Sambrook *et al.*, 1989) with Sequenase (USB) according to the protocol suggested by the manufacturer, using walking primers. The largest insert comprised an ORF of 2637 bp, corresponding to 97% of the complete ORF. The 5′- and 3′-ends were completed by the RACE method (Frohman *et al.*, 1988), using bovine mammary gland epithelial cell RNA as the template. RACE products were cloned into M13mp18 and several clones were sequenced as described above.

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Note added in proof

Harrison Lavoie *et al.* have independently identified the human cDNA of β′COP (see accompanying paper). The EMBL database accession number for β′COP is X72756.