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Identification and Characterization of Novel Isoforms of COP I Subunits[1]

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COP I–coated vesicles are involved in vesicular trafficking in the early secretory pathway. The COP I coat is composed of seven subunits, α-, β-, β′-, γ-, δ-, ε-, and ζ-COPs. Evidence suggests, however, that there may be isoforms of the COP I subunits. In the present study, we identified homologs of γ-COP (γ2-COP; original γ-COP is referred to as γ1-COP in this paper) and of ζ-COP (ζ2-COP; original ζ-COP is referred to as ζ1-COP). γ1- and γ2-COPs, and ζ1- and ζ2-COPs share 80 and 75%, respectively, of amino acids. mRNAs for γ2-COP and ζ2-COP are expressed ubiquitously, suggesting their fundamental role in cellular function. Immunofluorescence analysis shows that γ2-COP and ζ2-COP are colocalized with β-COP in the paranuclear *cis*-Golgi region. Yeast two-hybrid analysis indicates that γ1- and γ2-COPs can directly, albeit promiscuously, interact with ζ1- and ζ2-COPs. Like γ1-COP, γ2-COP can form a complex with β-COP *in vivo*. The γ1-COP-containing and γ2-COP–containing complexes can similarly interact with the cytoplasmic domain of p23. These results indicate that γ2-COP and ζ2-COP can form a COP I–like complex in place of γ1-COP and ζ1-COP, respectively, and suggest that the COP I complex and the COP I–like complex are functionally redundant.

Key words: COP I–coated vesicle, two-hybrid analysis, vesicular transport.

In eukaryotic cells, transport of proteins and lipids between membrane-bound compartments is mediated predominantly by carrier vesicles that bud from a donor compartment and fuse with an acceptor compartment to deliver cargo molecules. The generation of carrier vesicles from a donor compartment involves recruitment of cytosolic coat proteins onto the membrane. COP I–coated vesicles are thought to be responsible mainly for retrograde transport of recycled proteins from the Golgi complex and pre-Golgi compartments to the endoplasmic reticulum (for review, see Refs. *1–3*). The coat of COP I-coated vesicles is composed of seven subunits (α-, β-, β′-, γ-, δ-, ε- and ζ-COPs) of a stable cytosolic protein complex. Recruitment of COP I onto the membrane requires a small GTPase, ADP-ribosylation factor (ARF), which cycles between a GDP-bound inactive and a GTP-bound active form. The GTP-bound ARF triggers assembly of COP I onto Golgi membranes. COP I is also known to bind the dilysine motifs and the diphenylalanine motifs in the cytoplasmic domain of transmembrane cargo proteins, such as p24 family members (*1–3*). Studies using a photocross-linking technique have shown that ARF binds to β- and γ-COPs, and dilysine/diphenylalanine motifs bind to γ-COP (*4–6*).

Although COP I-coated vesicles are involved predominantly in the early secretory pathway, more recent studies suggested that they also play a role in the endocytic pathway (*7–10*, reviewed in Ref. *1*). It is of particular interest that, in these studies, γ- and δ-COP were not found on endosomes (*8*, *9*). This suggests that the composition of the endosomal COP I coat is simpler than that of the COP I coat that functions in the early secretory pathway, or that the endosomal homologs of γ- and δ-COPs have not been identified. Upon searching an expressed sequence tag (EST) database, we found cDNA fragments that potentially code for a γ-COP homolog. We also found ESTs potentially coding for a ζ-COP homolog. We therefore set out to characterize the homologs of COP I subunits.

EXPERIMENTAL PROCEDURES

cDNA Clones—All IMAGE consortium cDNA clones were

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Abbreviations: ARF, ADP-ribosylation factor; EST, expressed sequence tag; HA, hemagglutinin; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; BFA, brefeldin A.

purchased from the UK-HGMP resource center (Cambridge, UK). The IMAGE clone ID No. 34685 covered amino acids 358–874 of human γ1-COP. A cDNA fragment covering the missing NH_2-terminal region of γ1-COP was obtained by polymerase chain reaction of a human liver cDNA library (Life Technologies, Rockville, MD) using a primer synthesized on the basis of the sequence of an EST (accession No. AA853380) and a second primer synthesized on the basis of the determined sequence of the above partial cDNA. The IMAGE clone ID No. 858462 covered amino acids 600–871 of human γ2-COP. To obtain a cDNA clone covering the missing NH_2-terminal region of γ2-COP, a human hepatoma HepG2 cell cDNA library (*11*) was screened using a ^{32}P-labeled synthetic oligonucleotide, 5′-GGGAAGACAGGAGCCATAGCAAGAGGAATTGATTTCATGTCAG-3′, which covered a γ2-COP cDNA region significantly different from the corresponding γ1-COP cDNA region. One of the obtained cDNA clones covered the entire coding sequence of human γ2-COP. The IMAGE clones ID Nos. 487094 and 403024 covered the entire coding sequences of human ζ1-COP and mouse ζ2-COP, respectively. The IMAGE clone ID No. 205969 covered amino acids 55–210 of human ζ2-COP. A cDNA clone covering the entire coding sequence of human ζ2-COP was obtained by screening of the HepG2 library using the above partial cDNA as a probe. The IMAGE clone ID No. 649750 covered the entire coding sequence of human β-COP. The IMAGE clone ID No. 937216 covered amino acids 319–511 of human δ-COP. The missing 5′-terminal cDNA fragment of δ-COP was obtained by polymerase chain reaction of the human liver cDNA library using primers synthesized on the basis of the published sequence (*12*).

Plasmid Construction—For expression in mammalian cells as a fusion protein with an NH_2-terminal hemagglutinin (HA) tag, the γ1-, γ2-, ζ1-, or ζ2-COP cDNA was subcloned into the pcDNA3-HAN vector (*13*). For two-hybrid analysis, the γ1-, γ2-, ζ1-, ζ2-, β-, or δ-COP cDNA was subcloned into the pGBT9-BEN vector (*14*) or the pGAD10 vector (Clontech Laboratories, Palo Alto, CA). Construction of two-hybrid vectors for adaptor subunits was described previously (*14*). For expression in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST), a synthetic double-stranded oligonucleotide for the cytoplasmic domain of p23 (amino acid 204–219; Ref. *15*) or for its mutant with amino acid substitutions (FA, KA, or FA/KA) was subcloned into the pGEX-4T-3 vector (Amersham Pharmacia Biotech Buckinghamshire, UK).

Antibodies—Antibodies to γ1- or γ2-COP were raised in rabbits against a synthetic peptide for the COOH-terminal 19 amino acids of human γ1- or γ2-COP conjugated with keyhole limpet hemocyanin. Polyclonal rabbit anti–β-COP antibodies were purchased from Affinity Bioreagents (Golden, CO). Monoclonal rat anti-HA antibody (3F10) was from Roche Diagnostics (Indianapolis, IN). Monoclonal mouse anti-HA antibody conjugated with agarose beads (HA-probe) was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)–conjugated and Cy3-conjugated anti–rat IgG and HRP-conjugated anti–rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA) and Alexa488-conjugated anti–rabbit IgG was from Molecular Probes (Eugene, OR).

Northern Blot Analysis—A human multiple tissue RNA blot (Clontech Laboratories) was probed with a ^{32}P-labeled fragment for the 3′-noncoding region of human γ2- or ζ2-COP cDNA according to the manufacturer's instructions.

Cell Fractionation and Western Blot Analysis—hEK-293 cells grown at approximately 70% confluence on three 100-mm dishes were transfected with the expression vector for HA-γ1-COP or HA-γ2-COP using a FuGene6 transfection reagent (Roche Diagnostics) and incubated for two days. The cells were then washed twice with ice-cold phosphate-buffered saline (PBS), homogenized in 0.6 ml of ice-cold homogenization buffer A (10 mM HEPES-KOH, pH 7.2, 0.25 M sucrose) containing a Complete™ protease inhibitor mixture (Roche Diagnostics) by passage ten times through a 22-gauge needle and centrifuged at 2,500 rpm for 10 min at 4°C in a microcentrifuge to remove nuclei and unbroken cells. The postnuclear supernatant was then centrifuged at 120,000 ×*g* for 60 min at 4°C to obtain cytosol and membrane fractions. The pellet was rinsed with homogenization buffer A, dissolved in lysis buffer (50 mM Tris-HCl, pH7.5, 1% Triton X-100, 2.5 mM EDTA, 0.25 M NaCl) containing the Complete™ mixture, and centrifuged at 12,000 rpm for 10 min at 4°C in a microcentrifuge. The supernatant was used as a membrane fraction. The postnuclear supernatant and cytosol and membrane fractions containing 30 μg of protein were electrophoresed on a 7.5% SDS-polyacrylamide gel and electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA). The blot was incubated sequentially with monoclonal rat anti-HA antibody and with HRP-conjugated anti–rat IgG, and detected using a Renaissance Chemiluminescence reagent *Plus* (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions.

Establishment of Cell Lines Stably Expressing HA-Tagged COP—Rat Clone 9 hepatocytes grown at approximately 70% confluence were transfected with the expression vector for NH_2-terminally HA-tagged γ1-, γ2-, ζ1-, or ζ2-COP using the FuGene6 transfection reagent, and stable clones were selected in the presence of 0.8 mg/ml Geneticin (Life Technologies). The clones thus isolated were examined by immunofluorescence analysis, and those with moderate protein expression levels (Clone 9/HA-γ1-COP, Clone 9/HA-γ2-COP, Clone 9/HA-ζ1-COP, and Clone 9/HA-ζ2-COP) were used in the following experiments.

Indirect Immunofluorescence Analysis—The above cell lines grown in wells of 8-well Lab-Tek-II chamber slides (Nunc A/S, Roskilde, Denmark) were processed for indirect immunofluorescence analysis as described previously (*16*). Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. When indicated, the cells were incubated in the presence of 5 μg/ml brefeldin A (BFA) for 15 min or in the presence of 5 μg/ml nocodazole for 20 min or 2 h prior to fixation. The fixed and permeabilized cells were incubated sequentially with a combination of monoclonal rat anti-HA antibody and polyclonal rabbit anti–β-COP antibody and with a combination of Cy3-conjugated anti–rat IgG and Alexa488-conjugated anti–rabbit IgG. The stained cells were observed with a confocal laser-scanning microscope (TCS-NT; Leica Mikrosystems Vertrieb GmbH, Bensheim, Germany).

Two-Hybrid Analysis—Yeast two-hybrid analysis was performed as described previously (*14*). Briefly, yeast Y190 cells harboring a pGBT9-based bait vector was transformed with a pGAD10-based prey vector, grown for 8 h in synthetic medium lacking tryptophan, leucine and histidine,

and plated on synthetic medium lacking tryptophan, leucine and histidine and containing 25–50 mM 3-aminotriazole. After 9–10 days of incubation, colonies were picked up and subjected to a liquid culture assay using *o*-nitrophenyl-galactopyranoside as a substrate to determine β-galactosidase activity (*17*).

Coimmunoprecipitation Analysis—hEK-293 cells grown at approximately 70% confluence on a 100-mm dish were transfected with the expression vector for HA-γ1-COP or HA-γ2-COP using the FuGene6 transfection reagent and incubated for two days. The cells were washed twice with wash buffer (20 mM Tris-HCl, pH 7.5, 0.15M NaCl, 2 mM EDTA) and lysed in 0.5 ml of immunoprecipitation buffer (wash buffer containing 0.5% Triton X-100 and the Complete™ mixture) for 15 min on ice. The lysates were centrifuged at 15,000 rpm for 15 min at 4°C in a microcentrifuge. The supernatant containing 0.4 mg of protein was mixed with 30 μl of HA-probe and incubated overnight at 4°C on a rotating wheel. The beads were pelleted by centrifugation at 2,500 rpm for 5 min in a microcentrifuge and washed five times with immunoprecipitation buffer, then once with PBS. Proteins eluted from the beads by boiling in SDS-PAGE sample buffer were subjected to 6% SDS-PAGE and blotted onto an Immobilon-P membrane. The blot was incubated with either monoclonal rat anti-HA antibody or polyclonal rabbit anti–β-COP antibodies, then with HRP-conjugated secondary antibodies, and detected using the Renaissance Chemiluminescence reagent *Plus*.

Pull Down Assay—The cytoplasmic domain of p23 or its FA, KA, or FA/KA mutant fused to the COOH-terminus of GST was expressed in *E. coli* BL21(DE3) cells and purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech). Clone 9/HA-γ1-COP or Clone 9/HA-γ2-COP cells grown at approximately 90% confluence on four 150-mm dishes were incubated in the presence of 20 mM sodium butyrate for 8 h, then washed twice with PBS. The cells were then homogenized in 0.7 ml of homogenization buffer B (50 mM HEPES-KOH, pH 7.3, 90 mM KCl, 0.3 M NaCl) containing the Complete™ mixture by passage 15 times through a 22-gauge needle and centrifuged at 2,500 rpm for 10 min at 4°C in a microcentrifuge to remove nuclei and unbroken cells. The postnuclear supernatant was then centrifuged at 120,000 ×*g* for 60 min at 4°C, and the pellet was rinsed with homogenization buffer B and lysed in 0.7 ml of homogenization buffer B containing 0.5% Nonidet P-40 for 45 min on ice. The lysates were centrifuged at 12,000 rpm for 20 min at 4°C in a microcentrifuge. The supernatant containing 0.8 mg of protein was then incubated overnight at 4°C with 10 μg of the GST-fusion protein prebound to glutathione-Sepharose beads and centrifuged at 2,500 rpm for 1 min. The pellet was washed three times with homogenization buffer B containing 0.5% Nonidet P-40, then twice with homogenization buffer B. Proteins eluted from the beads by boiling in SDS-PAGE sample buffer were subjected to 10% SDS-PAGE and blotted onto an Immobilon-P membrane. The blot was incubated with monoclonal rat anti-HA antibody, then with HRP-conjugated anti–rat IgG, and detected using the Renaissance Chemiluminescence reagent *Plus*.

RESULTS AND DISCUSSION

Identification of γ- and ζ-COP Homologs—Although it is well known that the COP I complex functions in the early secretory pathway, several of evidence suggest that it is also present on endosomal compartments. However, γ- and δ-COP are not found in the endosomal COP I coat (*8*, *9*), suggesting that the endosomal COP I coat lacks these subunits or that the endosomal homologs of γ- and δ-COPs have not been identified. To identify the potential isoforms of the COP I subunits, we exploited the availability of large databases of ESTs. By searching the databases with the γ- and δ-COP sequences as queries, we found that several human ESTs potentially code for a part of a protein homologous but not identical to γ-COP. Sequence analysis of one of the EST clones (IMAGE ID No. 858462) and a cDNA cloned from human HepG2 cell library revealed that the deduced sequence of the γ-COP homolog is composed of 871 amino acids. We also cloned a cDNA for human γ-COP, which is composed of 874 amino acids. We hereafter refer to the γ-COP homolog as γ2-COP and original γ-COP as γ1-COP. Figure 1A shows an alignment of the human γ1- and γ2-COP sequences. They share approximately 80% overall amino acid identity. The γ2-COP sequence is completely identical to that reported while this study was in progress; the latter was identified in the course of a search of imprinted genes on chromosome 7 (*18*).

While this study was in progress, we also found mouse and human ESTs that potentially code for a ζ-COP homolog. We hereafter refer to the ζ-COP homolog as ζ2-COP and original ζ-COP as ζ1-COP. Figure 1B shows the amino acid sequences of human and mouse ζ2-COP along with that of human ζ1-COP deduced from the nucleotide sequences of the EST clones and cDNAs we cloned. Human and mouse ζ2-COP show approximately 75% overall amino acid identity with human ζ1-COP, and human and mouse ζ2-COP share approximately 89% of amino acids. A significant difference between ζ1- and ζ2-COPs is that the latter has an extra sequence of approximately 30 amino acids at the NH_2-terminus.

Distribution of γ2- and ζ2-COP mRNAs in Human Tissues—Northern blot analyses of a human multiple tissue RNA blot revealed that an approximately 3.0-kb transcript of γ2-COP is expressed in all tissues examined, although the expression level is relatively low in liver and lung (Fig. 2A), and that an approximately 1.0-kb transcript of ζ2-COP is expressed in all tissues examined, with relatively low expression levels in brain and lung (Fig. 2B). The ubiquitous distributions of the γ2-COP and ζ2-COP transcripts accord with the fact that there are many ESTs for them derived from a variety of tissues and cell lines in the databases. It is therefore likely that both γ2-COP and ζ2-COP play fundamental roles in cellular function like the conventional COP I subunits.

Western Blot Analysis—To characterize the γ1- and γ2-COP proteins, we attempted to raise antibodies that specifically recognize each protein. However, unfortunately, the antibodies raised against the COOH-terminal 19–amino acid peptide of γ1-COP recognized both endogenous γ1- and γ2-COPs, and those against the COOH-terminal 19–amino acid peptide of γ2-COP detected endogenous γ1-COP but not γ2-COP upon Western blot analysis (data not shown). Therefore, we transiently transfected hEK-293 cells with an expression vector for HA-tagged γ1- or γ2-COP. As shown in Fig. 3A, Western blot analysis of postnuclear supernatants from cells expressing HA-γ1-COP and from

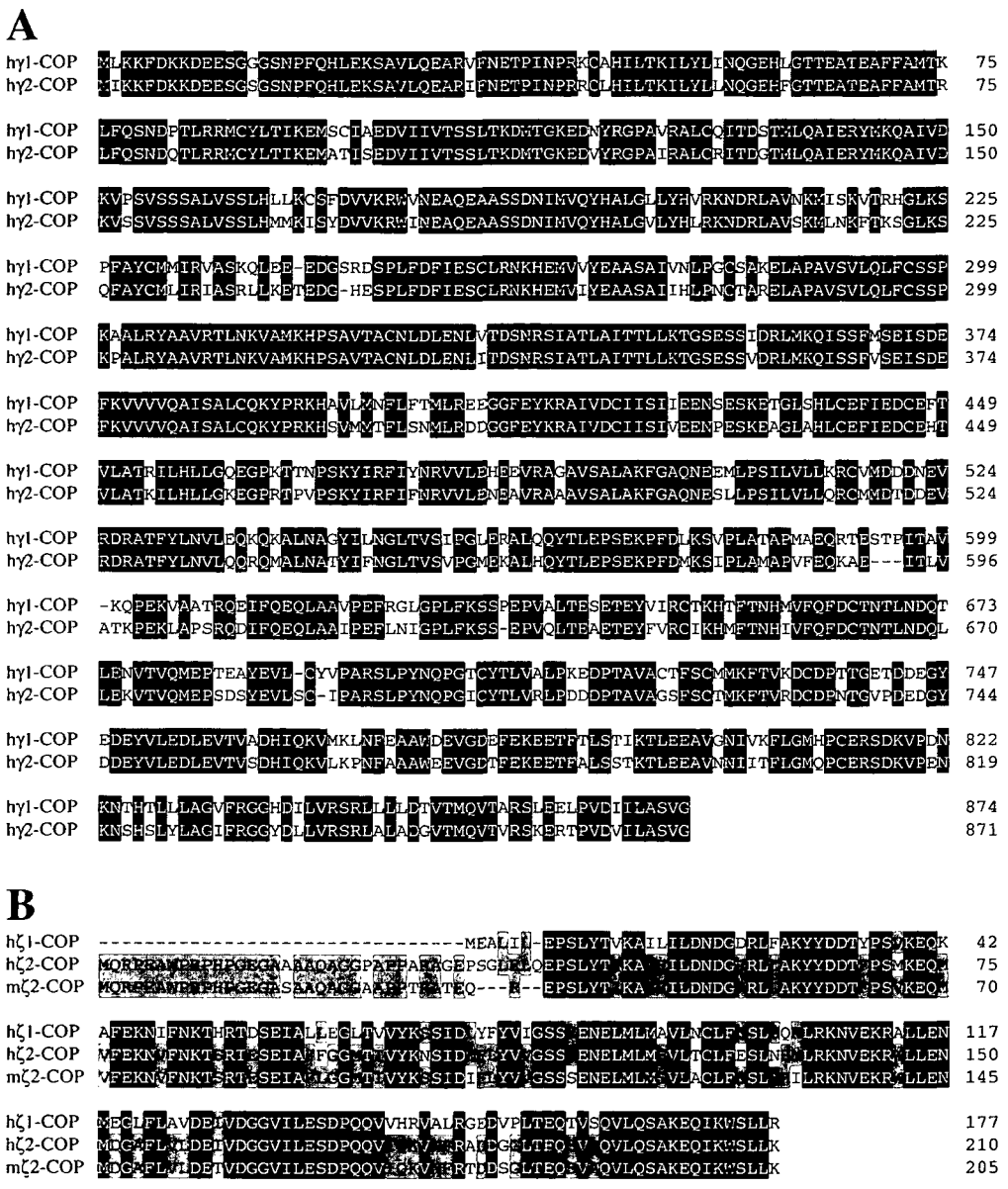


Fig. 1. **Primary sequences of γ- and ζ-COPs.** (A) Alignment of the deduced amino acid sequences of human γ1-COP (hγ1-COP) and γ2-COP (hγ2-COP). The alignment was prepared using the GENETYX-MAC Multi-sequences Program Ver. 10.1.1 (Software Development, Co., Ltd., Tokyo). Amino acid residues conserved between them are shown with black boxes. Gaps introduced into the alignment are indicated by hyphens. (B) Alignment of the deduced amino acid sequences of human ζ1-COP (hζ1-COP) and ζ2-COP (hζ2-COP) and mouse ζ2-COP (mζ2-COP). The alignment was prepared using the GENETYX-MAC Multi-sequences Program Ver. 10.1.1. Amino acid residues conserved among all of them are shown with black boxes and those conserved between two of them are shaded. Gaps introduced into the alignment are indicated by hyphens.

those expressing HA-γ2-COP with anti-HA antibody detected proteins of approximately 100 kDa (lane 2) and 97 kDa (lane 3), respectively, which approximate the calculated molecular weights of γ1-COP (M_r = 97,717) and γ2-COP (M_r = 97,621), respectively. When the postnuclear supernatants were fractionated into cytosol and membrane fractions (Fig. 3B), HA-γ1-COP and HA-γ2-COP were detected in both fractions (lanes 2 and 3, and 5 and 6, respectively), like endogenous β-COP (lanes 8 and 9). These results indicate that γ2-COP, like conventional COP I subunits, cycles between cytosolic and membrane-bound pools.

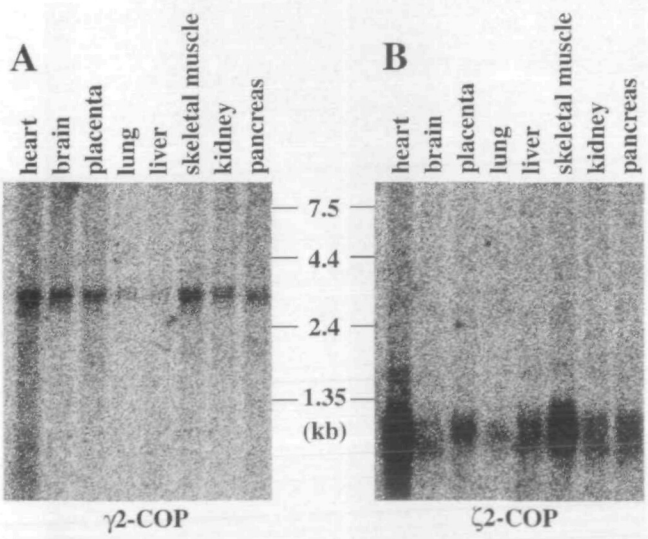
Immunofluorescence Analysis of γ2- and ζ2-COP Localization—To compare the subcellular localization of γ2-COP with that of γ1-COP, we first examined Clone 9 cells transiently transfected with a vector for HA-tagged γ1-COP or γ2-COP. However, when stained with anti-HA antibody, not only cells expressing HA-γ2-COP but also those expressing HA-γ1-COP showed intense cytoplasmic signals and often had cytoplasmic aggregates. These aberrant signals appeared to be generated by overexpression of the protein. We therefore established a cell line stably expressing HA-γ1-COP (Clone 9/HA-γ1-COP) or HA-γ2-COP (Clone 9/HA-γ2-COP) at a moderate level. The cells were then subjected to double staining with anti-HA and anti-β-COP antibodies (Fig. 4). Clone 9/HA-γ2-COP cells showed a typical Golgi-like paranuclear staining pattern for HA-γ2-COP (E), like that for HA-γ1-COP in Clone 9/HA-γ1-COP cells (A). Furthermore, the paranuclear staining for HA-γ2-COP was completely superimposed on that for endogenous β-COP (compare E and E′). We also established Clone 9 cells stably expressing HA-tagged ζ1-COP or ζ2-COP, and found that both were colocalized with β-COP in the paranuclear region (Fig. 5, compare A and A′, and B and B′, respectively).

We then set out to examine whether γ2-COP has similar properties to γ1-COP. To this end, we first treated Clone 9/HA-γ1-COP and Clone 9/HA-γ2-COP cells with BFA, which inhibits membrane association of COP I by inhibiting guanine nucleotide exchange on ARF (*19*). Consistent with this

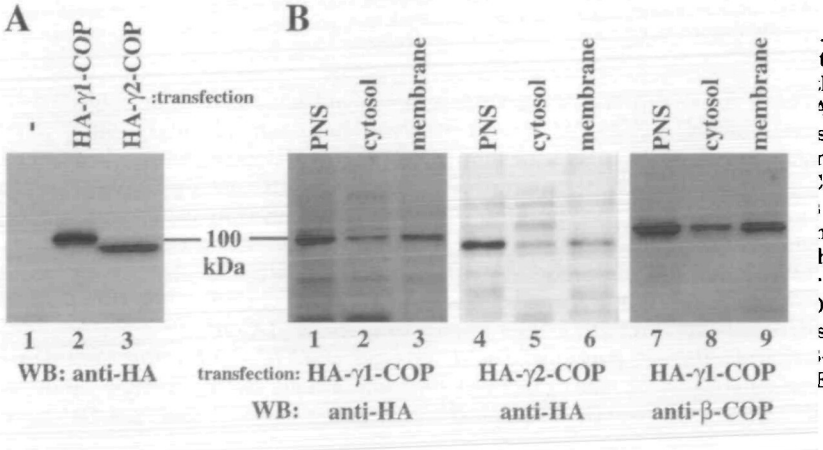
fact, β-COP and HA-γ1-COP were redistributed from Golgi membranes into the cytoplasm when Clone 9/HA-γ1-COP cells were treated with BFA (Fig. 4, B and B′). Similarly, HA-γ2-COP was redistributed into the cytoplasm upon the BFA treatment (F). Thus, like the conventional COP I subunits, association of γ2-COP with Golgi membranes appears to be regulated in an ARF-dependent manner.

We then treated Clone 9/HA-γ1-COP and Clone 9/HA-γ2-COP cells with nocodazole, a microtubule-disrupting reagent. Previous studies have shown that short-term (10–30 min) treatment of cells with nocodazole causes proteins recycling between the *cis*-Golgi and the endoplasmic reticulum to accumulate at peripheral endoplasmic reticulum exit sites but has a marginal effect on the distribution of Golgi-resident proteins. In contrast, long-term (1–2 h) treatment gives rise to redistribution of both the recycling and resident proteins into the peripheral sites (*20*, *21*). In addition, we have recently found that β-COP shows a behavior similar to that of recycling proteins upon nocodazole treatment (Kawamoto, K., Yoshida, Y., Shinotsuka, C., and Nakayama, K., manuscript in preparation; see also Fig. 4, C′, D′, G′, and H′). As shown in Fig. 4, treatment of Clone 9/HA-γ2-COP cells with nocodazole for 20 min caused accumulation of not only β-COP (G′) but also HA-γ2-COP (G) at the peripheral punctate structures. Furthermore, the scattered labeling for HA-γ2-COP was completely superimposed on that for β-COP. In contrast, a Golgi-resident protein, mannosidase II, remained in the Golgi-like paranuclear region under these conditions, and was redistributed into the peripheral sites by 2-h nocodazole treatment (data not shown). Thus, contrary to our initial expectation that γ2-COP may function on endosomal compartments in place of γ1-COP, these observations indicate that it functions mainly on the *cis*-Golgi like the conventional COP I subunits and suggest that it may form a complex with β-COP and other COP I subunits. However, these observations do not exclude the possibility that γ2-COP could also function on endosomal compartments.

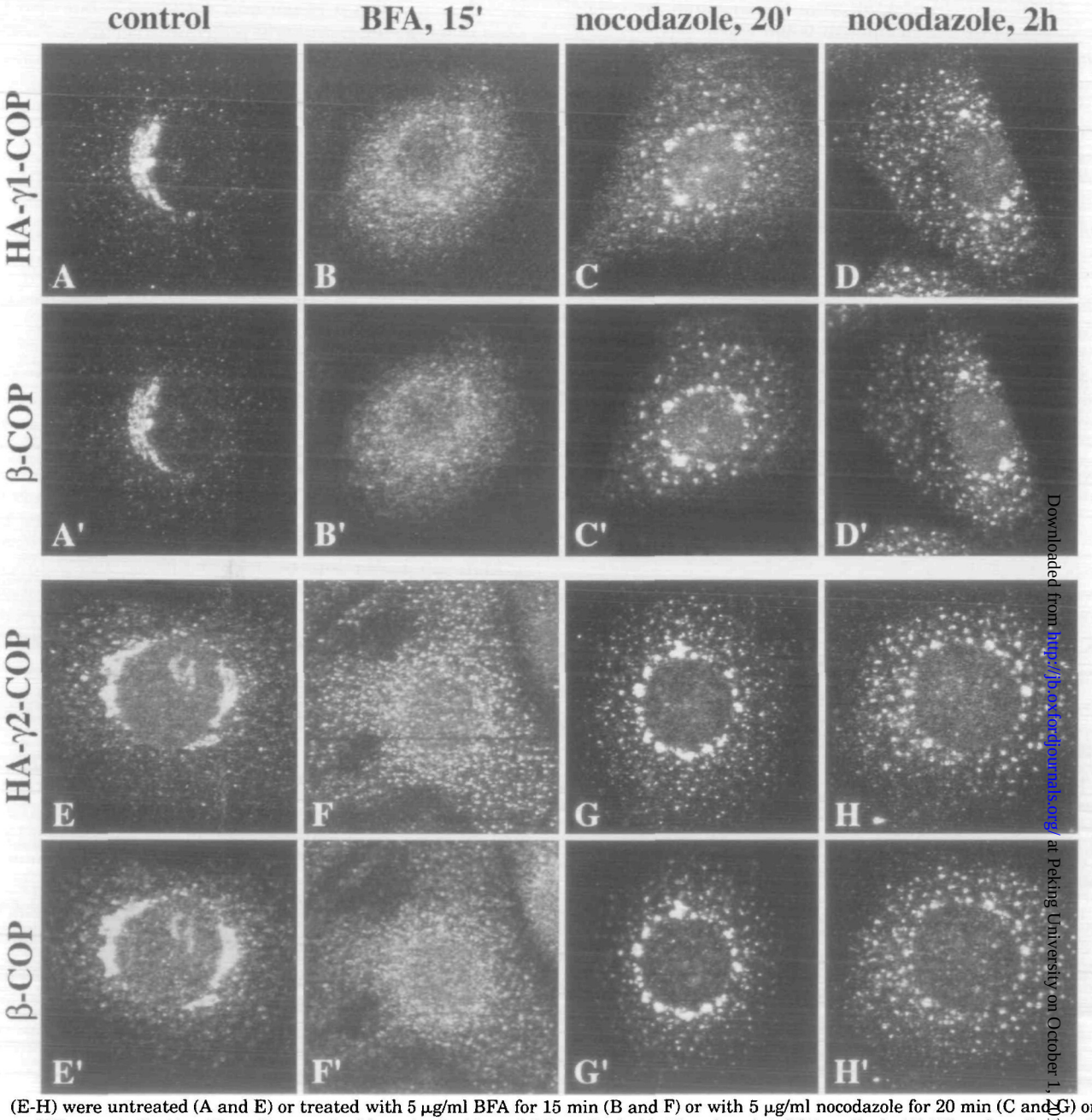
Interaction between γ- and ζ-COPs Revealed by Yeast Two-Hybrid Analysis—We next examined interactions of γ2- and ζ2-COPs with the known COP I subunits. To this end, we made use of the yeast two-hybrid system, because a previous study using this approach showed interactions between COP I subunits in various combinations: γ- and ζ-COPs, β- and δ-COPs, α- and β′-COPs, and α- and ε-COPs (*22*). Furthermore, the COP I complex can be disintegrated in high salt buffer into two subcomplexes: the F subcomplex composed of β-, γ-, δ-, and ζ-COPs, and the B subcomplex composed of α-, β′-, and ε-COPs (*23*, *24*; reviewed in *1*). We therefore examined interactions of γ2- and ζ2-COPs with the components of the F subcomplex. As shown in Fig. 6, when a bait vector for γ1- or γ2-COP was transformed into reporter yeast cells, the cells showed a high level of β-galactosidase activity when cotransformed with a prey vector not only for ζ1-COP but also for ζ2-COP, indicating that both γ-COPs can interact with both ζ-COPs. In contrast, neither γ1- nor γ2-COP interacted with the σ1 subunit of the AP-1 clathrin adaptor complex, which has a common ancestral origin with ζ-COPs (*25*). Furthermore, in agreement with the previous report (*22*), neither γ1- nor γ2-COP showed a significant interaction with β- or δ-COP. When ζ2-COP was used as a bait, it showed significant interactions with both γ1- and γ2-COPs, and not with β- or δ-COP, nor with the γ1-adaptin subunit of the AP-1 clathrin adaptor complex, which has a common ancestral origin with γ-COPs (*25*). However, our attempt to obtain two-hybrid data using ζ1-COP as a bait was unsuccessful, because yeast cells transformed with its bait vector showed an extremely high level of β-galactosidase activity in a



was probed for γ2-COP (A) and ζ2-COP (B) as described under "EXPERIMENTAL PROCEDURES."



. **Western blot analysis.** (A) Postnuclear sutant prepared from hEK-293 cells transfected he expression vector for HA-γ1-COP (lane 2) or A-γ2-COP (lane 3) or without transfection (lane s subjected to Western blot analysis with antintibody as described under "EXPERIMENTAL EDURES." (B) Postnuclear supernatant (PNS) 1, 4 and 7), cytosol fraction (lanes 2, 5 and 8) membrane fraction (lanes 3, 6 and 9) prepared hEK-293 cells transfected with the expression for HA-γ1-COP (lanes 1–3 and 7–9) or for HA-P (lanes 4–6) were subjected to Western blot sis with anti-HA antibody (lanes 1–6) or with -COP antibody (lanes 7–9) as described under ERIMENTAL PROCEDURES."

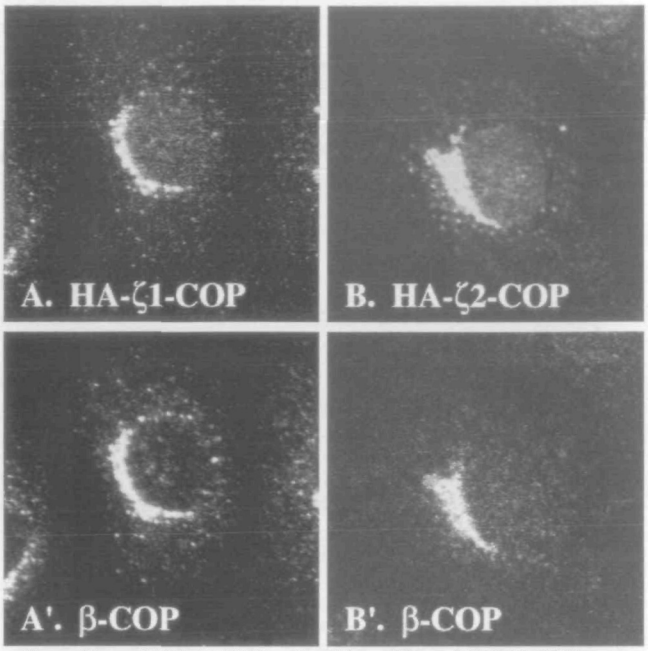


ells (E-H) were untreated (A and E) or treated with 5 μg/ml BFA for 15 min (B and F) or with 5 μg/ml nocodazole for 20 min (C and G) or 2 h (D and H) prior to fixation. The cells were double stained with anti-HA antibody (A–H) and anti-β-COP antibody (A′–H′) as described under "EXPERIMENTAL PROCEDURES."

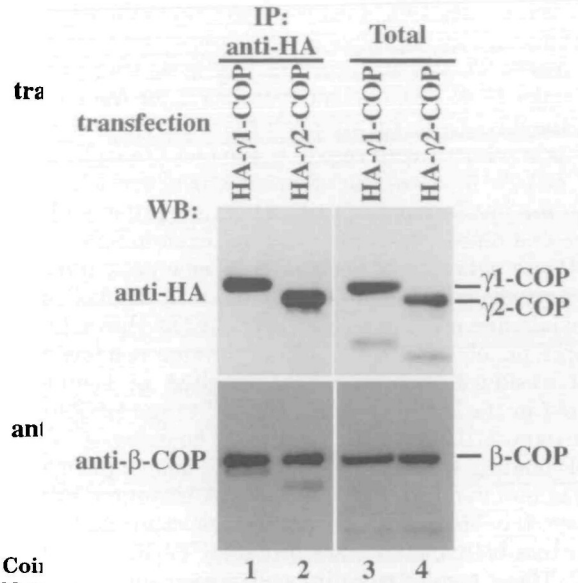
prey-independent manner (data not shown). These data indicate that γ1- and γ2-COPs can interact directly, albeit promiscuously, with ζ1- and ζ2-COPs, and do not interact directly with β- or δ-COP.

Both γ1- and γ2-COPs Form a Complex Containing β-COP In Vivo—Although the above two-hybrid analysis uncovered direct interactions between γ1- or γ2-COP and ζ1- or ζ2-COP in every combination, it remained unclear whether γ2- and ζ2-COP form a complex with other known COP I subunits or with unidentified counterparts. To address this issue, lysates prepared from cells transiently transfected with HA-γ1-COP or HA-γ2-COP were immunoprecipitated with anti-HA antibody and then subjected to SDS-PAGE and Western blot analysis with anti-β-COP antibody. As shown in Fig. 7, lower panel, the anti-HA antibody coimmunoprecipitated β-COP not only from lysates prepared from HA-γ1-COP–transfected cells (lane 1) but also from those prepared from HA-γ2-COP–transfected cells (lane 2). Taken together with the above two-hybrid data, these results indicate that γ2-COP is a component of a COP I-like complex in place of γ1-COP.

Binding of γ1-COP–and γ2-COP–Containing Complexes to the p23 Cytoplasmic Domain—To further address whether complexes containing γ1-COP and γ2-COP have



localization. Clone 9/HA-ζ1-COP cells (A) or Clone 9/HA-ζ2-COP cells (B) were fixed and double stained with anti-HA antibody (A and B) and anti-β-COP antibody (A′ and B′) as described under "EXPERIMENTAL PROCEDURES."



7. Coi
Ζ-293 cells transfected with the expression vector for HA-γ1-COP
es 1 and 3) or for HA-γ2-COP (lanes 2 and 4) were immunopre-
tated with anti-HA–conjugated agarose. The total lysates (lanes 3 and 4) and immunoprecipitates (lanes 1 and 2) were subjected to Western blot analysis with anti-HA antibody (upper panel) or with anti-β–COP antibody (lower panel) as described under "EXPERIMENTAL PROCEDURES." Positions of HA-γ1-COP, HA-γ2-COP, and β-COP are indicated.

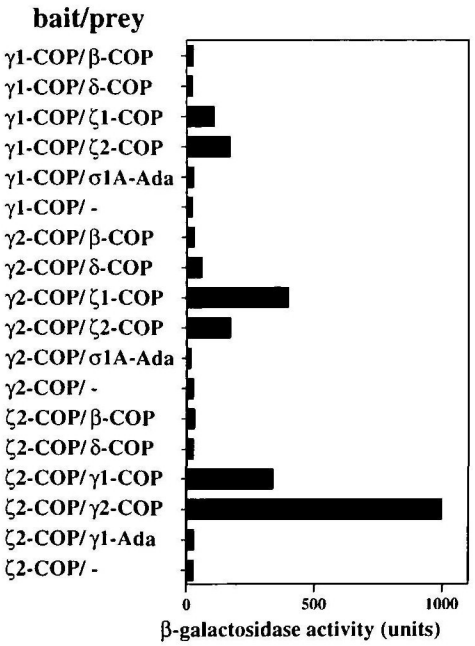


Fig. 6. **Two-hybrid analysis.** Reporter yeast cells were cotransformed with a pGBT9-based bait vector and a pGAD10-based prey vector as indicated and were subjected to liquid β-galactosidase assay as described under "EXPERIMENTAL PROCEDURES." σ1A-Ada, σ1A subunit of the AP-1 clathrin adaptor complex; γ1-Ada, γ1-adaptin subunit of the AP-1 complex.

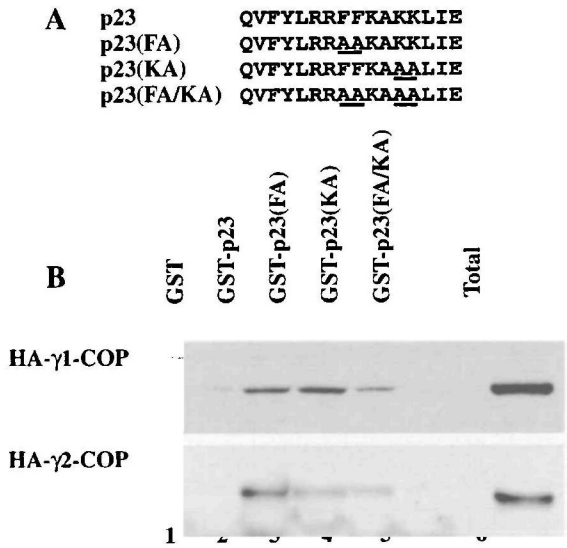


Fig. 8. **Pull down assay.** (A) Sequences of the cytoplasmic domain of p23 and its FA, KA, and FA/KA mutants fused to GST. Substituted residues are underlined. (B) Lysates prepared from Clone 9/HA-γ1-COP cells (upper panel) or Clone 9/HA-γ2-COP cells (lower panel) were incubated with GST (lane 1), GST-p23 (lane 2), GST-p23(FA) (lane 3), GST-p23(KA) (lane 4), or GST-p23(FA/KA) (lane 5) prebound to glutathione-Sepharose beads and centrifuged. The pellets and total cell lysates (lane 6) were subjected to Western blot analysis with anti-HA antibody as described under "EXPERIMENTAL PROCEDURES."

functional differences, we examined binding of these complexes to the cytoplasmic domain of p23. p23 is a member of the p24 family, which consists of putative receptors for cargo proteins transported between the endoplasmic reticulum and the Golgi complex, and is enriched in COP I–

coated vesicles (*26*). COP I is known to bind to the p23 cytoplasmic domain by recognizing the dilysine and diphenylalanine motifs (*26–28*). It is currently believed that γ1-COP is the major COP I component responsible for the recognition of the dilysine motif, although there have been contradicting observations with regard to the motif recognition (*4*, *24*, *27*, *28*). At first, we therefore set out to reproduce the data on the involvement of γ1-COP in interaction with the dilysine and diphenylalanine motifs by examining whether γ1-COP was pulled down with a GST fusion protein having the p23 cytoplasmic domain or its mutant at the dilysine or diphenylalanine motif or at both (Fig. 8A). As shown in Fig. 8B, upper panel, substitution of the dilysine residues with alanine residues (KA mutation) resulted in significant reduction in the binding of HA-γ1-COP to the GST fusion protein (lane 4). In contrast, apparently no decrease of HA-γ1-COP binding was observed when the double phenylalanine was mutated to double alanine (FA mutation, lane 3). However, the HA-γ1-COP binding was completely abolished when both motifs were mutated (FA/KA mutation, lane 5). These results were in good agreement with those reported by Dominguez *et al.* (*27*). When binding of HA-γ2-COP to the GST-p23 fusion proteins was examined under the same conditions, essentially the same results were obtained (Fig. 8B, lower panel); the FA mutation resulted in only a slight, if any, decrease of HA-γ2-COP binding (lane 3), the KA mutation resulted in greater decrease (lane 4), and the FA/KA mutation completely abolished the binding (lane 5). Thus, we could not observe any significant difference between γ1-COP–containing and γ2-COP–containing complexes in binding to the p23 cytoplasmic domain, suggesting their functional redundancy.

Concluding Remarks—In the present study, we identified homologs of COP I subunits, γ2-COP and ζ2-COP. Contrary to our expectation, however, we could find no significant difference between the homologs and the conventional COP I counterparts in tissue distribution, subcellular distribution, complex formation or function. Therefore, it seems likely that γ1-COP and γ2-COP, and ζ1-COP and ζ2-COP are functionally redundant, although a further study will be required to address this issue.

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