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Novel Isotypic γ/ζ Subunits Reveal Three Coatomer Complexes in Mammals

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In early secretory transport, coat recruitment for the formation of coat protein I (COPI) vesicles involves binding to donor Golgi membranes of the small GTPase ADP-ribosylation factor 1 and subsequent attachment of the cytoplasmic heptameric complex coatomer. Various hypotheses exist as to the precise role of and possible routes taken by COPI vesicles in the mammalian cell. Here we report the ubiquitous expression of two novel isotypes of coatomer subunits γ- and ζ-COP that are incorporated into coatomer, and show that three isotypes exist of the complex defined by the subunit combinations γ1/ζ1, γ1/ζ2, and γ2/ζ1. In a liver cytosol, these forms make up the total coatomer in a ratio of about 2:1:2, respectively. The coatomer isotypes are located differentially within the early secretory pathway, and the γ2/ζ1 isotype is preferentially incorporated into COPI vesicles. A population of COPI vesicles was characterized that almost exclusively contains γ2/ζ1 coatomer. This existence of three structurally different forms of coatomer will need to be considered in future models of COPI-mediated transport.

In the eukaryotic cell, a variety of protein-coated vesicles serve the sorting and transport of proteins (and lipids) to their different residencies within the endomembrane system (8, 24, 48, 51). Clathrin heavy and light chains were the first coat proteins discovered (26, 43, 44) and have turned out to serve as a quite general device to help form vesicles that mediate transport in endocytic and late secretory pathways, i.e., between the plasma membrane, endosomes, and the *trans*-Golgi. The origin of vesiculation and the direction clathrin-coated vesicles (CCVs) take is determined by another set of proteins, the adaptor (AP) complexes (AP1 to AP4) (5). Specific binding of an individual AP to a cytoplasmic membrane protein signature is thought to define the origin of the forming vesicle and represents the first layer of a forming CCV. Subsequent binding of clathrin completes the budding of a CCV. Thus, the individual APs in the various CCVs (and as more recently discovered, Golgi-derived γ-adaptin ear ADP-ribosylation factor binding proteins) (6, 47) rule the directionality of vesicular transport between plasma membrane, *trans*-Golgi, and the endosomal and lysosomal system.

In the early secretory pathway, two types of coats are known to date, COPI and COPII. In both cases, coat recruitment is preceded by binding of a small GTPase to the donor membrane in its GTP-bound form. Like CCVs, COPII-coated vesicles carry a coat made of two layers, the Sec23/24 complex that binds directly to the transitional elements of the endoplasmic reticulum (ER) and the Sec13/31 that binds on top of this layer (1, 2). COPII vesicles seem to serve exclusively the exit of proteins from the ER and thus travel unidirectionally. Although COPII vesicles need not distinguish between different origins, several isotypes of the COPII component Sec24 have been characterized. Whereas a redundant function was proposed for the Sec24p homologue Iss1p (28), Lst1p was suggested to participate in the sorting of distinct cargo (46, 55). In addition, Sec24p has been shown to be important for the recruitment of SNAREs into COPII vesicles (34).

Unlike COPII vesicles and CCVs, COPI vesicles recruit their coat protein, coatomer, in a single step. Coatomer is a cytosolic protein complex made up of seven subunits (COPs), some of which show homology to AP proteins (5, 11, 53). Individual COPs were first characterized as coat components of vesicles formed in vitro from Golgi-enriched membranes (53, 60). These COPI vesicles were shown to contain anterograde cargo proteins (35, 37). Subsequently, coatomer has been shown to bind to cytoplasmic tail sequence signatures (9, 30), known to serve retrograde transport from the Golgi back to the ER (25, 36), indicating a role in retrograde transport of COPI vesicles. Accordingly, electron microscopy revealed the presence of COPI vesicles that contain either anterograde or retrograde cargo (38, 39).

The resulting view that COPI vesicles can serve both anterograde transport through the Golgi stack and retrograde transport from the Golgi back to the ER is challenged by a model of biosynthetic transport by Golgi maturation. This hypothesis does not attribute any direct function in forward movement of cargo to COPI vesicles, but instead it attributes to COPI vesicles a role in back transport of Golgi resident proteins, which is necessary to maintain the identity of the individual Golgi stacks during anterograde-directed maturation (17, 45). This view suggests unselected (bulk) forward movement of cargo through the cisternae of the Golgi apparatus and is based on the analyzed transport of molecules and aggregates too large

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to be accommodated in the lumen of an average COPI vesicle (3, 7). On the other hand, megavesicles with a considerably larger diameter have been described previously (59).

Bidirectional movement of COPI vesicles was difficult to envisage given the presence of only one species of coatomer that is recruited to the donor membrane in a single step, especially in the light of a variety of adaptor proteins in CCVs. With the advance of the human genome project, however, evidence has emerged for the presence of additional coatomer subunits, γ2- and ζ2-COP (4, 15), and sequences encoding isotypes of γ- and ζ-COP were identified also in plants and in insects (reference 20 and unpublished database sequences). Although both proteins have not been shown to be endogenously expressed, tagged cDNAs for γ2- and ζ2-COP were expressed in rat hepatocytes. Immunoprecipitation (IP) studies with anti-β-COP antibodies revealed that hemagglutinin (HA)-tagged γ2-COP was incorporated into the coatomer complex, and both HA-γ2-COP and HA-ζ2-COP colocalize with endogenous coatomer as shown by immunofluorescence light microscopy (15). The γ2-COP gene is localized within an imprinted gene cluster in humans and mice and expressed in most fetal tissues paternally (4, 29).

These data have raised the question as to an incorporation into coatomer of the endogenous isotypic subunits γ2- and ζ2-COP, the stoichiometry of subunits within a given complex, and the number of coatomer complex isotypes that exist in a mammalian cell as well as the stoichiometry of these complexes and their functions.

We show here that three isotypes of coatomer exist in mammals, which are defined by the presence of three combinations of a γ/ζ subcomplex made of the COP isotypes γ1, γ2, ζ1, and ζ2. These heptameric coatomer isotypes contain either γ1/ζ1-, γ1/ζ2-, or γ2/ζ1-COP and occur in a ratio of about 2:1:2, respectively. The coatomer isotypes differ in their location within the endomembrane system and seem to serve diverse functions, as we have characterized a population of COPI vesicles that is coated by only one coatomer isotype, defined by γ2/ζ1-COP.

MATERIALS AND METHODS

Antibodies. The following antibodies were used in this study: monoclonal antibody CM1A10 (hybridoma cells donated by J. E. Rothman, Memorial Sloan-Kettering Center, New York, N.Y.), rabbit polyclonal anti-coatomer 883 (16) that recognizes α-, β′-, and γ1-COP (refer to supplemental material available at http://www.ub.uni-heidelberg.de/archiv/4012), rabbit anti-β′-COP 891 (21, 57), rabbit anti-β-COP B1.2 (42), rabbit anti-δ-COP 877 (14), rabbit γ1-R (42) and ζ1-R (produced as described by Kuge et al.) (27), both directed against the recombinant proteins, and a monoclonal mouse anti-HA antibody (BabCO, Richmond, Calif.). Hybridoma cells producing anti-β-COP antibody M3A5 were kindly donated by Thomas Kreis. Peroxidase-labeled secondary antibodies against mouse, rabbit, and guinea pig immunoglobulin G were purchased from Dianova (Hamburg, Germany), and Alexa 488- and 546-labeled antibodies directed against rabbit, guinea pig, and mouse immunoglobulin G were obtained from Molecular Probes Europe (Leiden, The Netherlands). Antibodies specific for either γ2-, ζ1-, or ζ2-COP were raised according to standard protocols in rabbits and guinea pigs. To this end, unique peptides for the different subunits (γ2-I, amino acids [aa] 237 to 248, SRLLKETEDGHE; γ2-II, aa 589 to 599, QKAEITLVATK; ζ1, aa 143 to 154, VHRVALRGEDVP; ζ2, aa 2 to 14, QRPE AWPRPHPGE) were coupled to keyhole limpet hemocyanin by glutaraldehyde cross-linking. Affinity purification of antibodies was carried out with columns containing the respective immobilized antigenic peptides. Bound antibodies were eluted with 0.1 M glycine-HCl (pH 2.8) and subsequently neutralized with 1 M Tris-HCl (pH 8.8).

Cell culture. HepG2 cells were grown in Dulbecco's minimal essential medium containing 10% (vol/vol) heat-inactivated fetal calf serum (FCS), 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine. Clone 9 rat hepatocytes, stably expressing either HA-γ1- or HA-γ2-COP were cultivated in Ham's F-12 medium containing FCS and antibiotics at the above concentrations. Additionally, G418 was added to a final concentration of 0.5 mg/ml. All cells were grown at 37°C and 5% CO_2.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the following gel systems: 6 and 7.5% gels were used for the detection of α-, β′-, β-, γ1-, γ2-, and δ-COP; 12% gels were used for the detection of ζ1- and ζ2-COP; and 7.5 to 15% gradient gels were used when γ1-, γ2-, ζ1-, and ζ2-COP were to be separated on one and the same gel. In 6 and 7.5% gels and gradient gels, the ratio of acrylamide to bisacrylamide was 100:1. Western blotting (semidry procedure) and enhanced chemiluminescence detection (Amersham) were performed according to standard protocols.

Preparation of cytosol from tissues. Cytosol from rat livers was prepared with all steps carried out at 4°C. Livers from six rats were collected in buffer H [165 mM KOH, 50 mM HEPES, 2 mM $Mg(OAc)_2$, 1 mM dithiothreitol (pH 7.55)] supplemented with protease inhibitors (1 μM leupeptin, 10 μM antipain, 100 μM phenylmethylsulfonyl fluoride), and a 1:10 homogenate (tissue weight/buffer volume ratio) was prepared. After removing nuclei and mitochondria (by centrifugation at 6,000 × *g* for 10 min), the supernatant was subjected to centrifugations at 100,000 × *g* for 60 min followed by 180,000 × *g* for 90 min (TFT 55.38 rotor; Kontron Instruments, Engolding, Germany). The resulting supernatant was concentrated about 10-fold with a MINITAN ultra concentration unit (MWCO, 10,000; Millipore). The concentrated cytosol was finally cleared at 100,000 × *g* for 60 min, and aliquots were stored at −80°C. The cytosol had a final concentration of 24.5 mg/ml. Cytosol from a human liver (22 g) was prepared as described above and had a final concentration of 16 mg/ml. Cytosols from fresh mouse tissues were prepared in 25 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.5), 150 mM NaCl, and 2 mM EDTA completed with protease-inhibitor cocktail (Roche, Mannheim, Germany). Homogenates (1:4 tissue weight/buffer volume ratio) of the various tissues were centrifuged for 10 min at 700 × *g* and 4°C, and the lipid layers were carefully removed. Thereafter, the supernatants were subjected to a further centrifugation step for 60 min at 100,000 × *g* and 4°C. Aliquots of the supernatant (cytosol) were stored at −80°C.

Preparation of cytosol from cultured cells. Cells grown to confluence on 20 15-cm-diameter dishes were washed with phosphate-buffered saline (PBS), trypsinized, and collected in 50 ml of PBS. The cells were washed three times in PBS and finally resuspended in 3 ml of homogenization buffer (25 mM HEPES-KOH [pH 7.4], 50 mM KCl) completed with EDTA-free protease-inhibitor cocktail (Roche). Then cells were passed 10 times each through 21-, 24-, and 27-gauge needles. The resulting homogenate was centrifuged at 700 × *g* for 10 min at 4°C, and the supernatant was subjected to a further centrifugation step at 100,000 × *g* for 60 min at 4°C. Typically, the resulting high-speed supernatant (cytosol) had a concentration of 4 mg/ml.

IP of coatomer. Antibodies were incubated with protein A-Sepharose (CL-4B; Amersham) on a rotor wheel in 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40 (IP buffer) for 60 min at room temperature (RT). After washing with IP buffer, cytosol was added and incubation continued for 60 min. Beads were collected by centrifugation, washed, and eluted in reducing SDS sample buffer at 70°C.

For IP with the ζ2-specific antibody and for analysis of immunoprecipitated coatomer for its ζ isotypes, the IP was performed as described above except the following modification: IP buffer was used without detergent and IP was carried out for 30 min at RT.

Metabolic labeling of cells. Cells grown to confluence on a 10-cm-diameter dish were washed with PBS and incubated for 90 min at 37°C in methionine-free RPMI 1640 medium containing 10% dialyzed FCS. Then 300 μCi of [^{35}S]methionine (Amershan) was added, and the incubation continued for 90 min. Labeling was stopped by washing the cells with ice-cold PBS and lysing them in 0.5% NP-40 buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA for 90 min on ice. After removal of insoluble material by centrifugation, the supernatants were directly used for IPs. To determine the stoichiometry of γ1- and γ2-COP with polyclonal antipeptide antibodies, a preclear of the lysates was performed prior to the IP. To this end, presera of the antibodies 891, B1.2, and 877 were incubated with protein A-Sepharose beads as described above. After 60 min at RT, the beads were washed three times, lysates were added, and the mixtures were incubated either for 60 min at RT or overnight at 4°C on a rotor wheel. The beads were removed, and the supernatant was added to antibody-coupled protein A-Sepharose beads. Proteins bound to the antibod-

ies were eluted with SDS sample buffer and analyzed after SDS-PAGE by either a phosphorimager (Bio-Rad, Munich, Germany) or autoradiography with Kodak X-Omat films. Signals were quantified with the QuantityOne software (Bio-Rad).

Indirect immunofluorescence. Clone 9 rat liver hepatocytes were plated on coverslips 3 days before the experiment. Cells were washed with PBS, fixed with 3% paraformaldehyde and 1% (vol/vol) of a saturated picric acid solution for 20 min at RT, and permeabilized with 1% Triton X-100 for 10 min at RT. Thereafter, cells were labeled with specific antibodies in PBS for 30 min at 37°C. The primary antibodies were reacted with Alexa 488- and 546-labeled secondary antibodies (30 min, 37°C), and the samples were mounted in Mowiol 4-88 (Calbiochem, San Diego, Calif.). All cells were imaged with a Zeiss LSM 510 confocal microscope with 488- or 546-nm excitation. Images were obtained with a 63× 1.4-numerical-aperture objective, and the pinhole diameter was set to 1 Airey unit. Immunostaining was analyzed with the Zeiss LSM software. Intensity profiles of the merged channels along randomly chosen lines were obtained with the profile tool of the LSM software.

COPI vesicle budding assay. COPI vesicles were generated from rat liver Golgi membranes, as described by Serafini and Rothman (52) by using 200 μg of rat liver Golgi membranes and 20 mg of rat liver cytosol in a final volume of 1.5 ml and 10 mM guanosine-5′-(di-γ-immido)-triphosphate instead of guanosine-5′-(γ-thio)-triphosphate. The continuous sucrose gradient was fractionated from the bottom into fractions of 250 μl each. A 15-μl aliquot of each fraction was subjected to SDS–12% PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and probed with the 883 antibody. The peak COPI vesicle fractions (38 to 42% sucrose) were pooled.

For analysis of the ζ2/ζ1 and γ2/γ1 ratios, aliquots of the COPI vesicles, primed Golgi (representing Golgi membranes after incubation with cytosol), Golgi, and cytosolic coatomer enriched with the CM1 antibody were subjected to SDS-PAGE and Western blotting. γ isotypes were immunostained with the γ1-R antibody, and ζ isotypes were immunostained with antibodies specific for either ζ1- or ζ2-COP with the enhanced chemiluminescence detection system. The signals obtained were quantified with the QuantityOne software (Bio-Rad).

Immunoisolation of HA-γ2-COPI vesicles. For immunoisolation of HA-γ2 COPI vesicles, the protocol described above was used with the following modifications: 100 μg of rat liver Golgi membranes and 4 mg of cytosol from the HA-γ2-COP-expressing cell line were used. The peak vesicle fractions were pooled and diluted 1:2 with HSSB [25 mM HEPES-KOH (pH 7.4), 250 mM KCl, 2.5 mM $Mg(OAc)_2$, 0.2 M saccharose]. Thereafter, one half of the COPI vesicles was added to 50 μl of anti-HA affinity matrix (Roche) and incubated on a rotor wheel for 60 min at RT. Thereafter, the matrix was washed three times with HSSB, and vesicles bound to the matrix were eluted with SDS sample buffer. The COPI vesicles of the other half were collected by a single centrifugation step at 100,000 × *g* for 60 min at 4°C and solubilized in SDS sample buffer. Various amounts of total COPI vesicles as well as the immunoisolated vesicles were subjected to SDS-PAGE and Western blot analysis.

RESULTS

Both endogenous isotypes of γ- and of ζ-COP are incorporated into coatomer and are ubiquitously expressed in various tissues. Human DNA sequence comparison between γ1- and γ2-COP reveals an overall identity of 80% for the proteins, with only a few stretches of marked heterogeneity. Peptides were synthesized according to two such sequences in γ2-COP (aa 237 to 248 and 589 to 599) and used to raise antibodies in rabbits and guinea pigs.

ζ2-COP has an N-terminal extension of about 30 aa that is missing in ζ1-COP. A peptide according to aa 2 to 14 of this N-terminal sequence was used to generate antibodies against ζ2-COP. In addition, an antibody was raised against a peptide analogous to an internal sequence of ζ1-COP that differs from ζ2-COP (aa 143 to 154). Sequence alignment of the corresponding peptides and characterization of the antibodies are described in the supplemental materials (Fig. A1 [available at http://www.ub.uni-heidelberg.de/archiv/4012]).

To assess whether endogenous isotypes are incorporated into coatomer, the complex was isolated from a human liver cytosol by IP with an antibody specific only for intact native coatomer (40) and analyzed by Western blotting. Blots were either stained with Coomassie blue or immunoreacted with an antibody directed against γ2-COP or recombinant γ1-COP (γ1-R). A typical pattern of COPs is obtained of around 100 kDa after Coomassie staining (Fig. 1A, lane 1), with an additional band that reacts with the anti-γ2-specific antibody (Fig. 1A, lane 2), whereas two bands within this apparent molecular mass range emerge after reaction with the polyclonal antibodies against recombinant γ1-COP (Fig. 1A, lane 3). To verify the nature of the band decorated with the anti-γ2-COP antibody, it was excised from a Coomassie-stained gel and submitted to peptide analysis by matrix-assisted laser desorption ionization mass spectrometry. The peptide masses characterized are listed in Table 1 and include both peptides of the extreme N and C termini, excluding a degradation product of γ1-COP, and thus independently establish this protein as representing full-length endogenous γ2-COP. Immunoblotting of the lower-molecular-mass range with an anti-ζ2-COP antibody revealed the presence of this endogenous subunit as well. In Fig. 1B, the blot was developed with the antibody ζ2-rb (lane 1), the ζ1-specific antibody (ζ1-rb) (lane 2), and an antibody directed against complete ζ1-COP (ζ1-R) (lane 3).

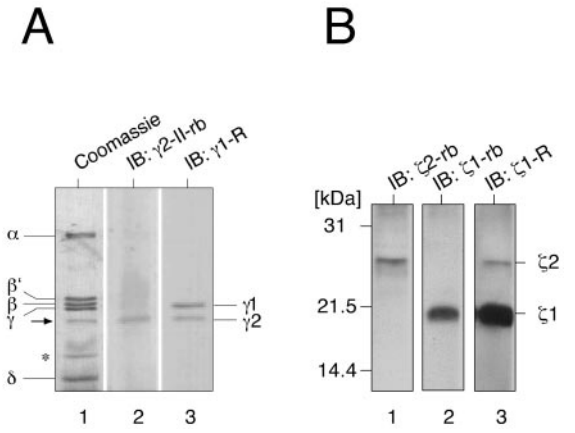

FIG. 1. Incorporation of endogenous subunits γ2- and ζ2-COP into coatomer. (A) Coatomer was immunoprecipitated with the antibody CM1 (directed against the intact complex) and analyzed by SDS–6% gel electrophoresis and Western blotting. Coomassie staining of the immunoprecipitate is shown in lane 1. An additional band close to the 100-kDa COPs is marked with an arrow (the band marked with an asterisk is due to antiserum background). Immunostaining with the antibody specific for γ2-COP and the antibody against both, γ1- and γ2-COP, respectively, is shown in lanes 2 and 3. (B) Separation of the immunoprecipitate as in panel A but on a 12% acrylamide gel. Western blotting was performed with the antibody specific for ζ2-COP (lane 1), the antibody specific for ζ1-COP (lane 2), and the antibody specific for both ζ1- and ζ2-COP (lane 3). IB, immunoblot.

Expression of γ2-COP and ζ2-COP was then analyzed in various mammalian species, showing a ubiquitous occurrence of the proteins (Fig. A2 in the supplemental material [http://www.ub.uni-heidelberg.de/archiv/4012]). We went on to analyze the expression of the γ and ζ isotypes in various tissues of the mouse. To this end, coatomer was isolated by IP from the tissue extracts indicated in Fig. 2, and the presence of isotypes was analyzed by Western blotting with the specific antibodies. As shown in Fig. 2, in all tissues investigated both isotypes of ζ- and of γ-COP are detected, possibly at various ratios.

TABLE 1. Peptides characterized in the γ2-COP band

Detected mass (kDa)	aa in:	
	γ2	γ1
873.67	25–32	25–32
881.48	214–220	
897.00	214–220	
1,046.74		591–600
1,111.75	143–151	143–151
1,135.70	833–842	
1,200.74	33–42	
1,232.82	455–465	
1,244.70	339–350	339–350
1,299.75	591–602	
1,352.81	528–538	
1,355.84	859–871	
1,446.80	578–590	
1,489.87	843–856	
1,570.93	857–871	
1,748.90	841–856	
1,863.96	127–142	
1,984.96	630–646	
2,050.07	561–577	
2,163.06	339–350	Trypsin

Stoichiometry of γ and ζ isotypes. To determine the stoichiometry of γ1- and γ2-COP in a cytosol, HepG2 cells were metabolically labeled with radioactive methionine, and coatomer was immunoprecipitated from the cell extracts with antibodies directed against various COPs. After separation by SDS-gel electrophoresis, the radioactivity in the bands was determined by autoradiography and subsequent scanning with a phosphorimager. For quantitative evaluation, the numbers of methionine residues present in an individual COP subunit were taken into account, their mass-to-radioactivity ratios were corrected accordingly, and α-COP was set to 100%. In Fig. 3A, a typical autoradiograph of an immunoprecipitate with an antibody specific for native intact coatomer is shown, as is the corresponding scanning profile. In Fig. 3B, the quantity of the large subunits α-, β′-, β-, γ1- and γ2-COP is shown as an average of the results from five independent experiments performed as outlined in Fig. 3A. These experiments indicate that γ2-COP comprises about 30% of total γ-COP and suggest a 1:1 stoichiometry of the sum of the γ isotypes with each of the

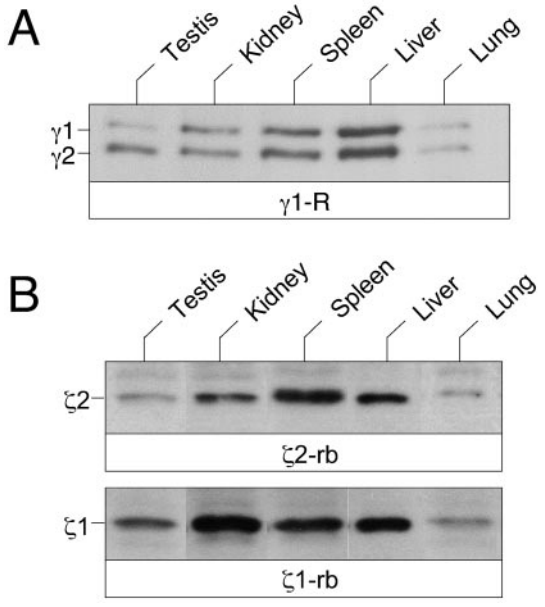

FIG. 2. Expression of γ- and ζ-COP isotypes in various tissues of the mouse. Coatomer was immunoprecipitated with antibody CM1 from various tissues of a mouse as indicated at the top of the lanes and analyzed by Western blotting. (A) Immunostaining with the antibody against recombinant γ1-COP that recognizes both γ isotypes (7.5% acrylamide). (B) Immunostaining with antibodies specific for ζ2-COP (upper panel) or ζ1-COP (lower panel) (12% acrylamide).

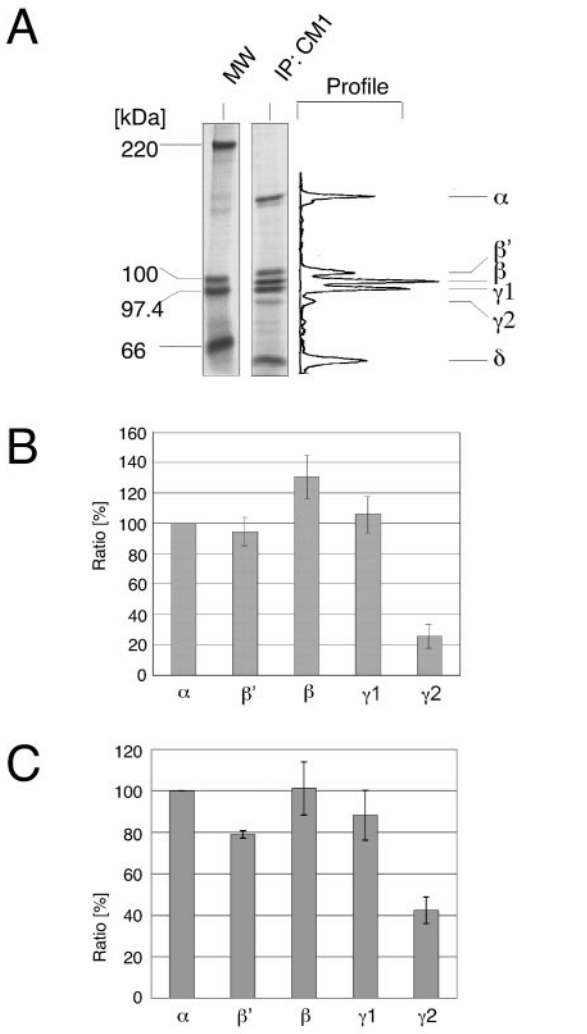

FIG. 3. Stoichiometry of γ-COP isotypes. Coatomer was immunoprecipitated from cytosol of HepG2 cells after metabolic labeling with [^{35}S]Met and analyzed by SDS–6% acrylamide gel electrophoresis and autoradiography. (A) Marker proteins indicating molecular masses are shown in the first lane, and an autoradiograph of precipitated coatomer with the antibody CM1 is shown in the second lane. (B) Quantitative evaluation of five experiments as in panel A, with the value for α-COP set to 100%. (C) Quantitative evaluation of IPs with antibodies directed against β-COP, β′-COP, or δ-COP. Each result is the average of the results from four independent experiments. Again, the value for α-COP was set to 100%. Individual protein masses were calculated by taking into account the number of Met residues in each subunit.

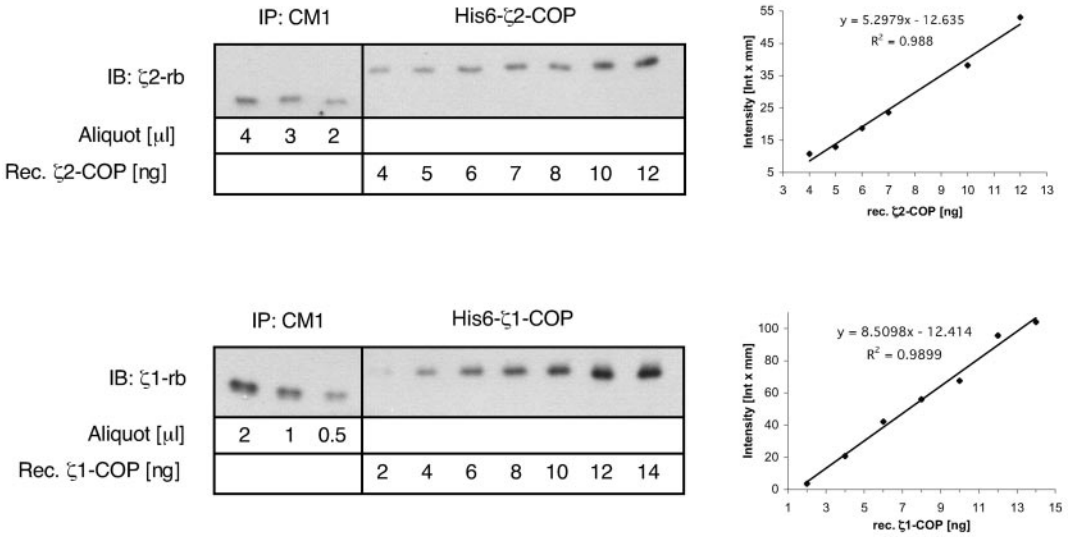

FIG. 4. Stoichiometry of ζ-COP isotypes. Coatomer was immunoprecipitated with the antibody CM1 from a mouse liver cytosol and analyzed by Western blotting (12% acrylamide). (Upper panel) Aliquots of the immunoprecipitate (left side, 4, 3, and 2 μl) were stained with the antibody specific for ζ2-COP, and their ζ2-COP contents were assessed by extrapolating from a standard mass curve derived from immunoblotting of recombinant His_6-tagged ζ2-COP (right lanes). (Lower panel) Experiments were performed as described for the upper panel. However, immunostaining was performed with antibodies against ζ1-COP, and the standard mass curve was derived from recombinant (Rec.) His_6-tagged ζ1-COP. The corresponding standard mass curves are depicted at the right side of each panel. Note that His_6-tagged ζ-COPs migrate more slowly than the endogenous proteins. IB, immunoblot.

other subunits in the complex. Part of the percentage above 100% obtained for β- and γ1-COP is likely due to the fact that, for these subunits, no baseline separation was achieved. To rule out a bias for subunits caused by the labeling time or by the antibody used for IP, similar experiments were performed with different incubation times for the metabolic labeling and antibodies directed against β-, β′-, or δ-COP for the precipitation. Different incorporation times of [^{35}S]methionine yielded similar stoichiometries of the coatomer subunits (refer to Fig. A3 in the supplemental material [http://www.ub.uni-heidelberg.de/archiv/4012]). The same holds for the use of different antibodies for the IP. In these experiments, the subunit against which the precipitating antibody is directed was not evaluated. The results from four experiments with each of the three antibodies were averaged, and from these data, the average for each subunit is depicted in Fig. 3C. Together, these results show that γ2-COP is present in coatomer at about 40%.

To assess the ratio of ζ1- and ζ2-COP in a mouse liver cytosol, quantitative immunoblotting experiments were performed. To this end, coatomer was immunoprecipitated with an antibody against the intact complex, and the concentrations of the two ζ isotypic subunits were evaluated by Western blotting, with known amounts of the recombinant subunits as mass standards. A typical experiment is shown in Fig. 4. From the average of the results from three independent experiments, we conclude that ζ2-COP represents about 20% of the total ζ-COP isotypes.

These data indicate but do not prove that only one isotype of γ- and ζ-COP, each, is present in a given coatomer complex. To further analyze the presence of γ1- and γ2-isotypes in a given coatomer, cell lines were used that stably express HA-tagged γ1- or γ2-subunits (15). To facilitate their detection, the proteins were metabolically labeled with radioactive methionine, and coatomer was immunoprecipitated by using either an antibody against β′-COP (to precipitate all coatomer species) or an antibody directed against the HA tag. Immunoprecipitates were then separated by SDS-gel electrophoresis, and the COPs were visualized by autoradiography. The results are shown in Fig. 5A. As expected, the anti-β′-COP antibody precipitates the complete coatomer, with both γ isotypes present independent of the cell line used (Fig. 5A, lanes 2 and 4). In contrast, anti-HA antibodies exclusively precipitate γ1-COP from the HA-γ1-COP-expressing cell line (Fig. 5A, lane 3), and likewise, when used for the HA-γ2-COP-expressing cell line, they do precipitate γ2-COP but not γ1-COP (Fig. 5A, lane 5). This finding establishes that the two γ isotypes do not occur in one and the same complex but that a given coatomer is characterized by the presence of either γ1- or γ2-COP.

The presence of ζ isotypes in purified rabbit liver coatomer was analyzed by IP with the ζ2-COP specific antibody ζ2-gp. To this end, the amount of immunoprecipitated coatomer was standardized by immunostaining of known amounts of coatomer with an anti-β-COP antibody (Fig. 5B, upper panel, lanes 1 and 2), revealing the total amount of coatomer in the precipitate (Fig. 5B, upper panel, lane 3). The ζ2-COP-specific antibody (lane 3) precipitated an amount of coatomer (between 0.5 and 0.2 μg) that allows a safe detection of ζ1-COP (Fig. 5B, lower panel, lanes 1 and 2). As a result, no ζ1-COP is detected in the ζ2-COP-specific precipitate (Fig. 5B, lower panel, lane 3). Thus, as with the γ isotypes, only one of the ζ isotypes is present in a given complex.

Together with the experiments shown in Fig. 3 and 4, this establishes that coatomer isotypes exist, each with seven subunits that contain either γ1- or γ2-COP and ζ1- or ζ2-COP.

Combinations of ζ- and γ-COPs define three distinct coatomer isotypes. Taking into account that an individual

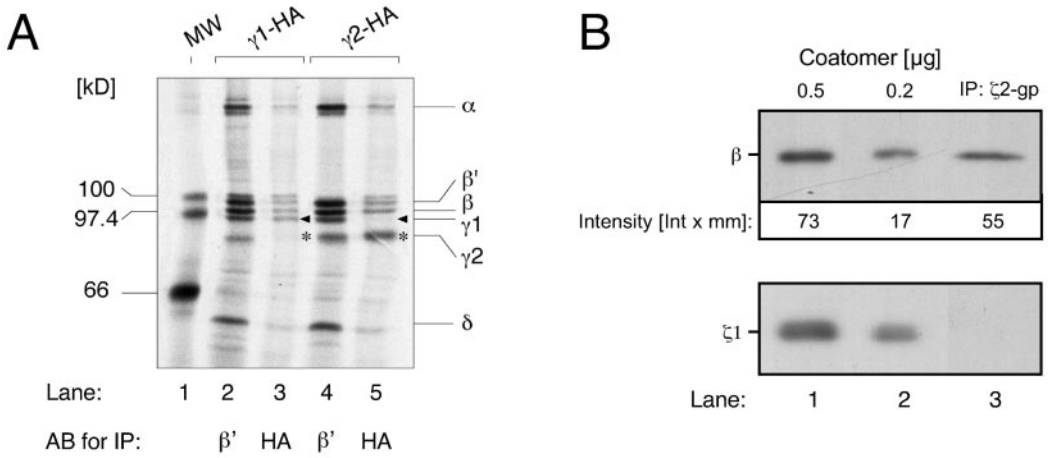

FIG. 5. An individual coatomer complex contains only one isotype of each, γ- or ζ-COP. (A) Cell lines stably expressing HA-tagged versions of either γ1-COP or γ2-COP were grown in the presence of [^{35}S]Met, and coatomer was isolated from their cytosols by IP and analyzed by SDS–6% acrylamide gel electrophoresis and autoradiography. IP with antibodies against β′-COP (lane 2) or against the HA tag (lane 3), both from a HA-tagged γ1-COP-expressing cell line, is shown. IP with a β′-COP-specific antibody (lane 4) and a HA-specific antibody (lane 5), both from a HA-tagged γ2-COP-expressing cell line, is shown. In lanes 3 and 5, the position of γ1-COP is marked with an arrowhead and the location of γ2-COP is marked with an asterisk. (B) IP of coatomer from rabbit liver cytosol (12% acrylamide) with an antibody directed against ζ2-COP (lane 3). Lanes 1 and 2, defined amounts of coatomer as mass standards. Immunostaining with antibodies (AB) against β-COP (upper panel) and against ζ1-COP (lower panel) is shown.

coatomer molecule contains only one copy of each subunit, we analyzed the combinations of ζ and γ isotypes, each of which would define a distinct complex. To this end, coatomer was immunoprecipitated from rat liver cytosol with a ζ2-specific antibody (ζ2-gp) and analyzed by Western blotting with an antibody that recognizes both γ1- and γ2-COP. As depicted in Fig. 6A, lane 2, only γ1-COP is coprecipitated with ζ2-COP. As a control, coatomer was immunoprecipitated with the antibody CM1 that recognizes native coatomer (Fig. 6A, lane 1), giving rise to both γ isotypes. This result establishes the existence of a coatomer isotype defined by the presence of γ1- and ζ2-COP, representing about 20% of total coatomer in liver cytosol (cf. Fig. 4).

A similar analysis for ζ1-COP was precluded because the specific anti-ζ1-COP antibody turned out to not be useful in precipitation experiments. Therefore, to assess coatomer isotypes defined by combinations with ζ1-COP, we performed the following depletion experiment. Rat liver cytosol was reacted three times in a series with the precipitating anti-ζ2-COP antibody. As shown in Fig. 6B, lane 4, after this treatment no ζ2-COP was detected in the precipitate. Total proteins were precipitated with trichloroacetic acid from an aliquot of the

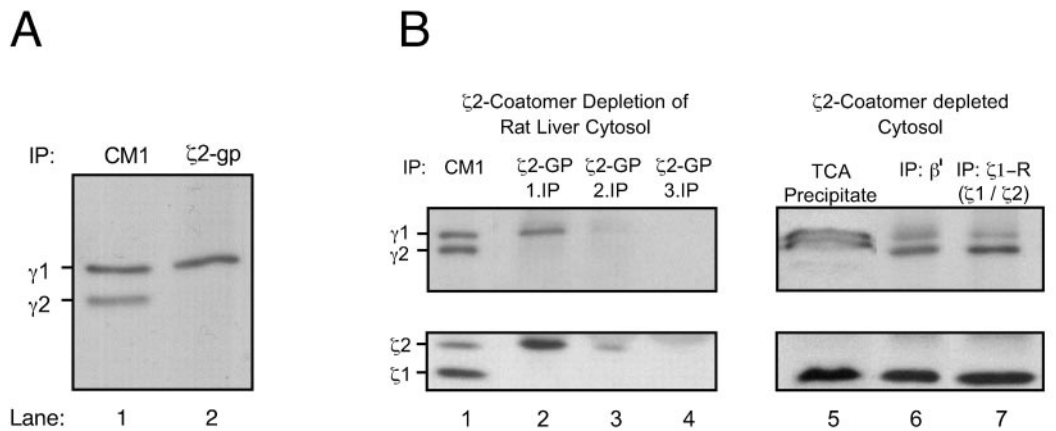

FIG. 6. Coatomer complexes defined by distinct combinations of γ- and ζ-COP isotypes. (A) IP of coatomer from rat liver cytosol with antibodies directed against complete coatomer (lane 1) or against ζ2-COP (lane 2). Western blotting was performed with the antibody γ1-R directed against both γ-subunits (7.5% acrylamide). (B) Immunodepletion of ζ2-COP in rat liver cytosol with subsequent analysis of coatomer remaining in the supernatant. Coatomer was immunoprecipitated with the antibody CM1 directed against the complete complex (lane 1) or subsequently immunoprecipitated three times with an antibody specific for ζ2-COP (lanes 2, 3, and 4). Trichloroacetic acid precipitate of an aliquot of the ζ2-COP-depleted cytosol (lane 5). IP of ζ2-COP-depleted cytosol with antibodies against all coatomer species (lane 6) or against recombinant ζ1-COP, recognizing both ζ1- and ζ2-COP (lane 7). Samples were analyzed by Western blotting (7.5 to 15% acrylamide) with antibodies directed against γ1- and γ2-COP (upper panels) or against ζ1- and ζ2-COP (lower panels).

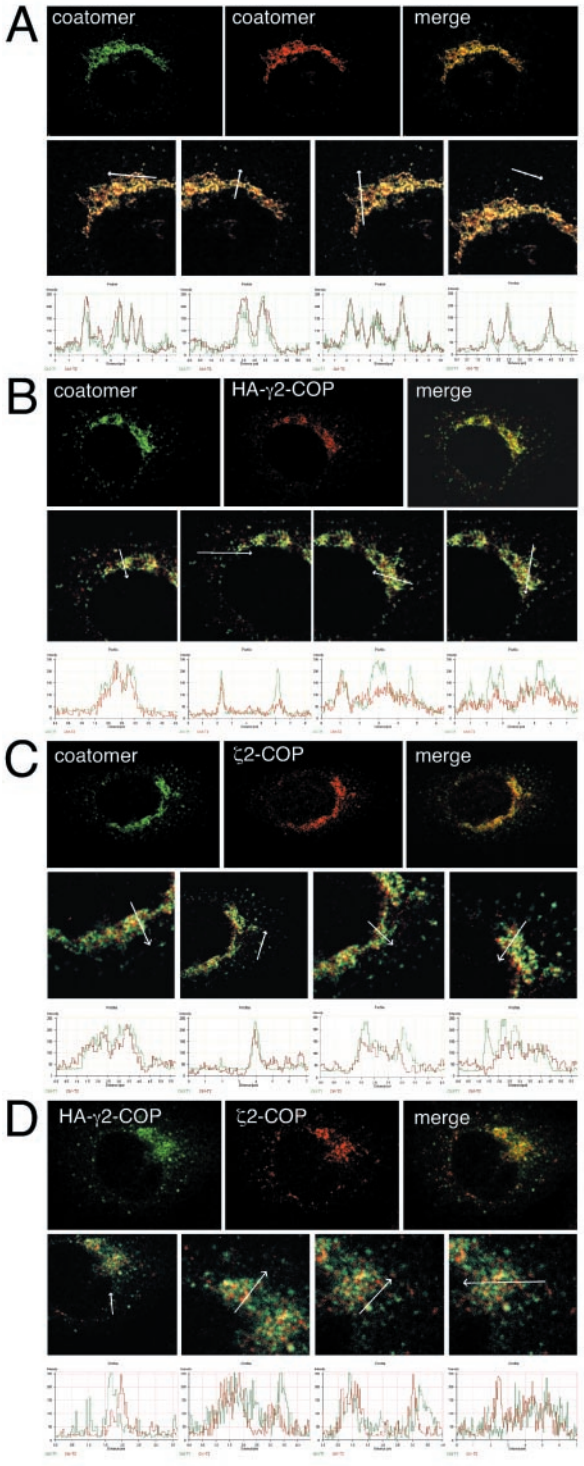

FIG. 7. Differential localization of different coatomer isotypes. Rat liver hepatocytes were grown at 37°C on coverslips. Cells were fixed, permeabilized, and indirectly immunostained with Alexa 488- and 546-labeled secondary antibodies. (A) Total coatomer was immunostained in clone 9 rat liver hepatocytes, with either the anti-coatomer antibody CM1 (green channel) or the anti-β′ antibody 891 (red channel). (B) Clone 9 rat liver hepatocytes stably transfected with HA-γ2-COP were immunostained with an antibody against all coatomer isotypes (891, green channel) and with an anti-HA antibody (red channel). (C) Clone 9 rat liver hepatocytes were immunostained with the anti-coatomer antibody CM1 (green channel) and with the anti-ζ2-COP antibody ζ2-gp (red channel). (D) Clone 9 rat liver hepatocytes stably transfected with HA-γ2-COP were stained with the anti-HA antibody (green channel) and with the anti-ζ2-COP antibody ζ2-gp (red channel). The upper right picture of each panel shows the merged image. Overlapping areas appear yellow. Each panel provides four different intensity profiles of both channels along randomly chosen lines (white arrows).

supernatant after the last round of IP. Residual γ and ζ subunits were visualized with specific antibodies, indicating complete depletion of ζ2-COP (Fig. 6B, lane 5, compare the relative staining intensities of the various subunits in lane 1 of Fig. 6B, where total coatomer was immunoprecipitated with the antibody CM1). From further aliquots of the ζ2-depleted cytosol, IPs were now performed with an anti-β′-COP antibody (to IP all isotypes of the complex) (Fig. 6B, lane 6) and with the antibody against both ζ isotypes (Fig. 6B, lane 7). Clearly, both antibodies give rise to similar patterns containing γ1- and γ2-COP, and in light of the absence of detectable amounts of ζ2-COP in the depleted cytosol, we must conclude that two isotypes of coatomer exist with ζ1-COP, one in combination with γ1-COP and one in combination with γ2-COP. With ζ2-COP coatomer comprising about 20% of the total complex (cf. Fig. 4), and γ2-COP comprising about 40% (cf. Fig. 3C), we conclude that the γ1/ζ1 isotype represents about 40% of the total. In summary, coatomer isotypes exist in a liver cytosol that are defined by their subunits γ1/ζ1, γ1/ζ2, and γ2/ζ1 at a ratio of about 2:1:2, respectively.

Differential subcellular localizations of coatomer isotypes. The appreciable amounts of each of the three isotypes present suggests the possibility that individual coatomers might indeed play specialized roles. As a first step to investigate this possibility, we analyzed the localization of individual isotypes by immunofluorescence microscopy. In Fig. 7A, localization of total coatomer species was visualized in rat liver hepatocytes with two different anti-coatomer antibodies, the anti-β′-COP antibody 891 and the monoclonal antibody CM1 that recognizes only the native coatomer complex. Both antibodies show a typical Golgi-like perinuclear staining pattern, and intensity profiles along randomly chosen lines reveal a complete colocalization. In Fig. 7B, localization of total coatomer is compared with the γ2/ζ1 subtype in rat liver hepatocytes stably transfected with HA-γ2-COP by using an antibody against the HA epitope tag. Consistent with previous results (15), HA-γ2-COP colocalizes predominately with total coatomer and thus resides in organelles of the early biosynthetic transport (49, 54). The same holds for the γ1/ζ2 coatomer subtype by using the ζ2-COP-specific antibody ζ2-gp (Fig. 7C). However, we observe substantially more overall coatomer signals in both cases that appear in addition to the colocalization with the isotypic complexes (refer to intensity profiles in Fig. 7B and C). These additional signals indicate isotypes other than γ2/ζ1 coatomer (Fig. 7B) or γ1/ζ2 coatomer (Fig. 7C), showing a differential localization of the isotypes. This observation was strengthened by a direct comparison of the immunostaining obtained with the γ1/ζ2 and γ2/ζ1 isotypes in the HA-γ2-COP-expressing cell line. In addition to a partial colocalization, a differential localization can clearly be observed (Fig. 7D).

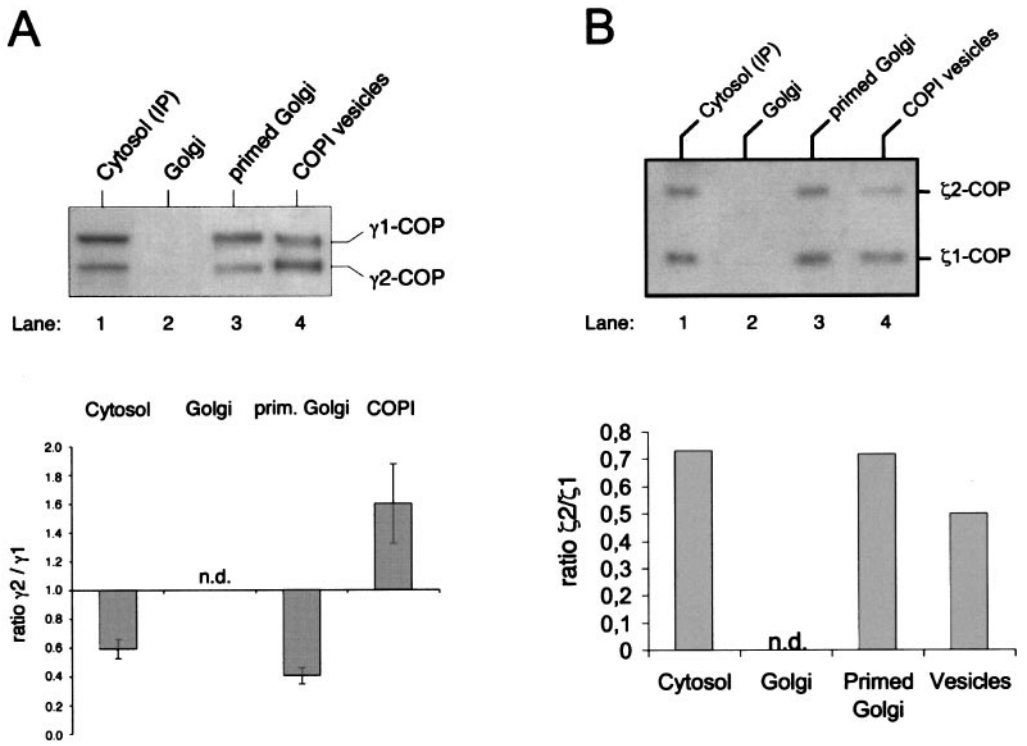

FIG. 8. The γ2/ζ1-coatomer isotype is preferentially incorporated into COPI-coated vesicles. (A) Analysis of γ-COP isotypes by Western blotting (6% acrylamide) with the antibody directed against both γ subunits (γ1-R). Lanes: 1, immunoprecipitate from a rat liver cytosol with an antibody against native coatomer (CM1); 2, aliquot of the rat liver Golgi used; 3, primed Golgi; 4, purified COPI vesicle fraction. (B) Analysis of ζ-COP isotypes (12% acrylamide). Samples are the same as in panel A; however, they are stained with antibodies directed against both ζ subunit isotypes (ζ1-rb and ζ2-rb). Quantitation of the experiments is depicted in graphs below panels A and B. n.d., not detectable.

Preferential incorporation into COPI vesicles of γ2/ζ1 coatomer. The differential localization within the endomembrane system of coatomer isotypes suggests specialized functions for the individual complexes. To address this possibility, we investigated the isotype composition of coatomer recruited to Golgi membranes and vesicles in an in vitro COPI vesicle budding assay. To this end, rat liver Golgi was incubated under the conditions established to prime vesiculation with rat liver cytosol (52). From such incubations, the primed Golgi fraction was isolated by centrifugation and treated with 250 mM KCl to release the COPI vesicles attached, and vesicles were purified by differential centrifugation. At each step, an aliquot of the samples was analyzed by Western blotting for the presence of γ1- and γ2-COP. The results are shown in Fig. 8A. In lane 1, the ratio of the staining intensities in the cytosol of γ1- and γ2-COP is shown. Whereas the Golgi-enriched fraction does not display detectable amounts of coatomer (as expected) (Fig. 8A, lane 2), the ratio of γ1- and γ2-COP coatomer recruited to the Golgi after priming is similar to that in the cytosol used (Fig. 8A, lane 3). In contrast, in the COPI vesicle population isolated from the primed Golgi, the ratio of γ1- to γ2-COP is markedly changed, with γ2-COP now prevailing (Fig. 8A, lane 4). A quantitative evaluation of three independent experiments is given in Fig. 8A, lower panel.

A similar experiment was performed to analyze whether a preference exists for the incorporation into COPI vesicles for ζ-COP isotypes. As shown in Fig. 8B, the ratio of ζ2- to ζ1-COP is indeed changed in the vesicle population (Fig. 8B, compare lane 4 with lanes 1 and 3). A quantitative evaluation of this experiment is depicted in the lower panel of Fig. 8B. The reduction of the γ1/ζ2 coatomer observed is consistent with the enrichment of the γ2/ζ1 subtype in COPI vesicles.

These results suggested that individual populations of COPI vesicles exist, each defined by a specific coatomer isotype. To address this possibility, immunoisolation experiments were performed. To this end, COPI vesicles were generated in vitro from rat liver Golgi membranes employing cytosol from the HA-γ2-COP-expressing cell line. After purification by differential centrifugation, the vesicle fraction was incubated with immobilized HA antibodies, and the immunoisolated fraction was analyzed by Western blotting for its content of γ1- and γ2-COP and compared with the input vesicle population. The result is shown in Fig. 9. The amount of COPI coat present was visualized by immunostaining with the anti-coatomer antibody (883) that recognizes α-, β′-, and γ1-COP (Fig. A4 in the supplemental material [http://www.ub.uni-heidelberg.de/archiv/4012]). γ2-COP was probed with the anti-HA antibody. In a sample of the immunoisolated vesicle fraction (Fig. 9, lane 1) that would allow a safe detection of γ1-COP (Fig. 9, compare lanes 2 and 3 with lane 1), γ1-COP is almost completely depleted. Given the technical limits of immunoisolation of

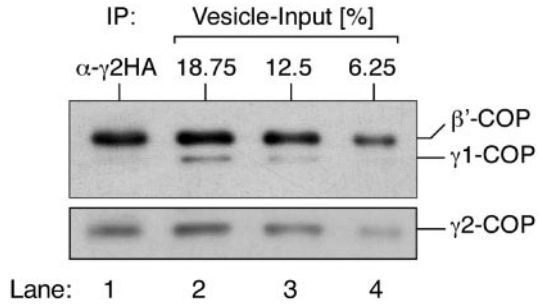

FIG. 9. Characterization of COPI vesicles immunopurified with antibodies against HA-tagged γ2-COP. Western blot analyses (7.5% acrylamide) of vesicles immunoisolated with an anti-HA antibody from a vesicle preparation generated with cytosol from a HA-tagged γ2-COP-expressing cell line (lane 1) and of various mass amounts of the vesicle preparation, as indicated at the top of lanes 2, 3, and 4, are shown. β′-COP and γ1-COP were stained with an antibody recognizing both proteins (883, upper panel), and γ2-COP was stained with the HA-specific antibody (lower panel).

membranes in the absence of detergent, this finding strongly supports the existence of an individual type of COPI vesicles coated with the γ2/ζ1 isotype of the complex.

DISCUSSION

Coatomer is a stable protein complex composed of seven subunits, α-, β-, β′-, γ-, δ-, ε-, and ζ-COP. Three subcomplexes have been characterized that make up its architecture. A trimeric subcomplex of α-, β′-, and ε-COP, a dimeric subcomplex of β- and δ-COP, and a dimeric subcomplex of γ- and ζ-COP (14, 32, 42). These subcomplexes form the overall complex by additional interactions of various subunits (13). Human genomic sequences have shown the presence of an additional gene for γ-COP, and the corresponding mRNA was described (4). Likewise, a cDNA for a second ζ-COP was found (A. Whitney, personal communication, group of the late Thomas Kreis) (SwissProt accession number Q9JHH9). The cDNAs of both genes were cloned with an HA tag in rat hepatocytes and found to be incorporated into coatomer. Additionally, the tagged versions of γ2-COP and ζ2-COP colocalize with coatomer as shown by immunofluorescence microscopy (15). In light of the fact that γ- and ζ-COP form within coatomer a dimeric subcomplex, which is involved in membrane binding of the overall complex (22, 23, 62), this concurrence of γ and ζ isotypes might indicate specialized functions of the respective complexes.

The presence of isotypic COPs in conventional preparations of coatomer may have escaped our (and other investigators') notice because the γ2 isotype migrates a little faster than the family of 100-kDa subunits and cross-reacts with most of the antibodies against γ1-COP. Therefore, γ2-COP was misleadingly addressed as a degradation product of γ1-COP. ζ2-COP, making up only about 20% of total ζ-COP, was frequently seen as a contamination.

The above findings raised questions as to the relative masses of COP isotypes, about their stoichiometry within a coatomer complex, and about specific combinations of γ- and ζ-COP that would define individual complexes. Here we have shown that the endogenous isotypes of both γ- and ζ-COP are incorporated individually into coatomer, i.e., into complexes, each with seven subunits. We find a total of three combinations, γ1/ζ1-, γ1/ζ2-, and γ2/ζ1-COP that define coatomer isoforms in a liver cytosol at a ratio of about 2:1:2 (Fig. 10). Thus, the novel isotypic complexes make up more than one half of total coatomer, and this remarkable amount suggested to us a possibility that different functions may be attributed to the individual isoforms.

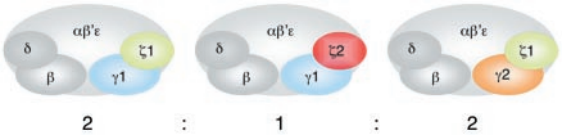

FIG. 10. Three coatomer isoforms in mammals defined by their γ/ζ combinations.

However, two hybrid studies have pointed to a functional redundancy of the isotypes because all four possible interactions of γ and ζ subunits were found (15). A structural discrimination of protein-protein interactions may, however, depend on the context of the overall structure of a complex, and thus, interactions of individual subunits do not necessarily reflect an in vivo situation. Moreover, these findings might be a consequence of incorporation of the subunits into yeast coatomer, a unique complex that has not evolved a mechanism to discriminate between individual COP isotypes.

At this point we can only speculate about a structural basis for the specific γ/ζ-COP interactions that define the three coatomer isotypes in mammals. At the level of primary structure, only two short sequences are found in γ1- and γ2-COP of marked heterogeneity (which were used to raise anti-γ2-specific antibodies) (cf. supplemental material [http://www.ub.uni-heidelberg.de/archiv/4012]). The ζ isotypes differ more markedly, with an N-terminal extension of about 30 aa in ζ2-COP. This additional sequence might be involved either in the specificity of ζ2-COP for γ1-COP and/or in differential functions that the γ1/ζ2 subcomplex might confer to its coatomer isotype.

Interestingly, our gene bank data mining has revealed a coexistence of γ and ζ isotypes, i.e., the existence of a second γ-COP in an organism is linked to the presence of a second ζ-COP, as evidenced in all vertebrates investigated as well as in plants and some insects. Altogether, this points to a specialized function of γ/ζ-COP dimeric subcomplexes within coatomer isotypes.

This view is strengthened by our finding of differential locations of the subunits in membranes of the early secretory pathway and, finally, in isolating a population of COPI vesicles quite homogeneously coated with a single isotype of coatomer, defined by γ2/ζ1-COP.

A variety of functions have been proposed for coatomer. The complex was localized to endosomal membranes, and a role has been discussed in retrieving proteins from endosomes back to the Golgi and the ER (10, 18, 61). Peroxisomes were described to bind coatomer in an ADP-ribosylation factor- and GTP-dependent manner, and consequently the COPI coat was suggested to be involved in a transport pathway between these organelles and the ER. This pathway might serve peroxisome biogenesis by mediating exchange of ER-derived proteins and

lipids (41, 58). As opposed to a role in biosynthetic anterograde protein transport (35, 37, 39), COPI vesicles are held to mediate retrograde transport, between the Golgi and the ER (9, 30, 56) or retrograde transport within the Golgi apparatus to maintain the identity of Golgi cisternae during maturation of this organelle system (7, 17, 33). Furthermore, a structural role was attributed to coatomer in the maintenance of the Golgi apparatus (19) and as a device to protect the Golgi membrane from uncontrolled fusion (12, 31, 50).

With the characterization of three isotypes of coatomer, the stage is now set for experiments with a focus on individual functions that individual isoforms of the complex may serve.

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