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A single binding site for dilysine retrieval motifs and p23 within the γ subunit of coatomer

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ABSTRACT **Coatomer, the major component of the coat of COPI transport vesicles, binds both to the dilysine motif of resident membrane proteins of the endoplasmic reticulum and to the cytoplasmic domain of p23, a major type I membrane protein of COPI vesicles. Using a photocrosslinking approach, we find that under native conditions a peptide analogous to the cytoplasmic domain of p23 interacts with coatomer exclusively through its γ subunit and shares its binding site with a KKXX retrieval motif. However, upon dissociation of coatomer, interaction with various subunits, including an α-, β′-, ε-COP subcomplex, of the photoreactive peptide is observed. We suggest that, under physiological conditions, interaction of coatomer with both endoplasmic reticulum retrieval motifs and the cytoplasmic domain of p23 is mediated by γ-COP.**

COPI-coated vesicles ferry proteins within the early secretory pathway (1). Their coat is composed of two components: coatomer (2), an oligomeric protein complex that consists of seven distinct subunits [α to ζ coat proteins, (COPs)] (3–14), and ADP-ribosylation factor 1 (ARF1) (15, 16). The major membrane proteins of COPI vesicles are p24 (17) and p23 (18), members of the p24 family of type I membrane proteins, characterized by a large luminal domain, a single transmembrane domain and a short cytoplasmic tail (17). The cytoplasmic domain of p23 is structurally similar but not identical to the KKXX retrieval motif of membrane proteins of the endoplasmic reticulum (ER) (19, 20) (Fig. 1*A*).

Coatomer is able to bind to both ER retrieval motifs (11, 21) and dibasic/diphenylalanine motifs of members of the p24 family (18, 22). This property was taken as an indication that the coat complex is involved in retrograde transport (21, 23)—e.g., in sorting of cargo into the Golgi-ER pathway (24, 25)—in addition to playing a role in anterograde transport (3, 25–27).

Interaction of coatomer with characteristic KKXX retrieval motifs (11, 13, 21, 28) as well as with cytoplasmic domains of members of the p24 family (18, 22) was studied by several groups with various results, using different experimental approaches. These include *in vitro* binding studies with chimeric proteins (21, 22, 28) as well as yeast genetics (11), and they suggest a trimeric subcomplex of α-, β′-, ε-COP to contain the binding site. Studies with cell lysates and fusion proteins containing various cytoplasmic tail domains led to a concept of a differential interaction of coatomer with these domains: a subcomplex of α-, β′-, and ε-COP was proposed to contain a binding site for dilysine motifs, whereas β-, γ-, and ζ-COP were suggested to mediate coatomer binding by means of diphenylalanine motifs (22). On the other hand, coatomer subunit γ was described as the COP that binds to a characteristic ER retrieval motif, the cytoplasmic peptide of Wbp1p (13) (Fig. 1*A*).

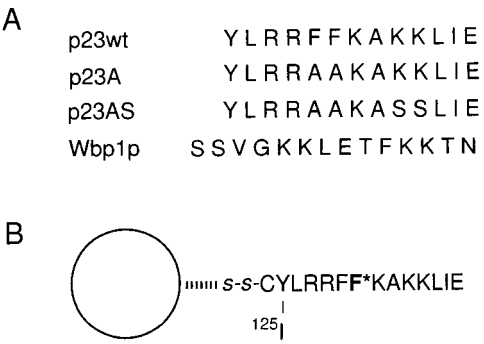

FIG. 1. Peptides used in this study. (*A*) Peptide sequences of p23wt, p23A, p23AS, and Wbp1p. p23wt represents the cytoplasmic domain of p23 known to bind coatomer. p23A binds coatomer less efficiently than the wild-type peptide, whereas the peptide p23AS has lost this capability. Wbp1p represents the cytoplasmic domain of a subunit of the yeast N-oligosaccharyltransferase complex and contains a characteristic KKXX ER-retrieval motif also known to interact with coatomer. (*B*) Immobilized, photoreactive p23wt peptide (^{125}I-F*-p23wt). The natural Phe at position −8 was replaced by the photoreactive analogue L-4-[3-(trifluoromethyl-3*H*-diazirin-3-yl)]phenylalanine (F*, Tmd-Phe). F*-p23wt peptide was immobilized by coupling to thiopropyl-Sepharose by a disulfide bond and was radioactively labeled with [^{125}I]iodine.

As the structural characteristics of ER retrieval motifs differ from those of the p23 cytoplasmic tail (Fig. 1*A*), we were interested whether both types of peptide share a common coatomer binding site or whether different sites exist in the complex. Therefore, and to eventually resolve the above mentioned discrepancy, photocrosslinking studies were performed with peptides analogous to the cytoplasmic tail of p23.

Here we show that in intact coatomer, it is exclusively its γ subunit that binds to the cytoplasmic tail peptide of p23. This binding can be inhibited by an excess of a typical KKXX peptide, indicating that both p23 and ER-resident membrane proteins share a common binding site within γ-COP. However, upon dissociation of coatomer, α-COP present in a subcomplex of α-, β′-, ε-COP as well as dissociated β-COP and γ-COP are photocrosslinked to the p23 peptide.

MATERIALS AND METHODS

Materials. Coatomer was isolated as described (29). Anti-COP antibodies against the individual coatomer subunits are

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Abbreviations: ARF, ADP-ribosylation factor; CHO, Chinese hamster ovary; COP, coat protein; COPI, protein coat composed of ARF1 and coatomer; ER, endoplasmic reticulum; Tmd, L-4-[3-(trifluoromethyl-3*H*-diazirin-3-yl)]; F*, Tmd-Phe; TX100, Triton X-100.
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described in ref. 29; anti-p23 cytoplasmic domain antibody is described in ref. 18. Hybridoma cells producing anti-β-COP antibody M3A5 were kindly donated by Thomas Kreis (University of Geneva, Switzerland).

Synthesis of Peptides. Synthetic peptides used in this study are shown in Fig. 1. Photoactivatable p23wt peptide was synthesized by replacing the natural Phe at position −8 by the photoreactive analogue L-4-[3-(trifluoromethyl-3*H*-diazirin-3-yl)]phenylalanine (Tmd-Phe, F*; Photoprobes, Knonau, Switzerland) as described (13). F*-p23wt peptide was coupled to thiopropyl-Sepharose (Amersham Pharmacia Biotech) by a disulfide bridge according to the manufacturer's instructions. For this purpose an N-terminal Cys was introduced (Fig. 1*B*). F*-p23wt in solution (10 nmol in 40 μl of 0.25 M sodium phosphate, pH 7.5) or coupled to Sepharose (10 nmol, ≈50 μl of sedimented beads in 150 μl of 0.25 M sodium phosphate, pH 7.5) was labeled with [^{125}I]iodine [1 mCi (37 MBq)] by using Iodo-Gen iodination reagent (10 μg, Pierce) according to the manufacturer's instructions. Iodide was removed by chromatography of the soluble peptide on a Sep-Pak C_{18} cartridge (Millipore). Peptide was eluted from the column with 75% (vol/vol) acetonitrile/0.1% trifluoroacetic acid in water. Upon addition of 0.5% (vol/vol) pyridine the solvent was evaporated in a Speed Vac. The peptide was dissolved in 70% (vol/vol) methanol/25 mM Tris·HCl, pH 7.5/5 mM dithiothreitol (DTT) and stored at −20°C. Iodinated immobilized peptide was dialyzed against phosphate-buffered salt solution to remove iodide and stored at 4°C. Specific radioactivity was 8×10^4 cpm/pmol. Iodinated peptides were used within 3 weeks.

Preparation of Cell Lysate. Chinese hamster ovary (CHO) cells (1×10^8) were lysed in 1.5 ml of lysis buffer A [0.5% Triton X-100 (TX100)/25 mM Tris·HCl, pH 7.4/100 mM NaCl/1 mM EDTA] containing 1 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), pepstatin A (1 μg/ml), and aprotinin (2 μg/ml). Insoluble material was removed by two centrifugation steps, at 1,500 × *g* for 10 min, and at 100,000 × *g* for 30 min at 4°C. High-salt extracts were prepared in buffer B (0.5% TX100/50 mM Tris·HCl, pH 7.4/300 mM NaCl) and the same set of protease inhibitors as in buffer A. For labeling of native coatomer, freshly prepared lysate was used. Freezing and thawing of cell lysate results in disassembly of coatomer, independent on the salt concentration used. Thus, for labeling of dissociated coatomer, lysate was frozen in liquid nitrogen, stored at −80°C, and thawed shortly before use. Identical results were obtained with cell lysate prepared in buffers A and B.

Photocrosslinking Experiments. ^{125}I-F*-p23wt Sepharose was pretreated with buffer C (0.5% TX100/50 mM Hepes, pH 7.5/300 mM NaCl/1 mM EDTA) containing 0.5 mg of bovine serum albumin (BSA) per ml for 1 h, followed by two washes with buffer C. Coatomer (4 μg, ≈7 pmol, centrifuged at 12,000 × *g* for 20 min shortly before use to remove any aggregated material) was incubated with ^{125}I-F*-p23wt Sepharose (1 nmol) in a total volume of 150 μl of buffer C for 90 min at room temperature on a rotator. Samples were washed three times with buffer C and once with buffer D (25 mM Tris·HCl, pH 7.4/100 mM NaCl/1 mM EDTA). Beads were resuspended in a total volume of 20 μl in this buffer and irradiated at 365 nm (100 W; Spectroline) for 2 min on ice. For immunoprecipitation of individual COPs, peptide was released from the Sepharose by treatment with buffer D containing 20 mM DTT, 1% SDS, 0.5 mg of BSA per ml for 15 min at 60°C in a total volume of 100 μl. Samples were dialyzed in collodion bags (Sartorius) against buffer D to remove DTT. For immunoprecipitation samples were transferred to microcentrifuge tubes and adjusted to a final concentration of 0.2% SDS, 1% TX100, 0.25 mg of BSA per ml in buffer D prior to addition of antibodies bound to staphylococcal protein A-Sepharose (CL-4B, Amersham Pharmacia Biotech). After incubation for 12 h at 4°C on a rotator, samples were washed three times with buffer A and once with this buffer without detergent. Immunoprecipitated material was released by heating the Sepharose beads in SDS-sample buffer under reducing conditions.

Photocrosslinking experiments with ^{125}I-F*-p23wt peptide in solution and with CHO lysates were performed as follows: ^{125}I-F*-p23wt peptide (1 nmol) was dried in a Speed Vac prior to addition of lysate (400 μg of protein) in a total volume of 33–35 μl of buffer A or B. Incubations were performed for 1 h on ice prior to photoactivation for 2 min on ice. Subsequently, coatomer was immunoprecipitated with an anti-γ-COP antibody bound to protein A-Sepharose without prior dissociation in buffer A. For analysis of individual COPs, immunoprecipitation was carried out in two steps: The first round was performed with an anti-γ-COP antibody and protein A-Sepharose (see above). After washing twice with buffer A, coatomer in the precipitates was dissociated by heating the samples in 1% SDS for 10 min at 95°C. Thereafter, individual COPs were immunoprecipitated in a second round with antibodies against β-, β′-, or γ-COP in buffer A containing final concentrations of 0.2% SDS and 1% TX100. For peptide competition experiments, freshly prepared CHO lysate (400 μg) was incubated with nonradioactive peptides (p23wt, Wbp1p, p23A, or p23AS) at concentrations of 0.3 mM (10-fold molar excess over ^{125}I-F*-p23wt) for 1 h on ice prior to incubation with the radioactive photolabile probe as described above.

SDS/PAGE, Autoradiography, and Immunoblot Analysis. Gradient gels of 7.5–16.5% were used when all COPs were to be separated on one gel. For the separation of α-, β′-, β-, γ-, and δ-COP 7.5% gels were used, and for ε- and ζ-COP, 15% gels. Electrophoresis was performed under reducing conditions on separating gels with a ratio of acrylamide to bisacrylamide of 100:1 (wt/wt). For detection of radioactive photocrosslinked products and immunoblot analysis, proteins were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Millipore) followed by autoradiography or by detection of COPs with peroxidase-conjugated antibodies (goat anti-mouse for M3A5 and goat anti-rabbit for all other antibodies) and enhanced chemiluminescence (ECL; Amersham).

RESULTS

Photocrosslinking of Coatomer to p23 Tail Peptide Results in Labeling of γ-COP. In an earlier study, we demonstrated that interaction of coatomer with a peptide analogous to the C terminus of Wbp1p, a characteristic ER retrieval motif, is mediated by the γ subunit of the complex (13). The cytoplasmic domain of p23, a major membrane protein of COPI vesicles, binds to coatomer with similar efficiency, however with different structural characteristics than ER-retrieval signals (18). We therefore investigated whether another binding site within coatomer exists for p23, or whether KKXX motifs and p23 share their binding site within the complex. To this end, we performed crosslinking experiments with peptides analogous to the cytoplasmic domain of p23. To orient the cytoplasmic domain of p23 on a surface similar to the situation on a membrane, the photolabile peptide was extended N-terminally by a Cys residue and covalently coupled to Sepharose beads by disulfide bond formation. Upon coupling, its Tyr residue was radioactively labeled with [^{125}I]iodine (Fig. 1*B*). Photoactivation after incubation of coatomer with ^{125}I-F*-p23wt Sepharose and analysis by SDS/PAGE yielded strong radioactive labeling of a protein with an apparent molecular mass of ≈100 kDa and some weak labeling of proteins migrating at 55 and 60 kDa (Fig. 2*B*, lane 1). Photocrosslinked radioactive COPs were immunoprecipitated upon cleavage of the peptide from Sepharose under reducing conditions in the presence of SDS (Fig. 2, lanes 2–9). Immunoblotting with antibodies against the various COPs and autoradiography show that on SDS treatment, each anti-COP antibody specifically precipitates the corresponding COP (Fig. 2*A*, lanes 2–6,

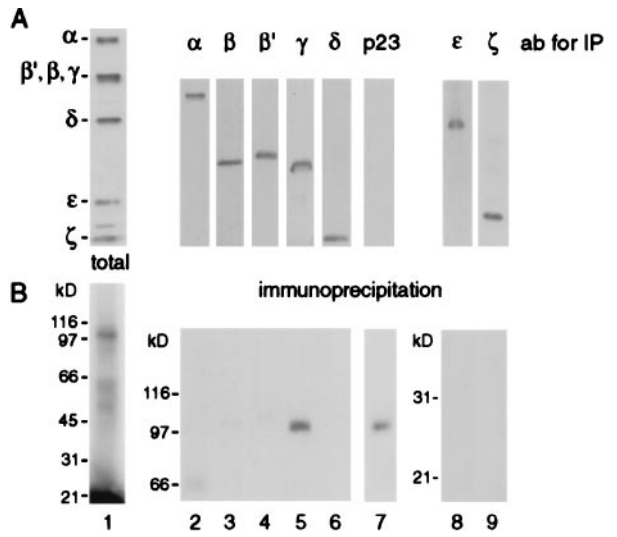

FIG. 2. Photocrosslinking of coatomer with immobilized ^{125}I-F*-p23wt. Coatomer was incubated with ^{125}I-F*-p23wt Sepharose for 90 min on ice prior to irradiation for 2 min on ice. Lanes 1, total photocrosslinked products. Lanes 2–9, samples immunoprecipitated with antibodies against individual COP proteins or the cytoplasmic domain of p23. (*A*) Immunoblot. (*B*) Autoradiogram of *A*. The samples were analyzed by SDS/PAGE, immunoblotting, and autoradiography. COPs were detected with a mixture of antibodies against all seven COPs. Total protein was separated on SDS/7.5–16.5% polyacrylamide gradient gels (lanes 1), α-, β-, β′-, γ-, and δ-COP were separated on 7.5% gels (lanes 2–7); ε- and ζ-COP were separated on 15% gels (lanes 8 and 9).

8, and 9) and that only antibodies against γ-COP are able to immunoprecipitate a radioactive protein with the size of γ-COP (Fig. 2*B*, lane 5). Thus, it is the γ subunit of coatomer that is specifically crosslinked to the immobilized p23 tail peptide. An antibody directed against the cytoplasmic domain of p23 indeed precipitates a radioactively labeled band with the size of γ-COP (Fig. 2*B*, lane 7). This finding confirms that the radioactivity introduced upon irradiation into the γ subunit represents reaction with the photolabile analogue of the p23 tail peptide. However, the amounts precipitated cannot be detected by immunostaining (Fig. 2*A*, lane 7). This result is expected even in case of photocrosslinking of the p23 peptide to COPs because of the yield of 1–3% of photocrosslinked product, characteristic for this technique (30).

Because in previous studies photolabeling of γ-COP was observed with a KKXX retrieval peptide in solution (13), we performed crosslink experiments with a soluble photolabile peptide analogous to the cytoplasmic domain of p23 (Fig. 1*A*). This method allows us to compare the results obtained with a retrieval peptide and with the p23 tail peptide. Radioactive photolabile peptide was incubated with freshly prepared cell lysate and irradiated. As expected, a variety of cellular proteins are radioactively labeled (Fig. 3*B*, lane 1). To detect photocrosslinked coatomer, immunoprecipitation with an anti-γ-COP antibody was performed without prior dissociation of the complex. This yielded all COPs (except ζ-COP, which tends to dissociate from the complex under the conditions used for immunoprecipitation) (Fig. 3*A*, lane 2). Only a single radioactive band was detected (Fig. 3*B*, lane 2), likely to represent β′-, β-, or γ-COP according to its migration. Therefore, α-, δ-, and ε-COPs can be excluded as binding partners. Again, the photocrosslinked COP was identified upon SDS treatment by immunoprecipitation with antibodies against β′-, β, and γ-COPs (Fig. 3*A*, lanes 3–5). Clearly, only the γ-COP antibody immunoprecipitates a radioactive protein with the size of γ-COP, revealing γ-COP as the sole binding partner of the p23 tail peptide also in solution in a whole cell lysate (Fig. 3, lane 5).

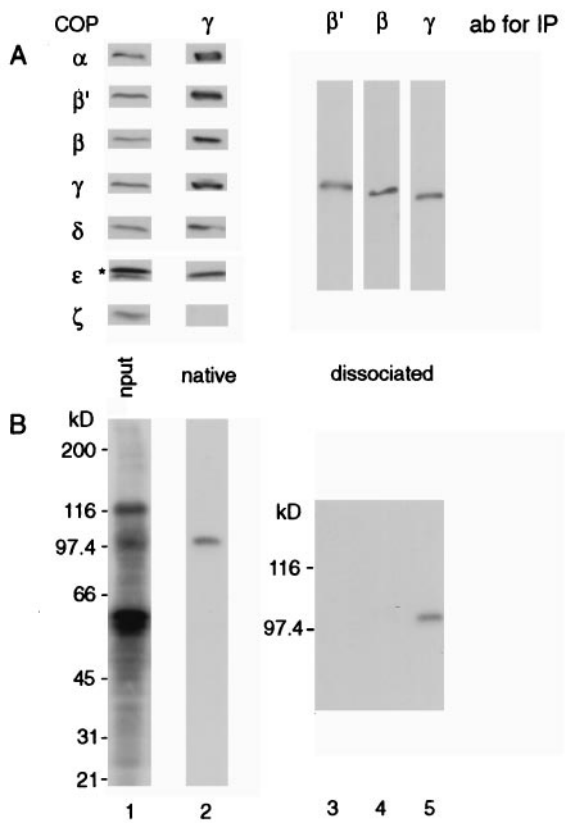

FIG. 3. Photocrosslinking of cell lysate with ^{125}I-F*-p23wt in solution. CHO cell lysate was incubated with ^{125}I-F*-p23wt in solution for 1 h on ice prior to photoactivation for 2 min on ice. (*A*) Lane 1, immunoblot of total cell lysate analyzed with a mixture of antibodies against all seven coatomer subunits. The asterisk indicates a cross-reactivity of the anti-ε-COP antiserum with a protein present in the lysate. Lane 2, immunoblot analysis of coatomer immunoprecipitated with an anti-γ-COP antibody without prior dissociation. Lanes 3–5, immunoblot analysis of β′-, β-, and γ-COPs individually immunoprecipitated (IP) upon dissociation of coatomer by SDS treatment. Samples were separated on 7.5–16.5% (lanes 1 and 2) or 7.5% polyacrylamide gels (lanes 3–5) in the presence of SDS. (*B*) Autoradiogram of *A*.

KKXX and p23 Peptides Share a Common Binding Site in γ-COP. The observation that both peptides Wbp1p (13) and p23wt (this study) interact with coatomer by means of γ-COP opens the question whether both peptides share the same binding site within this subunit. If so, an excess of nonradioactive Wbp1p peptide should compete with the p23 tail peptide and therefore reduce radioactive labeling of γ-COP by ^{125}I-F*-p23wt. To test this possibility, photocrosslinking experiments were performed with freshly prepared CHO lysate and ^{125}I-F*-p23wt in the presence of competing peptides. As expected for a specific interaction, labeling is markedly reduced in the presence of an excess of nonradioactive p23wt (Fig. 4*B*, lane 2). Importantly, photocrosslinking with ^{125}I-F*-p23wt in the presence of an excess of Wbp1p (Fig. 4, lane 3) also results in reduced radioactive labeling. Thus, both the Wbp1p and the p23 cytoplasmic tail peptides share a common binding site in γ-COP. Since the dilysine and diphenylalanine residues of p23 are involved in its interaction with coatomer, the presence of an excess of a peptide lacking both motifs, p23AS (Fig. 1*A*), should not affect labeling. This is indeed the case (Fig. 4, lane 5), and it underlines the specificity of the interaction of ^{125}I-F*-p23wt with coatomer. The Phe residues in positions −8 and −9 were shown to be required for efficient binding to coatomer of the p23 cytoplasmic domain (18, 31), and therefore a peptide with these residues mutated (Fig. 1*A*)

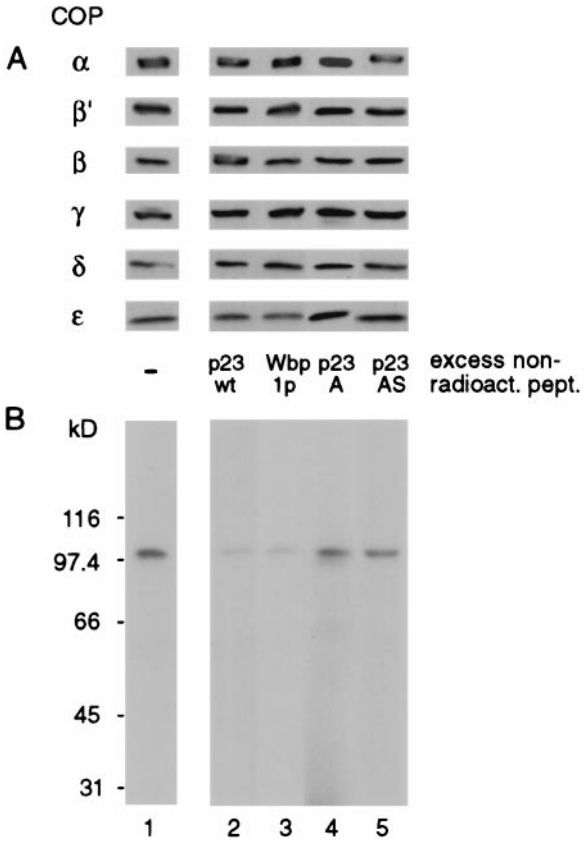

FIG. 4. Peptide competition experiments. CHO lysate was incubated with ^{125}I-F*-p23wt in solution in the absence (lane 1) or presence (lanes 2, 3, 4, and 5) of a 10-fold excess of nonradioactive peptides, and irradiated. Coatomer was immunoprecipitated with an anti-γ-COP antibody. Samples were separated on SDS/7.5–16.5% polyacrylamide gradient gels. (*A*) Immunoblot with antibodies against α-ε-COPs. (*B*) Autoradiogram of *A*.

is expected not to compete effectively. In fact, excess of this peptide does not significantly suppress the photolabeling of γ-COP (Fig. 4*B*, lane 4).

Specificity of the Interaction of p23 with Coatomer Depends on the Structural Integrity of the Complex. Our finding of an interaction between γ-COP and a Wbp1p peptide (13) is in disagreement with results obtained by others (11, 21, 22, 28). For these experiments lysates from mammalian cells (21, 22) or yeast mutants (11) as a source for coatomer, or disassembled coatomer from cytosol (28), were used. Lysates from mammalian cells were prepared in the presence of detergent, such as TX100, at high salt concentrations and were stored frozen (22). As it is known that some protein complexes are dissociated by freezing and thawing (32, 33), we investigated the state of assembly of COPs after freezing and thawing of cell lysates—i.e., in the presence of detergent. Clearly, after freezing and thawing, only a subcomplex of α-, β′, and ε-COP or even monomeric COPs are precipitated (Fig. 5*A*, lanes 2–4) with the same antibodies that precipitate the complete set of α-ε COPs from lysates that have not been frozen (Fig. 5*A*, lanes 5–7). However, in the absence of detergent, coatomer remains intact after being frozen and thawed (data not shown). Thus, freezing and thawing of a detergent-containing cell lysate leads to dissociation of coatomer, whereas the unfrozen complex remains intact in the presence of detergent. To investigate whether binding of p23wt peptide to coatomer is altered under such conditions, we performed photocrosslinking experiments with cell lysates stored frozen before use. As shown in Fig. 5*B*, unfrozen and frozen lysates indeed yield different results: immunoprecipitation with the antibody against α-COP identifies α-COP as the photocrosslinked product present in the α-, β′-, ε-COP trimer (Fig. 5*B*, lane 2). Likewise, monomeric β-COP (lane 3) as well as monomeric γ-COP (lane 4) is labeled with the photoreactive peptide. In contrast, control experiments with the unfrozen lysate exclusively yielded the radioactive band identified in Fig. 3, lane 5, as γ-COP throughout (Fig. 5*B*, lanes 5–7).

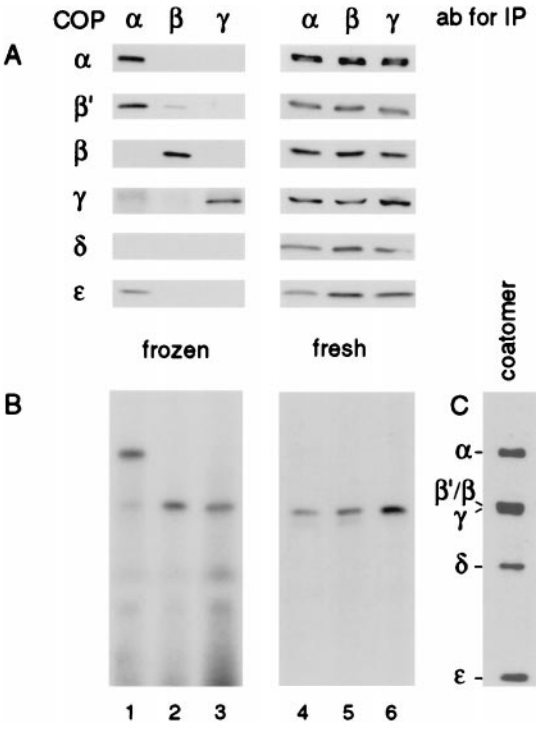

FIG. 5. Photocrosslinking of frozen CHO lysate with ^{125}I-F*-p23wt in solution. Incubations and photocrosslinking were performed as described in the legend to Fig. 3 with the exception that lysates used for the experiments shown in lanes 1–3 were frozen in liquid nitrogen, stored at −80°C, and thawed shortly before use. (*A*) Immunoblot. Lane 1, immunoprecipitation of COPs with an antibody against α-COP; lane 2, against β-COP; lane 3, against γ-COP. Lanes 4–6, immunoprecipitations analogous to those shown in lanes 1–3 but from freshly prepared cell lysates. (*B*) Autoradiogram of *A*. Coatomer as a standard, with COPs visualized by immunoblotting.

DISCUSSION

Formation of COPI vesicles is thought to involve interaction of coatomer with proteins of the p24 family (17, 18, 22). A member of this family, p23, is highly enriched in COPI vesicles and its interaction with coatomer depends on a dilysine and a diphenylalanine motif in its cytoplasmic domain (18). The cytoplasmic tail of p23 is structurally similar but not identical to the KKXX retrieval motif of ER resident membrane proteins (19, 20). The two structures bind coatomer with similar efficiency (18). Although binding to coatomer of the two types of peptide has been studied by several investigators (11, 13, 18, 21, 22, 28), the molecular detail underlying these interactions was still unclear. Particularly, it was not known to which site in coatomer the cytoplasmic domain of p23 would bind. Therefore, we investigated whether the cytoplasmic domain of p23 and a characteristic KKXX motif use different binding sites within the coatomer complex or share the same binding site.

We have previously shown that coatomer is able to bind to a characteristic KKXX retrieval motif by means of its γ subunit (13). Here, we demonstrate that in native coatomer it is the same subunit that mediates interaction of coatomer with the cytoplasmic domain of p23, and that the two peptides share a

common binding site within γ-COP. However, in partially dissociated coatomer, α-COP, β-COP, and γ-COP are able to bind to the p23 peptide.

To what extent does the introduction of the photoreactive group (Tmd) in F* interfere with the specificity of binding? Two lines of evidence indicate that the modification of the Phe residue in position −8 in p23wt did not significantly alter the binding of the peptide: (*i*) the observed labeling is efficiently decreased by competition with a 10-fold excess of the nonradioactive p23wt peptide, and (*ii*) introduction of a photolabile Phe residue at positions away from the diphenylalanine sequence (position −12: LmF* or position −6: AmF*) did not lead to labeling of COPs in addition to γ-COP (data not shown). However, with F* in those positions the overall efficiency of the photocrosslinking was significantly decreased to about 10–20%. This underlines an essential role in binding of coatomer of the Phe residues in positions −8 and −9 (18, 31). In general, interference of the Tmd residue in F* with biological activity of proteins does not seem to cause a basic problem, since this photolabile group has been successfully used for site-directed photolabeling of proteins in the past (34–36). Remarkably, γ-COP was the only coatomer subunit labeled not only regardless as to which position the photolabel was placed in the p23wt peptide, but also independently of the way the peptide was presented, either in solution or on the surface of a Sepharose bead, mimicking protrusions of a cytoplasmic domain from a membrane.

Our result may explain the following seemingly contradicting observations concerning binding of coatomer to KKXX-like signals: (*i*) *in vitro* binding of an α-, β′-, ε-COP subcomplex to a KKXX chimera (21, 22, 28) and to dilysine-containing cytoplasmic domains of some p24 members (22); (*ii*) binding of the same subcomplex to Golgi membranes (28); (*iii*) binding of β-, γ-, ζ-COP to diphenylalanine motifs of some p24 proteins (22); and (*iv*) lack of binding to an immobilized KKXX motif of coatomer from yeast mutants defective in α- or β′-COP (11). The main reason for this discrepancy most likely is a dissociation of coatomer into subcomplexes, specifically a stable α-, β′-, ε-COP trimer that has been reported by various groups (21, 22, 28, 29). This dissociation may have been caused by a variety of experimental conditions used, a major cause being the storage of detergent-containing samples in a frozen state. Some protein complexes are known to dissociate after freezing and thawing, and this has been used as a method to disassemble complex protein structures (32, 33). Freezing and thawing in the presence of detergent in fact leads to disassembly of coatomer as shown above. Likewise, it cannot be excluded that mutations within coatomer subunits lead to incorrect folding and therefore to improper assembly of the complete protein complex. This possibility would explain why coatomer from some yeast mutants is unable to bind to KKXX motifs (11). Since coatomer is recruited to membranes as an intact unit (10), it is unlikely that the binding observed with partially disassembled coatomer is of physiological relevance.

The finding that interaction of coatomer with the major membrane proteins of COPI vesicles, p23 and p24, depends on two motifs in their cytoplasmic domains—a dilysine and a diphenylalanine motif (18, 22)—has led to a concept of differential binding of coatomer to these different motifs: binding to the dilysine motif was suggested to regulate retrograde transport, whereas the diphenylalanine motif would be involved in anterograde transport (22). However, this concept is difficult to reconcile with our finding that in native coatomer γ-COP is the only binding site for the various peptides and that this interaction involves both the diphenylalanine and the dilysine sequence of p23.

Two different binding sites within coatomer have recently been suggested for dilysine motifs on the basis of the observation that interaction of coatomer with certain aminoglycoside antibiotics, which would mimic a pair of dilysine residues, leads to precipitation of the complex (37). This, however, would imply more than one binding site for amino group-containing structures within coatomer and is in contrast to the results of this study, which provide strong evidence for a single binding site for both dilysine retrieval motifs and the cytoplasmic domain of p23. The observed aggregation of coatomer by aminoglycosides is likely to be induced by another mechanism. In this context it is of note that coatomer upon binding to the p23 cytoplasmic peptide undergoes a conformational change that leads to aggregation of the complex, whereas related KKXX-containing structures are unable to alter the conformation of coatomer upon binding (C. Reinhard, M. Weidler, C.H., M. Bremser, K. Sohn, J. B. Helms, P. Roesch, and F.T.W., unpublished work). Thus, one and the same binding site may serve two different functions: binding to γ-COP of KKXX motifs is likely to mediate sorting of retrograde cargo into COPI-coated vesicles (11, 21, 38), whereas binding of p23 triggers a conformational change of coatomer that leads to aggregation of the coat of COPI vesicles. This concept underlines a function of p23 as part of the membrane machinery rather than as cargo of these vesicles.

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