A Major Transmembrane Protein of Golgi-derived COPI-coated Vesicles Involved in Coatomer Binding

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Abstract. Formation of non–clathrin-coated vesicles requires the recruitment of several cytosolic factors to the Golgi membrane. To identify membrane proteins involved in this budding process, a highly abundant type I transmembrane protein (p23) was isolated from mammalian Golgi-derived COPI-coated vesicles, and its cDNA was cloned and sequenced. It belongs to the p24 family of proteins involved in the budding of transport vesicles (Stamnes, M.A., M.W. Craighead, M.H. Hoe, N. Lampen, S. Geromanos, P. Tempst, and J.E. Rothman. 1995. *Proc. Natl. Acad. Sci. USA.* 92:8011–8015). p23 consists of a large NH_2-terminal luminal domain and a short COOH-terminal cytoplasmic tail (-LRRFFKAKKLIE-CO_2^-) that shows similarity, but not identity, with the sequence motif -KKXX-CO_2^-, known as a signal for retrieval of escaped ER-resident membrane proteins (Jackson, M.R., T. Nilsson, and P.A. Peterson. 1990. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3153–3162; Nilsson, T., M. Jackson, and P.A. Peterson. 1989. *Cell.* 58:707–718). The cytoplasmic tail of p23 binds to coatomer with similar efficiency as known KKXX motifs. However, the p23 tail differs from the KKXX motif in having an additional motif needed for binding of coatomer. p23 is localized to Golgi cisternae and, during vesicle formation, it concentrates into COPI-coated buds and vesicles. Biochemical analysis revealed that p23 is enriched in vesicles by a factor of ~20, as compared with the donor Golgi fraction, and is present in amounts stoichiometric to the small GTP-binding protein ADP-ribosylation factor (ARF) and coatomer. From these data we conclude that p23 represents a Golgi-specific receptor for coatomer involved in the formation of COPI-coated vesicles.

TRANSPORT of proteins along the secretory pathway is mediated by transport vesicles and can be reconstituted in vitro (41). From such incubations, Golgi-derived COPI-coated transport vesicles can be isolated (29). The coat of these vesicles consists of the small GTP-binding protein ADP-ribosylation factor (ARF)[1] and of coatomer, a cytosolic complex made up of seven subunits, the COPs (for coat proteins) (47, 48, 57). During budding of the vesicles, ARF in its GTP-bound form (ARF–GTP) binds to the Golgi membrane, with subsequent recruitment of coatomer (7, 35, 37). A direct GTP-dependent binding of ARF1 to coatomer in this budding process recently has been shown (Zhao, L., J.B. Helms, B. Brügger, C. Harter, B. Martoglio, R. Graf, J. Brunner, and F.T. Wieland, manuscript submitted for publication). However, interaction of coatomer with an additional binding partner in the membrane is likely to occur during the budding reaction, because, in addition to the ARF–GTP-dependent binding, a basal binding of coatomer to Golgi membranes is observed in the absence of GTP (37). Moreover, in vitro experiments have revealed binding of the complex to peptides analogous to the cytoplasmic tails of ER-resident membrane proteins (6). These tails contain a sequence motif KKXX-CO_2, previously described as an ER-retrieval signal (19, 30). Genetic experiments have implicated such interactions in retrograde vesicular transport from the Golgi to the ER (25) in addition to the well-established role of COPI-coated vesicles in anterograde transport (3, 33, 39, 41). Recently, a membrane protein from CHO cells was found as a component of COPI-coated vesicles that belongs to a novel family of type I membrane proteins (50). The p24 family comprises at least 13 members (five to seven homologues within one species) and shares the same type I membrane topology with a large luminal domain and a short cytoplasmic tail. Many of the p24 homologues have the COOH-terminal dilysine motif, and it has

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1. *Abbreviations used in this paper:* ARF, ADP-ribosylation factor; EST, expressed sequence tag; KLH, keyhole limpet hemocyanin.

therefore been speculated that they function as ARF or coatomer receptors (50). Alternatively, the large luminal domain of p24 homologues led to the speculation that they might be involved in cargo recognition. A yeast member of the p24 family, Emp24, was characterized as a component of COPII-coated vesicles (2, 45). In a yeast mutant lacking Emp24, ER to Golgi transport of a subset of proteins was reduced. It was therefore proposed that Emp24 might be involved in the sorting and/or concentration of cargo molecules into COPII-coated vesicles.

We began a search for transmembrane proteins of COPI-coated vesicles to identify a possible Golgi-derived coatomer receptor. As part of the budding machinery, such a receptor would be predicted to fulfill the following criteria: (a) high abundancy in COPI-coated vesicles, (b) Golgi localization, and (c) ability to bind coatomer.

Here we describe a novel type I membrane protein of COPI-coated transport vesicles with functional properties that meet these characteristics.

Materials and Methods

Antibodies

Antibodies against various peptides of p23 were generated in rabbits: polyclonal sera were raised against (a) a peptide containing the first 11 amino acids (ISFHLPVNSRK) of the NH$_2$ terminus of the mature p23, synthesized on a polylysine backbone (KAI2/3); (b) a peptide within the luminal domain of p23 (KITDSAGHILYSK) coupled to keyhole limpet hemocyanin (KLH) (#1327); (c) a peptide corresponding to the cytoplasmic tail of p23 (YLRRFFKAKKLIE) also coupled to KLH (#1402). Coupling of peptides to KLH for immunization and to CNBr-activated beads for affinity purification was performed according to standard protocols (14).

Subcellular Fractionation

Golgi-enriched membranes were isolated according to (52) without performing the final salt wash (galactosyltransferase activity was 35-fold enriched compared with starting postnuclear supernatant [21]). COPI-coated vesicles were generated by incubating rabbit liver Golgi with bovine brain cytosol in the presence of GTPγS and purified according to (48).

Protein Chemical Methods

SDS-PAGE was performed on 16% (44) or on 12% (24) acrylamide gels and proteins were visualized by silver (5). Two-dimensional gel electrophoresis was performed according to standard protocols (31) with 1.9% ampholines, pH 5–7, and 0.7% ampholines, pH 3.5–10, (ampholines from Pharmacia, Uppsala, Sweden) using the Mini Protean II 2-D Cell (Bio Rad Laboratories, Hercules, CA). Second dimension was performed on 12% SDS-PAGE. Western blotting was done according to (22). Microsequencing was performed according to (9).

Cloning and Sequencing of p23

p23 cDNA was cloned from a rabbit liver 5′-stretch plus cDNA library (λgt10; Clontech, Palo Alto, CA) using nondegenerated primers, obtained from two human expressed sequence tag (EST) sequences (accession numbers Z43865; T32238): 5′-ATG TCT GGT TTG TCT GGC CCA CCA GCC CGG CGG-3′, 5′-CTT CTC AAC TTT TGC AAT CTC TTC GTA ATT TTT-3′, 5′-AAT CAA TTT CTT GGC CTT GAA GAA GCG TCG-3′. Cloning was performed using filter hybridization standard techniques (43). The open reading frame of one full-length clone was sequenced, which comprised 657 base pairs. The corresponding sequence is accessible at the EMBL/GenBank/DDBJ database (accession number X98303).

Homologies and Structure Prediction of p23

For comparison of rabbit p23 with members of the p24 family, the BESTFIT program (Genetics Computer Group) was used, with the following peptide and cDNA sequences (accession numbers in brackets): rabbit p23 (this work; X98303), gp25L (p27869.swiss), Emp24 (em24_yeast.swiss; p32803.swiss), chop24 (p49020.swiss), yp24c (yhr0_yeast.swiss), yeast homologue of p23 (s55107.pir2); frog p23 cDNA (X90517), human p23 cDNAs (L40397, N30987, R20989, H62112), and fish p23 genomic sequence (U40761) were translated into peptide sequences. Homologies were determined for different domains of p23 (Ile^{32}-Val^{141}, Glu^{142}-Arg^{185}, Val^{186}-Tyr^{207}, Leu^{208}-Glu^{219}) and expressed in percentage identity to rabbit p23. Hydropathy plot of the p23 precursor protein was obtained according to (23), using a window of 11 amino acid residues.

Protease Digestion and Alkaline Treatment of Golgi Membranes

For protease treatment, Golgi-enriched membranes (52) were incubated with pronase E (6 μg pronase E, 60 μg membrane protein in 120 μl 10 mM Tris-HCl buffer for 20 min at 37°C). Mock incubations were performed under identical conditions without pronase E. Where indicated, Triton X-100 was added as a detergent to give a final concentration of 1% (wt/vol). Thereafter, the samples were precipitated by the addition of TCA (10% final concentration) and analyzed by SDS-PAGE on gels containing 12% acrylamide. p23 was visualized by Western blotting with a polyclonal rabbit antiserum directed against the NH$_2$ terminus of p23 (KAI2/3). Alkaline treatment was performed with 0.1 M Na$_2$CO$_3$ for 30 min at 0°C and subsequent centrifugation at 100,000 g for 30 min (under these conditions some soluble proteins [e.g., ζ-COP] tend to precipitate partially and therefore are recovered with the membrane fraction. However, no protein regarded as membrane bound is found in the supernatant).

Coatomer Binding to Beads

CHO cell lysates were prepared by extraction of the cells with Hepes detergent buffer (50 mM Hepes-KOH, pH 7.3, 90 mM KCl, (300 mM) NaCl, 0.5% NP-40, 1 mM PMSF, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 1 mM EDTA) and diluted to a final protein concentration of 1 mg/ml. Peptides were linked to Thiopropyl Sepharose 6B (Pharmacia) via their NH$_2$-terminal Cys residues. Coupling yields were determined after release of the peptides in weighed aliquots of the Sepharose beads by addition of 2-mercaptoethanol (50 mM), acid hydrolysis of the supernatants (6 M HCl) for 12 h at 110°C, and amino acid analysis according to (11). For incubation with cell lysates, the amounts of beads added were adjusted to give identical absolute amounts of peptides present in the incubations. Beads were added to a final volume of 175 μl containing 150 μg cell lysate protein and 3.3 nmol peptide. Incubation was at 4°C for 2 h or overnight. Thereafter, the beads were washed four times with Hepes detergent buffer and once with buffer without detergent, elution was performed by the addition of 100 μl of SDS sample buffer, and identical aliquots were separated on a 10% acrylamide SDS gel. For Western blotting, the following antibodies were used: rabbit anti–α/γ-COP (10), rabbit anti–β′-COP (51) and anti–δ-COP (15), and monoclonal mouse anti–β-COP antibody M3A5 (8). Western blots were developed by the enhanced chemiluminescence (ECL) system (Amersham Intl., Little Chalfont, UK) and quantitatively evaluated by scanning. To this end, the films were developed for various times, allowing the scanning of all bands in a linear range. As standards for calibration, various amounts of cell lysate were quantitated.

Immunocytochemistry

Immunofluorescence studies were performed as described earlier (36) using the following affinity-purified antibodies against p23, and antibodies against coatomer: KAI2/3, #1327, #1402, and CM1 (against coatomer). For colocalization, cells were fixed with paraformaldehyde. For immunelectron microscopy, thin cryosections of rat liver Golgi fractions (49) were fixed with 1% glutaraldehyde and processed for immunolabeling by cryosectioning (55) and by the protein A–gold method (40). Affinity-purified antibody against p23 (#1327) was diluted 1:2. Gold particles were 8–12 nm in size. Quantitation of gold particles was performed as described earlier (36).

Relative Amounts of p23 in Golgi Membranes and Vesicles

Golgi membranes (isolated as described under subcellular fractionation) used for the generation of COPI-coated transport vesicles and the resulting vesicles were separated by SDS-PAGE and visualized by Western

blotting using the polyclonal antiserum directed against the NH₂-terminal peptide of p23 (KAI2/3). Blots were developed with 3,3'-diamine benzidine (54) and quantified by computer scanning analysis.

Stoichiometry of p23 to Coatomer and ARF

COPI-coated vesicles were analyzed by Western blotting for their absolute amounts of p23, ARF, and ε-COP using polyclonal rabbit antibodies directed against these proteins and developed with the ECL system (Amersham Intl.) (59). The relative intensities of the corresponding bands were quantified by computer scanning analysis within a linear range of intensity and compared to defined amounts of recombinant standard proteins: ε-COP (M_r = 36,000) (13), ARF1 (~20 kD) (58), and the luminal domain of p23 (amino acid residues Ile[32] to Arg[185], M_r ~20 kD, expressed as (His)₆-tagged protein in *Escherichia coli* [pQE30; QIAGEN Inc., Chatsworth, CA]). The amounts of these proteins used for calibration of the Western blots were determined using the Lowry method (28). The molar ratios of ε-COP to p23 and to ARF were calculated as:

$$\frac{x \cdot \text{ng p23 (or ARF)} \cdot 36}{y \cdot \text{ng } \varepsilon\text{-COP} \cdot 20}. \tag{1}$$

Antibodies used were: polyclonal rabbit antibodies directed against (*a*) a peptide of the luminal domain of p23 (#1327), affinity purified using this peptide; (*b*) against ARF (37); and (*c*) against recombinant ε-COP (13).

Results

Isolation and Molecular Characterization of p23, a Major Membrane Protein from COPI-coated Vesicles

In vitro–generated COPI-coated vesicles were purified according to (48) and analyzed for their membrane protein constituents by SDS gel electrophoresis. Fig. 1 *A* (lane *1*) depicts the pattern of a purified vesicle fraction. A membrane protein fraction was prepared by alkaline treatment of the vesicles and subsequent centrifugation (Fig. 1 *A*, lane 2). Only a few prominent bands are observed in a molecular mass range between 14 and 30 kD: some of which were identified as ζ-COP and ARF, and another faint band visible at ~25 kD, which represents Erd2, the KDEL receptor, as revealed by immunoblotting (data not shown). An additional prominent band was detected with an apparent molecular mass of 23 kD. This band consists of two proteins as shown by two-dimensional gel electrophoresis (Fig. 1 *B*). By immunoblotting (data not shown), one was identified as the homologue of CHOp24 (*small arrow*), and the other one is a so far unknown protein (*large arrow*). Besides p23 and p24, only ζ-COP and ARF were found to be abundant in COPI-coated vesicles in the range between 14 and 31 kD. The material of the unknown protein was analyzed for its NH₂-terminal amino acid sequence (Fig. 1 *C*, lane *1*). To obtain larger amounts for internal peptide sequence information, a protein of the same apparent molecular mass and with an identical NH₂-terminal sequence was isolated from a Golgi membrane preparation. This protein provided the molecular structure of three additional peptides (Fig. 1 *C*, lane 2). Database research using the TBLASTN program revealed two human ESTs, one containing the information coding for three of the peptides and the other for the remaining one. Nondegenerated oligonucleotide probes were designed according to the EST cDNA sequences and used for screening a λgt10 rabbit liver cDNA library. The deduced amino acid sequence from the obtained cDNA is shown in Fig. 2 *A*. The cDNA encodes a protein of 219 amino acid residues and includes the four peptide sequences determined by
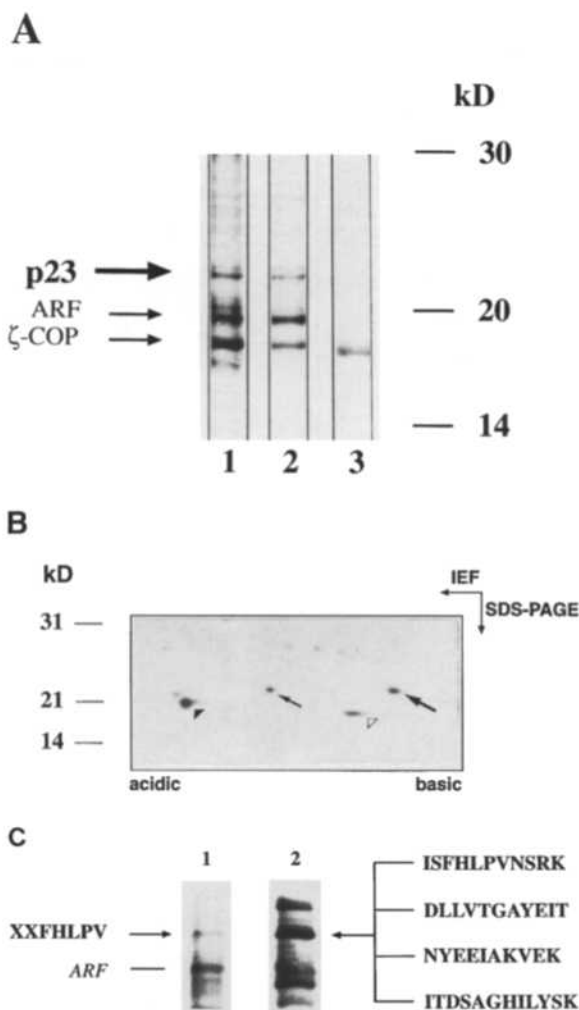

Figure 1. Isolation and characterization of p23 from isolated COPI-coated transport vesicles and from Golgi-enriched membranes. (*A*) COPI-coated vesicles analyzed by SDS gel electrophoresis (SDS-PAGE, 16% acrylamide) before (lane *1*) and after treatment with sodium carbonate (lane 2). In lane 3, the supernatant after carbonate treatment is analyzed. A low molecular mass window between 30 and 14 kD is shown. (*B*) COPI-coated vesicles analyzed by two-dimensional gel electrophoresis (12% acrylamide in the second dimension). Arrows indicate the spots corresponding to p23 (*large arrow*) and to p24 (*small arrow*). The most acidic spot at 21 kD is ζ-COP (*filled arrowhead*); the spot between p23 and p24 at ~20 kD is ARF (*open arrowhead*). (*C*) SDS-PAGE (12% acrylamide) analysis of total vesicles (lane *1*) and of a Golgi membrane fraction after carbonate treatment (lane 2). A low molecular mass window between ~20 and 30 kD is shown. The material depicted with arrows was analyzed by microsequencing and the peptide sequences obtained are shown. (*A–C*) Proteins were visualized by silver staining.

microsequencing of p23. From a hydropathy plot (Fig. 2 *B*), the structure of a type I membrane protein is suggested, with a large NH₂-terminal luminal domain and a short cytoplasmic tail. A suggested membrane-spanning domain comprises 22 amino acid residues containing four Phe residues. The NH₂-terminal hydrophobic stretch of 31 amino acid residues represents the signal peptide of p23, as the mature protein (188 amino acid residues) starts with Ile[32].

A

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MSGWSGPLAR  RGPGPLALLF  LFLLGPSSVL
A*ISFHLPVNS RKCLREEIHK  DLLVTGAYEI
TDQSGGAGGL  RTHLKITDSA  GHILYSKEDA
SKGKFAFTTE  DYDMFEVCFE  SKGTGRIPDQ
LVILDMKHGV  EAKNYEEIAK  VEKLKPLEVE
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STNTRVLYFS  IFSMFCLIGL  ATWQVFYLRR
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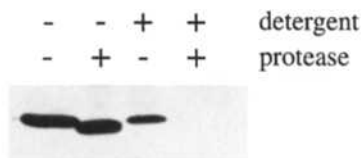
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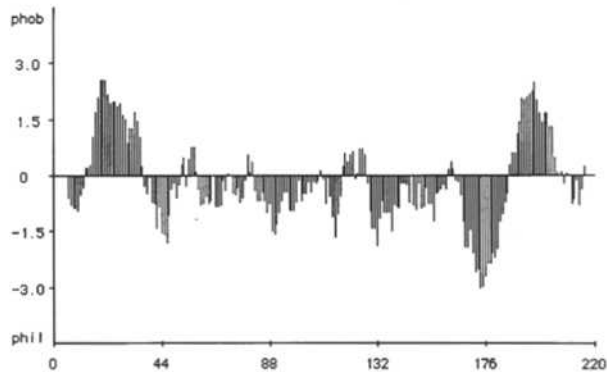
C

Figure 2. cDNA-derived amino acid sequence and topology of p23. (*A*) Amino acid sequence of the precursor protein. The mature protein starts with Ile[32], as indicated with an asterisk. Peptide sequences obtained by microsequencing of isolated p23 are underlined. A putative membrane-spanning domain is underlined in bold. The sequence data are available from EMBL/Gen-Bank/DDBJ database under the accession number X98303. (*B*) Hydropathy plot of p23 precursor protein. (*C*) Topology of p23. Purified Golgi-enriched membranes were digested with pronase E with or without detergent. p23 was analyzed by SDS-PAGE and subsequent Western blotting with an antibody against the NH₂ terminus of p23 (KAI2/3).

This predicted topology is confirmed by proteolytic digestion: after treatment with pronase E of an intact Golgi membrane fraction, a slightly smaller protein fragment (22 kD) is detected, consistent with the loss of a short cytosolic tail (Fig. 2 *C*). This overall structure is characteristic for a variety of related type I membrane proteins, the so-called p24 family (50). Members of this family with the overall highest identities to p23 (from 23–98%) are gathered in Table I. Homologues in various species of p23 are listed in the upper panel. Conservation within the animal homologues is observed throughout the complete sequence, including an ~100% identity of the COOH-terminal tails. The yeast

homologue yp24g exhibits the most striking homology in the luminal domain, next to the membrane span (63%). The lower panel shows additional members (relatives) of the family, with lower overall sequence identity and higher level of heterogeneity in the COOH-terminal tails. One striking feature shared by the whole family is a conserved Phe residue upstream of a dibasic motif.

The Complete Tail of p23 Represents a Novel Coatomer Binding Motif

The COOH-terminal tail of p23 ends with a sequence (KKLIE) reminiscent of but not identical with the dibasic ER retention/retrieval motifs KKXX or KXKXX (19, 30) known to bind coatomer directly (6, 15, 27). In addition, conservation of amino acid residues upstream of this sequence is observed, as shown in Table I. The functionality of the complete tail to bind coatomer was analyzed using a synthetic peptide linked to Sepharose via an NH₂-terminally introduced Cys residue. Total cell lysates were incubated with these beads, and binding of coatomer was quantitated by Western blotting. The efficiency of coatomer binding was quantitated by comparison of input signal with the amount bound to the beads. As shown in Fig. 3 *A* (lanes *1*, *7*, and *9*), the tail peptide of p23 does bind coatomer with an efficiency (~25% of the input material) comparable to the binding of the established retention/retrieval motifs of the adenoviral protein E19 (30) (31%), or the yeast oligosaccharyl transferase subunit Wbp1 (53) (36%). Binding to the established motifs was reported to strictly depend on the presence of the two Lys residues (6), as confirmed in lanes *8* and *10* in Fig. 3 *A*. In contrast, exchange of Lys residues 5 and 4 in the tail peptide of p23 with serine residues did not abolish binding of coatomer, but only caused a reduction to ~50% (Fig. 3 *A*, lane *2*). Thus, an upstream part of the tail sequence must contribute to this binding. Therefore, the conserved Phe residues in positions 8 and 9 (see Table I) were exchanged for Ala residues. As a result (Fig. 3 *A*, lane *3*), coatomer binding was completely abolished. Evidently, the presence of these conserved Phe residues is obligatory for coatomer binding. Accordingly, exchange of both the dibasic and the di-Phe motifs leads to a complete loss of the capability to bind coatomer as well (Fig. 3 *A*, lane *4*). Interestingly, the exact position of these Phe residues is not critical for the function of the cytosolic tail peptide of p23, because changing their position with the two upstream Arg-residues does not cause a significant decrease of binding capacity, as shown in lane *5*. In summary, we have defined, in addition to the dibasic motif, a new structural element in the tail peptide of p23 that is essential for the binding of COPI coat proteins.

p23 Is Localized to the Golgi and Is Highly Enriched in COPI-coated Vesicles

The presence of a unique KKXX-like motif in COPI-coated vesicles prompted us to analyze the intracellular localization of p23. Immunofluorescence microscopy with an antibody directed against an internal peptide of p23 shows a distinct perinuclear pattern typical of the Golgi complex of three different mammalian cell types (Fig. 4, *a–c*). The Golgi localization was confirmed in a double-labeling ex-

Table I. Homologues and Relatives of p23

Homologues of p23	Overall identity	Ile^{32}-Val^{141} (lumen)	Glu^{142}-Arg^{185} (lumen)	Val^{186}-Tyr^{207} (transmembrane domain)	Leu^{208}-Glu^{219} (cytoplasmic tail)	Tail structure
	%			%		
Rabbit	100	100	100	100	100	LRRFFKAKKLIE
Human	97.9	96.4	100	100	100	LRRFFKAKKLIE
Fish	88.3	80	100	100	100	LRRFFKAKKLIE
Frog	68.6	62.7	84.1	81.8	91.7	LRHFFKAKKLIE
Yeast	39.3	27.3	63	45.5	41.7	LKNYFKTKHII
Other members of the p24 family						
gp251	32.3	30.9	31.8	18.2	58.5	LKNFFIAKKLV
Emp24	27.9	21.8	31.8	31.8	50	LRRFFEVTSLV
chop24	23.6	17.3	29.5	27.3	33.3	LKRFFEVRRVV
yp24c	22.8	20	25	27.3	33.3	LKNFFVKQKVV

p23 is a member of the p24 family. The upper panel lists putative homologues of rabbit p23, based on their striking sequence identities. In the lower panel, putative relatives of p23 are shown, with a lower but significant degree of conservation. The cytoplasmic tails of the proteins are listed with their amino acid sequence under "Tail structure". For more members of the p24 family, see (50).
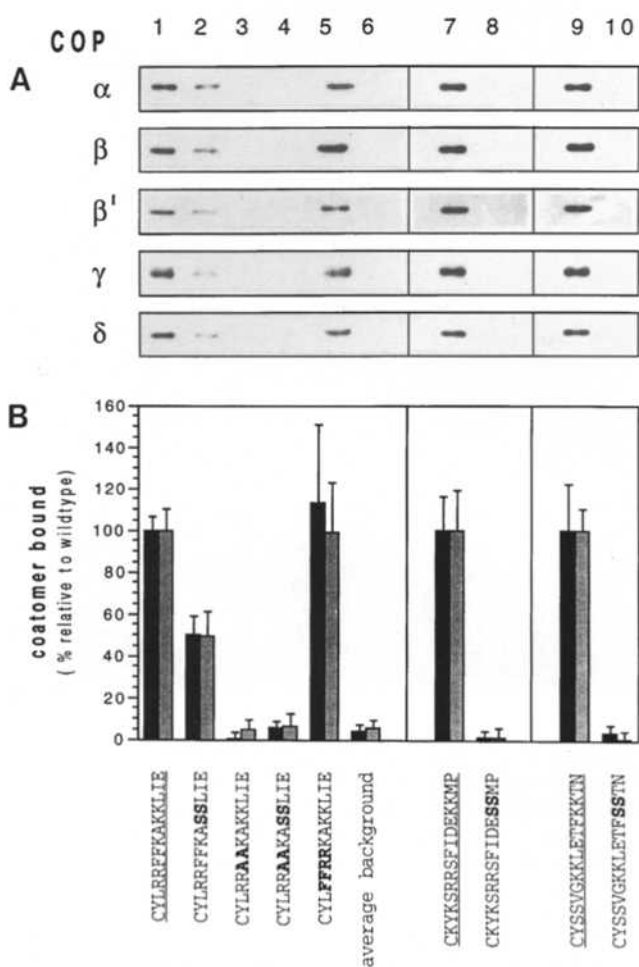

Figure 3. Binding of coatomer to the cytoplasmic tail peptides and various mutations of p23 (lanes *1–5*), adenoviral protein E19 (30) (lanes *7* and *8*), and yeast oligosaccharyltransferase subunit Wbp1 (53) (lanes *9* and *10*). Total CHO cell lysates were incubated with the various peptides (as indicated) covalently linked to Sepharose beads. Bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE and Western blotting with antibodies directed against different coatomer subunits indicated. Three independent quantitations were performed. (*A*) A typical Western blot obtained. (*B*) Quantitative evaluation of α-COP (*dark bars*) and β-COP (*light bars*) from Western blots. Values are means of +SD; "average background" depicts the signal obtained with the average amount of beads used but without peptide. Binding of coatomer to each wild-type peptide (p23, E19, and Wbp1) was set to 100%.
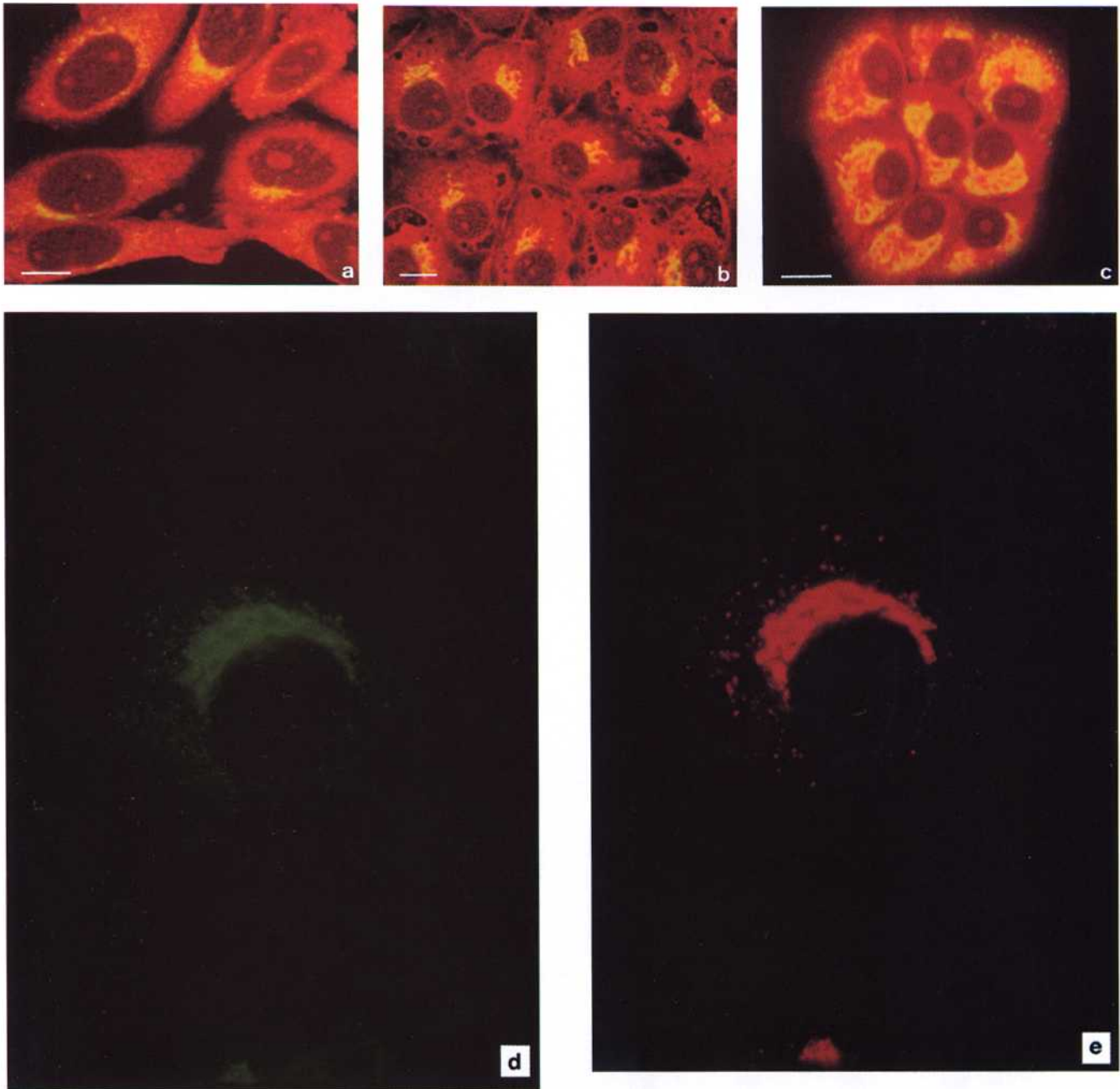
periment, where p23 colocalizes with coatomer, bound to the Golgi complex (32) (Fig 4, *d* and *e*).

The function of p23 in binding of coatomer, together with its predominant presence in the Golgi, suggested a role of p23 as a Golgi-derived coatomer receptor for the formation of COPI-coated vesicles. Therefore, its quantitative distribution was assessed by immunoelectron microscopy in control Golgi fractions ("unprimed") and in Golgi fractions incubated under the conditions defined to form COPI-coated vesicles in vitro (29) ("primed Golgi"). The results are shown in Fig. 5, *a–c*. In primed Golgi, a large fraction of p23 immunolabeling is observed on 70–90-nm coated vesicle profiles (Fig. 5, *a* and *c*). In unprimed Golgi, the budding of coated vesicles is virtually undetectable, and most immunolabeling is found on the cisternal elements (Fig. 5, *b* and *c*). In primed Golgi, the concentration between coated vesicle profiles and cisternal elements is ∼fourfold higher (Fig. 5 *c*). Thin-sections of intact insulin cells were also qualitatively assessed. p23 immunolabeling was predominant on Golgi-associated vesicles and found also, in sparse quantity, on transitional elements of the ER (data not shown).

p23 Is a Structural Component of COPI-coated Vesicles

The striking concentration in buds and vesicles observed by immunoelectron microscopy was estimated biochemically. To this end, the relative immunostaining of donor Golgi fractions and purified vesicles was compared by Western blotting. As shown in Fig. 6, 50 μg of donor Golgi protein (lane *2*) yielded a signal comparable to the one obtained by 2 μg of vesicular protein (lane *1*). Thus, we conclude that p23 is enriched in COPI-coated vesicles by a factor of ∼20. This is only a rough estimation, as both the

Figure 4. Immunolocalization of p23 to the Golgi complex. Immunofluorescent labeling of p23 in CHO-cells (*a*), NRK cells (*b*), and insulin cells in monolayer culture (*c*). A distinct perinuclear reticular-punctate labeling is elicited in all three cell types: (*a* and *c*) antibody directed against the NH₂ terminus of p23 (KAI2/3); (*b*) affinity-purified antibody against an internal peptide of the luminal domain of p23 (#1327). Monospecificity of these antibodies was assessed by Western blotting. Colocalization of p23 with coatomer in NRK cells: immunofluorescent labeling of p23 (*d*) with an antibody against the COOH terminus of p23 (#1402), and of coatomer (*e*) with an antibody recognizing native coatomer (CM1). Bars, 10 μm.

donor Golgi membranes and the vesicles are only 30–50% pure. However, given the fact that the protein to lipid ratio in vesicles is four times higher as compared with that of the Golgi donor membrane (Brügger, B., and F.T. Wieland, unpublished data), the actual enrichment of p23 into COPI-coated vesicles is likely to be underestimated. This made p23 a strong candidate for a coatomer receptor as part of the machinery for the formation of COPI-coated

vesicles. Such a role would require the presence in vesicles of p23 in amounts stoichiometric to coatomer. This was investigated by quantitation of the absolute amounts in the vesicles of ε-COP (for coatomer [13]), ARF, and p23 by Western blotting, using the purified recombinant proteins as calibration standards. The results of this analysis are given in Table II. From quantitation of four independent vesicle preparations, an average of ∼5 mol of p23 per 1 mol
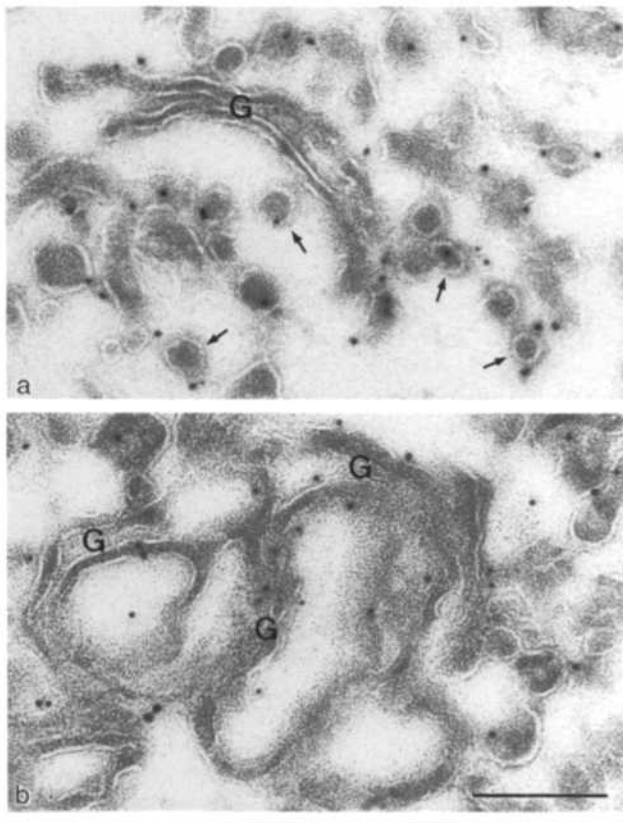
	Cisternae	70-90nm coated vesicle profiles
Control Golgi	75 ± 9	not detectable
GTPγS treated Golgi	33 ± 3	128 ± 13

D

Figure 5. p23 concentrates into coated buds and vesicles. (A) GTPγS–treated Golgi membranes with immunogold particles situated preferentially on 70–90-nm coated vesicle profiles (arrows). G, Golgi cisternal elements. (B) Control Golgi membranes (without GTPγS) with no detectable budding, but with a distinct p23 labeling on the cisternae (G). (C) Number of p23 immunogold particles per μm² of Golgi compartment (mean ± SEM). n = 10 Golgi areas evaluated. Total number of gold particles per μm² of Golgi area: GTPγS-treated, 23 ± 2; control, 29 ± 5. Bar, 200 nm.

of ε-COP is found. In the same preparations, an average of ~10 mol of ARF1 per 1 mol of ε-COP is observed, probably an overestimation (47) as a result of variations of the known unsaturable amounts of ARF that bind to Golgi membranes depending on the amount of active ARF present in various cytosol preparations (16). Thus, p23 is present in COPI-coated vesicles in amounts that allow stoichiometric binding to coatomer, and its stoichiometry points to an oligomeric complex as the binding partner.
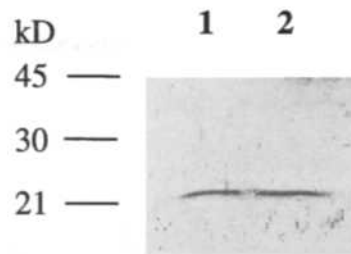

Figure 6. p23 is enriched in COPI-coated vesicles. The amounts of p23 in Golgi membranes and in isolated COPI-coated vesicles were compared by Western blotting. 50 μg of Golgi membranes (lane 2) and 2 μg of COPI-coated vesicle fraction (lane 1) were separated by SDS-PAGE and visualized by Western blotting using the polyclonal antiserum directed against the NH₂-terminal peptide of p23 (KAI2/3).

Discussion

We have characterized a novel membrane protein of COPI-coated vesicles that most likely represents a component of the membrane machinery involved in the formation of COPI-coated vesicles. The properties of p23 are consistent with a function as a coatomer receptor: p23 is a major membrane protein of COPI-coated vesicles; it is present in these vesicles in amounts stoichiometric to coatomer; and its cytoplasmic tail peptide binds coatomer efficiently, with structural elements involved in this binding that are different from the established dibasic motifs known to interact with the complex. It is localized to the Golgi and, upon priming, is concentrated into COPI-coated buds and vesicles. Taken together, these data suggest that p23 serves as a receptor for coatomer in Golgi membranes.

In vivo evidence for a role of p23 in biosynthetic protein transport comes from Xenopus, in which a homologue of the protein has been characterized at the mRNA level (17): p23 homologue expression, together with constituents of the ER translocation machinery, is coordinately increased when secretion of pro-opiomelanocortin is stimulated in the intermediate pituitary cells of the frog.

p23 belongs to the p24 family of type I membrane proteins that share short cytoplasmic tails and large luminal domains (50), and they have been discussed to serve as ARF/coatomer receptors and/or cargo receptors for COPII-coated vesicles that bud off the ER (45), as well as for COPI-coated vesicles (50). Currently, seven p24 family members are known within yeast and five members within

Table II. Stoichiometry of p23 to Coatomer and ARF

Vesicle prep	Molar ratio ε-COP:p23	Molar ratio ε-COP:ARF
I	1:4.9	–
II	1:4.8	–
III	1:6.9	1:9.6
IV	1:3.9	1:9.8

Stoichiometry of p23 to coatomer (represented by its subunit ε-COP) and ARF in isolated COPI-coated vesicle fractions. Four independent COPI-coated vesicle preparations were examined.

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mammals. It has been suggested that they are involved in vesicle formation at various intracellular locations (50). gp251 and Emp24 have been found to reside in the ER (45, 56). In COPI-coated vesicles only two members of this family are found to be abundant: p23 and p24. Mammalian p24 was described as a constituent of COPI-coated vesicles; however, its steady state localization in the cell is not yet defined.

It is not known at present what makes the various p24 family members localize to different intracellular membranes. In this context, it may be of note that a COOH-terminal double FF-containing dibasic motif is required and sufficient for pre-Golgi localization of ERGIC-53 (18). A possible role of the upstream FF of p23 in its steady state localization remains to be investigated. It was shown that the Golgi localization of a yeast protein, Emp47, is dependent on the presence of its KXKXX motif, but independent on intact α-COP (46). Subsequent work, however, revealed that in a γ-COP mutant (sec21), the steady state localization of Emp47 is perturbed, and the protein travels to the vacuole (26). These seemingly conflicting results indicate that individual subunits of the coatomer might play a role in governing the direction a COPI-coated vesicle may take.

Here we restrict our speculations on functions of p23, the member of the p24 family most abundant in COPI-coated vesicle preparations and clearly localized to the Golgi complex at steady state.

Machinery for Budding?

As in the binding of ER-retrieval motifs to coatomer, interaction of the complex with the COOH-terminal tail of p23 in this study was determined in vitro, impeding analysis of any impact ARF–GTP might have on this interaction. However, evidence for a direct interaction of Golgi-bound ARF–GTP with coatomer specifically via the β-subunit of the complex exists (Zhao, L., J.B. Helms, B. Brügger, C. Harter, B. Martoglio, R. Graf, J. Brunner, and F.T. Wieland, manuscript submitted for publication). This indicates that coatomer is bound to membranes by a bivalent interaction with ARF and p23. Thus, our present model for priming of COPI-coated vesicle formation includes (a) binding of ARF–GTP to Golgi membranes (7, 37) via an (at present uncharacterized [16]) ARF-receptor, and (b) binding of coatomer to membrane-anchored ARF via β-COP (Zhao, L., J.B. Helms, B. Brügger, C. Harter, B. Martoglio, R. Graf, J. Brunner, and F.T. Wieland, manuscript submitted for publication), with (c) concomitant recruitment of receptor-bound ARF (47) and p23 into budding regions of the membrane that would lead to binding of the cytoplasmic tail of the protein with coatomer subunits different from β-COP (alternatively, binding of ARF–GTP to coatomer may occur in specialized areas of the Golgi, with subsequent recruitment of p23 via its binding to coatomer). Independent studies have suggested either a trimeric complex formed from the α,β' and ϵ subunits of coatomer (6, 27), or γ-COP (15) to specifically bind to dibasic cytoplasmic tail motifs. γ-COP has also been identified as the predominant binding partner of a peptide analogous to the cytoplasmic tail of p23 (Harter, C., and F.T. Wieland, unpublished results). As shown

here, this tail binds coatomer with similar efficiency as compared with the established KKXX motifs. Given the high abundancy of p23 and p24 in COPI-coated vesicles and the stoichiometry between p23 and coatomer, this protein is likely to be part of the budding machinery and to function in the binding of coatomer in COPI-coated vesicles. Alternatively, the membrane layer of a COPI-coated vesicle for the binding of coatomer might be established by a mixture of a multitude of different K(X)KXX-containing membrane proteins, each of which is present in the vesicle at concentrations too low to be detected. Given the function of p23's tail peptide and the presence of this membrane protein in the vesicles in amounts stoichiometric to coatomer, this mechanism remains unlikely.

Various Types of COPI-coated Vesicles?

Although the scope of this study was to characterize membrane machinery involved in the formation of COPI-coated vesicles, it is tempting to speculate about the functions these vesicles serve. Experimental evidence exists for a role of Golgi-derived COPI-coated vesicles in anterograde biosynthetic protein transport through the Golgi apparatus (3, 34, 39, 41). More recent findings by Cosson and Letourneur showed that COPI-coated vesicles are implicated in retrograde transport from the Golgi to the ER (25) — a transport needed not only for the retrieval of luminal and membrane proteins of the ER, but also likely for the reshuffling of membrane lipids and constituents of forward transporting machinery (38).

Next, we would like to speculate on possible mechanisms for the formation of anterograde and retrograde vesicles. In the Golgi, p23 is present at concentrations far exceeding the concentrations of KKXX-containing cargo for retrieval to the ER, because retrieval tagged proteins at steady state are predominantly localized to the ER (20). As mentioned above, the manifold concentration of ARF–GTP and p23 in COPI-coated buds and vesicles upon priming indicates that these proteins form layers within the Golgi membrane that represent a scaffold for efficient binding of coatomer, allowing the formation of coated buds. For retrieval, the few escaped ER-type I membrane proteins present in the Golgi at any given time would then easily be accomodated within the forming coat structures, as a result of their K(X)KXX signals binding to coatomer with an affinity comparable to the tails of p23. This would provide a simple mechanism for selective retrieval of escaped ER-resident type I membrane proteins.

In contrast with retrieval, signals for anterograde transport through the Golgi have not been found. This is in accordance with the observation that cargo does not seem to be concentrated on its way through the Golgi apparatus (4, 12, 33, 34, 60). Therefore, in contrast with the exit from the ER (1), cargo receptors may not be needed for transport of proteins through the Golgi in an anterograde direction. Alternatively, the involvement of cargo receptors might not necessarily lead to a concentration of cargo within the Golgi above the level already achieved in the ER. In this case, members of the p24 family might well serve as cargo receptors for intra-Golgi transport. Localization of the individual members of this protein family to their various steady state residencies will be needed as a basis to clarify

this issue. As to the presence of p24 and p23 in COPI-coated vesicles, there are mainly two possibilities: (*a*) both proteins reside in one and the same vesicle; in this case, both membrane proteins together might form the scaffold for coatomer binding; or (*b*) p24 and p23 define different types of COPI-coated vesicles within a heterogeneous population, mediating either anterograde or retrograde transport. Thus, a major challenge for future work will be to further characterize COPI-coated vesicles with respect to their homogeneity.

Note Added in Proof. During submission of the manuscript, the sequence of p23 was published by Blum, R., P. Feick, M. Puype, J. Vandekerckhove, R. Klengel, W. Nastainczyk, and I. Shulz. 1996. Tmp21 and p24A, two type 1 proteins enriched in pancreatic microsomal membranes, are members of a protein family involved in vesicular trafficking. *J. Biol. Chem.* 271:17183–17189.

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