Abstract. Five mammalian members of the gp25L/emp24/p24 family have been identified as major constituents of the cis-Golgi network of rat liver and HeLa cells. Two of these were also found in membranes of higher density (corresponding to the ER), and this correlated with their ability to bind COP I in vitro. This binding was mediated by a K(X)KXX-like retrieval motif present in the cytoplasmic domain of these two members. A second motif, double phenylalanine (FF), present in the cytoplasmic domain of all five members, was shown to participate in the binding of Sec23 (COP II). This motif is part of a larger one, similar to the F/YXXXXF/Y strong endocytosis and putative AP2 binding motif. In vivo mutational analysis confirmed the roles of both motifs so that when COP I binding was expected to be impaired, cell surface expression was observed, whereas mutation of the Sec23 binding motif resulted in a redistribution to the ER. Surprisingly, upon expression of mutated members, steady-state distribution of unmutated ones shifted as well, presumably as a consequence of their observed oligomeric properties.

The elucidation of the transport machinery of the secretory pathway has benefited from both biochemical and genetic approaches (Rothman and Wieland, 1996; Schekman and Orci, 1996). This has allowed for the identification of two types of transport vesicles that can be distinguished by their coat, coat protein (COP) I and II coatamer. Whereas COP I vesicles can be seen budding from membranes throughout the pathway, COP II vesicles are formed exclusively at ER exit sites (e.g., transitional elements) where they export packed and condensed cargo from the ER (Aridor and Balch, 1996; Bednarek et al., 1996). After budding, COP II vesicles quickly shed their coat (Bednarek et al., 1996) and are subsequently delivered to (or transformed into) a tubular vesicular network usually referred to as the ER-to-Golgi intermediate compartment (ERGIC) (Schweizer et al., 1990). This intermediate compartment is transported along microtubules towards the more centrally located Golgi apparatus where it forms part of the cis-Golgi network (CGN) (Saraste and Svensson, 1991; Presley et al., 1997). During this process, budding of COP I transport vesicles takes place, and these either bring cargo forward (Rothman and Wieland, 1996) and/or recycle components in a retrograde manner (Pelham, 1994; Bednarek et al., 1996). The COP I coatamer has a predominant concentration on and around the CGN, but can also be seen in later compartments such as the trans cisterna of the Golgi and the TGN (Oprins et al., 1993) as well as in the endocytic pathway (Whitney et al., 1995).

Recruitment of coatamer onto membranes argues for the presence of coat binding proteins and candidates for these have recently emerged, mainly through the realization that COP I coatamer directly binds to the ER retrieval motif, K(X)KXX, present in some resident proteins of the ER and the ERGIC. This motif, first identified in a viral protein, E3/19k, encoded by the human adenovirus 2, suffices to maintain reporter molecules in the ER through
a constant retrieval from post-ER compartments. It was shown to be functional only if its second lysine was in a -3 position, and addition or deletion of amino acids COOH-terminal to this lysine abolished its ability to fully maintain proteins in the ER (Nilsson et al., 1989; Jackson et al., 1990). Remarkably, even the conservative substitution of lysines to either arginines or histidines led to loss of ER localization, and the same stringent criteria could be demonstrated in its ability to bind to COP I coatamer in vitro (Cossom and Letourneur, 1994). This strong functional correlation between ER retrieval and COP I coatamer binding placed COP I vesicles firmly onto the retrograde pathway and recently, several proteins with K(X)KXX-related motifs have been implicated as major coat binding receptors in vivo. Notably, ERGIC53 (p58 in rat), which is a major constituent of the ERGIC with lectin-like properties, displays a functional K(X)KXX in its cytoplasmic domain (Tisdale et al., 1997). Antibodies to this part of the molecule effectively compete for coatamer binding in permeabilized cells, showing that this molecule serves as a major receptor for the coat. Furthermore, such tail antibodies also inhibit anterograde transport, implicating this protein as a facilitator of cargo transport.

In a similar fashion, members of the gp25L/emp24/p24 (p24) family have also been implicated in bringing cargo forward from the ER and to bind coat proteins via their cytoplasmic domains. The two first members of this family were identified as a calnexin-associated integral membrane protein of the ER, (gp25L; Wada et al., 1991) and an endosome membrane protein in yeast (emp24; Singer-Krueger et al., 1993). Subsequent members were identified through genetic (erv25; Belden and Barlowe, 1996) and biochemical studies (CHOp24; Stannes et al., 1995) and p23 (Blum et al., 1996; Sohn et al., 1996), as well as as homology searches yielding a total of eight members in yeast and at least six in mammalian cells. Many of these display typical K(X)KXX motifs in their cytoplasmic domains, and genetic as well as biochemical studies have established links between these proteins and cargo export from the ER as well as being concentrated in COP II (Schimmoller et al., 1995; Elrod-Erickson and Kaiser, 1996; Belden and Barlowe, 1996) and I transport vesicles (Stannes et al., 1995; Sohn et al., 1996). Another member, a putative ligand for the T1/ST2 receptor, was isolated as a cell surface protein (Gayle et al., 1996).

The (function(s) of p24 members is yet to be demonstrated, but we have here undertaken a comparative study of five mammalian members and show that these proteins are highly abundant and reside, at steady state, in the CGN. Two of these members display typical K(X)KXX-like retrieval motifs and, as predicted, mediate binding of COP I coatamer in vitro. A second motif, composed of the two conserved phenylalanines conserved in all five members, mediates binding of Sec23 (COP II). Mutation of the retrieval-like motif redistributes mutated members as well as nonmutated ones to more distal parts of the secretory pathway including the cell surface. Mutation of the conserved double phenylalanine (FF) motif in only two members equally redistributed mutated and nonmutated family members to an ER-like location. This indicates a reliance on their association with each other to define their steady-state distributions.

Materials and Methods

Membrane Preparation, Subcellular Fractionation, Deglycosylation, Gel Filtration, Rate Zonal Centrifugation and NH₂-terminal Sequencing

Membranes were isolated from rat liver and HeLa cells (Sonnichsen et al., 1994) as described previously. For analytical fractionations, rat liver was homogenized as described by Bergeron et al. (1982). The homogenates were subjected to successive centrifugation at 1570 g_{max} for 10 min, the pellet washed once, and then both supernatants were pooled and centrifuged at 280,000 g_{max} for 40 min (fraction multiple large platforms [MLP]). MLP fractions were then loaded onto 0.5-2.5 M sucrose gradient and centrifuged at 80,000 g_{max} for 18 h. Fractions were collected and analyzed for their content of GalT, calnexin, GMP25, and p58. GalT assays were as described previously (Bergeron et al., 1982). After SDS-PAGE and Western blotting, filters were incubated with antibodies (see below) to reveal corresponding p24 members as well as other endogenous markers. HRP-conjugated secondary antibodies (Tago Inc., Burlingame, CA) using the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK) or 32P-labeled goat anti-rabbit (NEC, Mississauga, CA) were used to visualize immunoblots.

For rate zonal centrifugation, Golgi fractions were solubilized in 20 mM Hepes, pH 6.8, 50 mM NaCl, and 2% sodium cholate. After incubation for 1 h at 4°C, the lysate was centrifuged at 15,000 rpm for 10 min to remove insoluble membrane, and then the supernatant was loaded onto a 5-35% sucrose gradient containing 20 mM Hepes, pH 6.8, 50 mM NaCl, and 0.3% sodium cholate, and then centrifugated at 35,000 rpm for 17 h using a SW40 rotor (Beckman Instruments, Palo Alto, CA). Fractions were then collected and subjected to SDS-PAGE and immunoblotting.

Deglycosylation experiments were performed according to the recommendations of the supplier (Boehringer Mannheim, Mannheim, Germany). For pronase treatment, fractions were incubated for 1 h at 4°C with 1 M KC1/0.25 M sucrose/4 mM imidazole (KCI-SI buffer), centrifuged at 15,000 g_{max} for 5 min, and then resuspended in SI buffer. The fractions were then incubated with different amounts of pronase, with or without 0.1% Triton X-100, for 30 min at 0°C. The reaction was stopped by adding Pefabloc (Boehringer Mannheim) and EDTA (final concentrations, 5 and 10 mM, respectively) and boiled for 5 min. Sample buffer was then added, and the samples processed for SDS-PAGE and Western blotting.

For NH₂-terminal sequencing, fractions were subjected to Triton X-114 extraction carried out by the method of Bordier (1981), and then subjected to SDS-PAGE and transferred onto polyvinyl idene difluoride (PVDF) membranes. Membranes were rinsed three times with 1 M NaCl, once with water, and then stained for 30 s with 0.1% Coomassie brilliant blue R in 50% methanol and destained. Protein bands of interest were cut out and subjected to NH₂-terminal sequence analysis by automated Edman degradation according to the protocol of Hewick et al. (1981). Sequences were obtained by searching the following databases: GenBank, Swiss-Prot, and TIGR.

Cloning

For αβ, overlapping expressed sequence tag (EST) nucleotide sequences were used to design the primers used for the synthesis of the PCR probe from reverse transcriptase (RT) rat liver RNA. Total RNA was extracted from rat liver using the method of Chomczynski and Sacchi (1987). Total RNA (4 μg) was used with the RT kit ( Gibco BRL, Gaithersburg, MD). Template cDNA and 100 pmoles of primers were used for the PCR reaction. The products were separated in a 3% agarose gel, and then the band corresponding to the predicted size was excised and electrophoresed, followed by ethanol precipitation, and then cloned into the Smal site of plasmid pT7Z19R and sequenced using a (Applied Biosystem, Foster City, CA) sequencing kit. To clone αβ, a human brain cDNA was diluted, amplified, and then the plates were rinsed with Luna-Bertani (LB) medium and the phage DNA was precipitated with ethanol. A PCR reaction was carried out using S and A5 primer, and the positive pools sequenced using the RT-PCR fragment labeled by nick translation. One positive clone was then amplified, cut with EcoRI, and then a 1.3-kb fragment was subcloned into the EcoRI site of pT7Z19R and sequenced.

For the p24 family members, overlapping EST nucleotide sequences were used to design primers for nested PCRs. In each case, two sets of primers were designed and used to amplify full-length cDNAs using the Marathon cDNA amplification system (Clonetech Laboratories).
Inc., Palo Alto, CA). In all cases, a 3' race was combined with a 5' race to ensure that full-length cDNAs were obtained. The results of each pair was then used to design a forced-nested PCR to amplify full-length cDNAs from human placenta marathon-ready cDNA. All full-length cDNAs were designed to carry flanking BamHI sites to allow insertion into the pCMU [expression vector (Nilsson et al., 1989) for sequencing and transient transfection.

**Antibodies**

Rabbit polyclonal antibodies were generated against synthetic peptides corresponding to internal sequences of each p24 family member. Peptides used were TPGLGMCVEVKDPC (α, C), CVEVKPDPEKIVLAREY (α), DVEITIQPDKGGYDGC (β), CRLEGPDKVLYK (γ), CVYEDPQNTYRET (γ), STLEFPQITGHYDVDC (γ3), CKRTDSHILYKSEDA (β1) and RHLLLLSEKAVKL (cytoplasmic domain–α). Peptides were coupled to keyhole limpet hemocyanin (KLH) or BSA carrier and then injected into rabbits together with Friends Adjuvant (Sigma Chemical Co., Deisenhofer, Germany). Affinity purification was carried out essentially as described (Harlow and Lane, 1985). Rabbit polyclonal and mouse monoclonals against calnexin (Ou et al., 1993), KDEL receptor (Sonnichsen et al., 1994), myc (Evan et al., 1985), and vesic- 

**Electron Microscopy**

Preparation of tissue for cryoimmunoelectron EM was carried out as previously described (Dahan et al., 1994). Briefly, male Sprague-Dawley rats (100–125 g body weight) were fasted overnight and anesthetized. Livers were perfusion-fixed with Ringer’s lactate solution for 30 s, followed by freshly prepared 4% paraformaldehyde/0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at a flow rate of 10 ml/min for 10 min. Small pieces of liver were immersed in fixative for 1 h at 4°C, and then washed several times in 4% sucrose/0.1 M phosphate buffer. Liver pieces were then equilibrated, cryoprotected for 30 min–1 h with 2.3 M sucrose as described by Tokuyasu (1980), and then frozen directly in liquid nitrogen. Tissue sectioning, immuno- 

**Results**

**Identification of Golgi Membrane Proteins**

To identify abundant Golgi integral–membrane proteins, we purified rat hepatic membranes enriched for the Golgi marker, β1,4-galactosyltransferase (GaIT) but relatively diminished in endosomal contamination (Dominguez et al., 1997; manuscript submitted for publication). Morphological analysis of the Golgi fraction revealed stacked saccules with a mottled lipoprotein content, especially in saccular compartments (i.e., mitochondria, peroxisomes, lysosomes, multivesicular endosomes, and plasma membrane) was much lower than that observed over secretory apparatus compartments by respective primary antibody followed by species-specific secondary antibodies conjugated to either FITC (Tago Inc.) or Texas red (Vector Labs, Inc., Peterborough, United Kingdom). Fluorescent signals were visualized with a 24-red-green-blue (RGB) charge-coupled device (CCD) (Hamamatsu Photonics, Hamamatsu City, Japan) on an Axiovert 100TV (Carl Zeiss, Inc., Thornwood, NY) using a 100× lens and then recorded directly onto a disk using the Open Lab system (Improvision, Coventry, UK).

**Cell Culture, Transfection, and Immunofluorescence**

HeLa cells stably expressing N-acetylglucosaminyltransferase (NAGT) I tagged with a myc epitope (Nilsson et al., 1993) were kept in DME (GIBCO BRL, Paisley, Scotland) supplemented with 10% fetal calf se- rum, 10 μg/ml Geneticin (G4–18 sulphate) (Sigma Chemical Co., Deisenhofer, Germany) (450 μg/ml). Cells were transfected using the calcium phosphate technique as previously (Pálbo et al., 1986), except that 30 μg plasmid DNA was used for per 10 cm Petri dish of monolayer cells. At 72 h after transfection, cells were fixed and then processed for immunofluorescence as described previously (Nilsson et al., 1993; Peranen et al., 1993). Proteins were visualized using appropriate antibodies conjugated to species-specific secondary antibodies conjugated to either FITC (Tago Inc.) or Texas red (Vector Labs, Inc., Peterborough, United Kingdom). Fluorescent signals were visualized with a 24-red-green-blue (RGB) charge-coupled device (CCD) (Hamamatsu Photonics, Hamamatsu City, Japan) on an Axiovert 100TV (Carl Zeiss, Inc., Thornwood, NY) using a 100× lens and then recorded directly onto a disk using the Open Lab system (Improvision, Coventry, UK).
presented a significant proportion of the total integral membrane protein complement of the fraction.

cDNA Cloning, Sequencing, and Proposed Nomenclature

To clone the human cDNA of one of them (GMP25), oligonucleotide primers were synthesized based on overlapping sequences in two of the dbEST entries. These were used to generate a probe from rat liver RNA by RT-PCR. With this probe, screening of a human brain library led to the full-length sequence illustrated in Fig. 2 A, with the rat liver–deduced protein sequence corresponding to residues 1–43 of human GMP25. EST databases aided in the identification of the protein sequences in Fig. 1 B as constituents of the p24 family and also revealed a fifth member, p26.

For the other family members, full-length cDNAs were isolated (Fig. 2 B) as described in Materials and Methods. Despite sharing a homology of only 20%, the same amino acids were found at similar positions for all five family members (Fig. 2 B, highlighted by shading) (Blum et al., 1996). Interestingly, most of the conserved amino acids cluster towards the membrane-spanning domain (Fig. 2 A, highlighted by shading) and, when modelled in a helical wheel, face one side. Searching the database (Fig. 2 C) revealed that these sequences clustered into four major groups. Each group was represented by at least one member found in the purified hepatic Golgi fraction (Fig. 1 B). Two of the sequences (α2 and δ1) revealed the dilysine ER retrieval sequence (Jackson et al., 1990) at their carboxy termini (Fig. 2 B). Referring to their expected molecular weights or database accession numbers does not allow for easy comparison. We suggest they be referred to as p24 preceded by a single letter referring to the species as proposed by Fiedler and co-workers (1996), and subscripted by the Greek letter α, β, γ, or δ, followed by a number according to their position in the cluster tree and their relative time of publication. We suggest they be referred to as p24 preceded by a single letter referring to the species as proposed by Fiedler and co-workers (1996), and subscripted by the Greek letter α, β, γ, or δ, followed by a number according to their position in the cluster tree and their relative time of publication. The human sequence of GMP25 is denoted with number 2 since this member does not relate well to the gp25L (cp24α1). We suggest, therefore, that the ones examined in this study are termed hp24α2 (α2), hp24β1 (β1), hp24δ1 (δ1), hp24γ1 (γ1), and hp24γ4 (γ4) (Fig. 2 C).

Membrane Orientation of α2

The amino acid sequence of α2 as well as the other family members suggests a type I integral membrane protein with

Figure 1. (A) Random view of the purified hepatic Golgi fraction. Random sampling of the fraction (160-fold purification over the homogenate for the Golgi marker GalT) visualized after cross sectioning of filtered fraction reveals an abundance of stacked Golgi saccules (G) with a mottled content (Lp), especially in saccular distensions identified previously in Golgi apparatus in situ as apoE-containing lipoprotein particles (Dahan et al., 1994). Fenestrated structures (open arrowheads) are also evident. Bar, 400 nm. (B) Identification and NH2-terminal sequence of Golgi integral membrane proteins. The membrane proteins of the Golgi fraction were extracted with Triton X-114, subjected to SDSPAGE, transferred to PVDF membranes, stained by Coomassie brilliant blue, and then processed for NH2-terminal sequencing. Upper and lower case residues represent, respectively, certain and less certain amino acids. Repeated sequencing at five different regions of the broad band, indicated by the square bracket, has revealed only the indicated sequence. Four proteins of the p24 family were identified: Rat p23, the homologue to human p23; rat p24, the rat homologue of CHOp24; rat GMP25, the rat homologue to human GMP25, and a fourth previously uncharacterized family member, p26.
a predicted cleaved signal sequence (von Heijne, 1986), the cleavage of which was confirmed from NH$_2$-terminal sequencing (Fig. 1 $B$). Synthetic peptides deduced from the sequence of the $\alpha_2$ protein were used to raise antibodies. Protease protection assays and immunoblotting with affinity-purified antibody to residues 43–55 (Fig. 2 $A$) were used to probe the predicted intraluminal location of these residues of the $\alpha_2$ protein in purified hepatic Golgi fractions. This protein was sensitive to proteinase digestion only in the presence of detergent (Fig. 3 $A$), showing that the major portion of the molecule resides in the lumen. The sequence of the $\alpha_2$ protein predicted a single site of N-linked glycosylation at residue 88. Digestion with neuraminidase resulted in a slight shift in mobility on SDS-PAGE gels, consistent with terminal sialylation and a post-ER location for the $\alpha_2$ protein (Fig. 3 $B$). No additional molecular weight shift on SDS-PAGE was affected when samples were treated with both neuraminidase and O-glycosidase (Fig. 3 $B$), indicating that the protein was unlikely to be O-glycosylated. Treatment with peptide N-glycosidase F (Gpase F) led to an increase in mobility of ~3-kD consistent with a single site of N-glycosylation. The sensitivity to neuraminidase predicted an endoglycosidase H (endo H)-resistant glycoprotein, and this was confirmed (Fig. 3 $B$).

**Immunofluorescent Localization**

Conventional indirect immunofluorescence was attempted in HeLa cells stably expressing NAGT I with a myc epitope convenient for double labeling (Nilsson et al., 1993). Endogenous staining was weak, and transient expression was necessary for a stronger immunofluorescent signal. All five members were coexpressed, since transfection of individual members resulted in the formation of large, membranous structures of unknown origin (data not shown). It is presently unclear whether this was caused by high levels of expression, or if coexpression was required to circumvent formation of such structures. Upon coexpression, the $\alpha_2$ protein showed an apparent colocalization with the
Golgi marker (Fig. 4 A) as well as an apparent nuclear envelope and cytoplasmic reticular pattern typical of the ER. For the β₁ (Fig. 4 B), γ₁ (Fig. 4 C; revealed by antibodies recognizing both forms), and δ₁ (Fig. 4 D; revealed by antibodies recognizing both forms), apparent localization of the majority of α₂, β₁, γ₁ and δ₁ immunofluorescence (data not shown).

**EM Localization of α₂ in Rat Liver Cryosections**

The requirement of transfection for clear colocalization necessitated additional approaches. Using affinity-purified antisera to residues 186–198 of the α₂ sequence, localization in cryosections of rat liver was attempted. The results revealed antigenicity that was concentrated in smooth tubular membranes (Fig. 5, arrowheads) around Golgi apparatus and along the length of cisternal membranes of rough ER (Fig. 5, A and B, arrows). Quantitation of gold particle labeling within the secretory pathway compartments revealed 59% of labeling within smooth membranous networks, 35% within rough ER cisternae, and 6% within the Golgi apparatus (Fig. 5). Golgi apparatus was identified in cryosections of 18 hepatocytes from livers of two rats. Remarkably, the labeling distribution within Golgi stacks and closely surrounding regions revealed that α₂ protein was heterogeneously distributed, i.e., being concentrated in membranous tubulovesicular profiles immediately surrounding the Golgi apparatus, and the first sacule on one side of a given Golgi stack (Fig. 5, B–D, see brackets), suggesting a CGN localization. Few Golgi apparatus profiles were labeled over central Golgi sacules.

**Subcellular Distribution**

The EM immunolocalization of the α₂ sequence suggested a lack of colocalization with Golgi markers. This was tested by subcellular fractionation. Analytical fractionation of rat liver MLP fractions (total membranes minus nuclei; Fig. 6) revealed a median density of the Golgi marker GalT of 1.122, with a distribution different to that of the ER marker calnexin, with a median density of 1.178. The distribution of the intermediate compartment marker p58 (median density of 1.149) was intermediate to that of GalT and calnexin. The overall distribution of the α₂ protein was similar to that of p58. However, the α₂ protein revealed a slightly greater median density (1.153) and a peak (as determined by quantitative Western blotting) displaced to higher density to that of p58. Hence, as predicted from the EM studies, the α₂ protein was not in a compartment coincident with the GalT Golgi marker.

The subcellular distribution of all family members in rat liver membranes (Fig. 7, left) as well as in HeLa cells (Fig. 7, right), was compared. For rat liver homogenates, similar analytical gradients and a starting preparation of total membranes (MLP) similar to those used for the studies in Fig. 6 were used. Although overlapping, the distribution of the α₂ and δ₁ family members were displaced towards higher densities than the γ₂ and β₁ members. The distribution of p58, the intermediate compartment protein, was similar to the latter proteins, whereas syntaxin 5 (a Golgi resident target-soluble N-ethylmaleimide–sensitive factor attachment protein receptor (t-SNARE), Banfield et al., 1994) and the Golgi marker mannosidase II (Man II) were distributed towards lower densities. Calnexin was found in high density fractions. The heterogeneity in distribution of the p24 family members was confirmed in similar fractionation studies of HeLa cells (Fig. 7, right). This protocol used Nycodenz (Life Technologies, Eggenstein, Leopold Shafen, Germany) gradients instead of sucrose, and the use of a monoclonal antibody to GalT instead of Man II as a marker of the intermediate compartment. Taken together, a distinct pattern emerged in density gradients for both rat liver and HeLa cells for the α₂ and δ₁ family members. They revealed a distribution skewed more towards the ER marker than did the γ₂, γ₃, and β₁ family members.
Both δ₁ and α₂ show COOH-terminal sequences similar to the previously identified ER retrieval motif known to bind COP I coatamer (Cosson and Letourneur, 1994), K(X)KXX. Whereas the latter displays sequences fitting this motif, the former has one additional amino acid known to partially impair its function (Jackson et al., 1990). A third member, the β₁, shows sequences reminiscent of the K(X)KXX motif, but with its critical lysines substituted to arginines. Substitution of these two lysines is known to eliminate the ability of the K(X)KXX motif to prevent reporter molecules from reaching late Golgi compartments (Jackson et al., 1990), and to effectively abolish binding to COP I coatamer (Cosson and Letourneur, 1994). The two additional members, γ₁ and γ₂, showed no sequences that could be related to known motifs. To investigate the ability of each member to bind COP I coatamer, synthetic peptides corresponding to their cytoplasmic domains were attached to thio-Sepharose, and then incubated with HeLa cytosol. Their binding was compared to that of peptides corresponding to the cytoplasmic domains of a protein of the CGN, i.e., the KDEL receptor (erd2) (Lewis and Pelham, 1990), as well as peptides corresponding to the type II cytosolic domains of the Golgi resident enzymes N-acetylglucosaminyltransferase I (GalNac-T1) (Hagen et al., 1993; Homa et al., 1993) and Man II (Moremen and Robbins, 1991). As positive controls, peptides corresponding to the cytosolic domain of E3/19k (Cladaras and Wold, 1985) and UDP-glucuronyltransferase (UDP-GT) (Jackson et al., 1987) were included. These peptides contain the K(X)KXX motif. A mutant, E3/19k kk/ss, having the two lysines known to be critical for Golgi-to-ER retrieval (Jackson et al., 1990) and COP I coatamer binding (Cosson and Letourneur, 1994), mutated to serines was incorporated as a negative control.

Fig. 8A shows that sequences corresponding to the cytoplasmic domains of δ₁ and α₂ associated with COP I components (as tested for α, β, β', γ, and 8 COP subunits) as did those of E3/19k and UDP-GT. Little, if any, association of COP I subunits was observed to the other peptides under the conditions used. Interestingly, a small but significant binding of COP I components was observed to the peptide corresponding to the cytoplasmic domain of GalNac-T1 that might be explained by the presence of two basic amino acids immediately following the methionine.
of this type II transmembrane protein. Whereas a similar motif composed of double arginine (RR) has been shown to be sufficient for Golgi-to-ER retrieval of proteins of type II topology (Schutze et al., 1994), binding of such a motif to COP I subunits has yet to be established.

The surprising finding of these experiments was the failure of COP I subunits to bind $\beta_1$, $\gamma_3$, and $\gamma_4$. This would have been predicted from earlier studies (Fiedler et al., 1996; Sohn et al., 1996) that showed significant binding to both $\beta_1$ and to a mutated version of the $\delta_1$ where its COOH-terminal sequence had been changed from KKLIE to SSLIE. Therefore, we examined the influence of using fresh versus frozen cell and tissue lysates under different buffer conditions, but were unable to reveal any detectable binding to the other members (data not shown). However, upon prolonged exposures, a weak but significant binding to $\beta_1$ could be observed, and this was further increased when supplying purified COP I coatomer (data not shown). Under these conditions, no binding to $\gamma_3$ and $\gamma_4$ was observed, suggesting a weak but reproducible binding of COP I coatomer to $\beta_1$.

The unexpected finding that COP I coatomer could bind cytoplasmic domains as partial complexes (Cosson and Letourneur, 1994), rather than a complete unit, was recently extended by Fiedler and co-workers who showed that two subcomplexes of COP I coatomer either bound to a putative but conserved FF motif present in the cytoplasmic domain in most p24s members, or to the K(X)KXX-like motif. This was suggested to provide the mechanistic explanation for how COP I coatomer could be present on both forward (coated by COP I subunits $\alpha$, $\beta'$, and $\epsilon$) and retrograde (coated by COP I sub units $\alpha$, $\beta'$, and $\epsilon$) moving transport vesicles (Fiedler et al., 1996; Orci et al., 1997). Under no conditions were we able to reproduce subcomplex formation of the COP I coatomer ($\zeta$; and $\epsilon$ not assayed for). Rather, our findings are more in line with those observed by Sohn and co-workers (1996) (Fig. 3 A, lanes I and 5), who showed clear binding of all COP I subunits to $\delta_1$ ($\zeta$ and $\epsilon$ not assayed for). However, given that a blotting assay has clear technical limitations in quantitative terms, we do not exclude the possibility of individual protein members of the COP I coatomer complex to ex-
Analytical fractionation of the α₂ protein in rat liver homogenates. The MLP fraction from rat liver homogenates was centrifuged on linear sucrose gradients as described in Materials and Methods. Equal volumes of each fraction were evaluated for their content of GalT as evaluated by enzyme assay, calnexin, p58, and α₂ protein as recognized by immunoblots, and quantification was evaluated by densitometry. The number of separate fractionations (n) is indicated. Results were normalized according to the methodology of Beaufay et al. (1964).

In Vivo Mutational Analysis and Oligomeric Properties of p24s

Since only two out of the five chains bound COP I in relatively high amounts, and this coat is implicated in retrograde transport (Cosson and Letourneur, 1994), eliminating the Golgi-to-ER retrieval motif of the α₂ and δ₁ family members may alter their distribution. The α₂ and δ₁ sequences were accordingly mutated with two S residues, respectively, to alter the S domain to either α₂(1–4) or δ₁(1–8). In contrast, binding of Sec23 was greatly reduced upon changing the FF to AA but was completely unaltered when changing downstream sequences that included the KK. An influence of upstream sequences on binding Sec23 was also observed, suggesting that the FF motif is part of a larger one. This was examined in greater detail using the mutant, E3/19k ss/ss that binds Sec23 at relatively low levels but no COP I. This cytoplasmic domain (outlined in green) shows that the KK-like motifs of the p24s respond to the cytoplasmic domain of δ₁ were mutated so that amino acids encoding this domain were substituted two by two. Where possible, hydrophobic residues were substituted to alanines and hydrophilic ones to serines. The result of such an experiment is shown in Fig. 8 B. Both COP I and II binding was examined and as expected, when changing the KKLIE to SSSLIE, COP I binding was reduced significantly. This is consistent with the observations of Sohn and co-workers (1996). However, only a slight, if any, decrease of COP I binding was observed when the FF motif was mutated to double arginine (AA), and this was unexpected since Sohn and co-workers (1996) showed a greater dependency on this motif for binding to all COP I subunits (γ and ε not assayed for). Instead, we detected the influence of this motif in modulating COP I binding, since changing both the FF and the double lysine (KK) to AA and double serine (SS), respectively, completely abolished binding to COP I (Fig. 8 B, lane 9). In contrast, binding of Sec23 was greatly reduced upon changing the FF to AA but was completely unaltered when changing downstream sequences that included the KK. An influence of upstream sequences on binding Sec23 was also observed, suggesting that the FF motif is part of a larger one. This was examined in greater detail using the mutant, E3/19k ss/ss that binds Sec23 at relatively low levels but no COP I. This cytoplasmic domain (outlined in green) shows that changing this F to A did not result in a decrease in the already relatively low level of Sec23 binding, whereas introduction of an additional F next to the preexisting one increased Sec23 binding significantly. Furthermore, when introducing FY upstream of this F residue resembling those found in δ₁, Sec23 binding increased even further. Therefore, we tentatively conclude from these studies that whereas the KK residues present in δ₁ and α₂ mediate binding of COP I subunits, the FF residues, presumably part of a larger motif (see Discussion) allow for the binding of Sec23. However, a minor influence of the latter residues on COP I binding was also detected.
Family members to the cell surface can be readily ex-
in COP II vesicles (Belden and Barlowe, 1996). The ob-
servation that p24 family members redistribute as a
complex of all five p24 family members. Since the FF motif
was shown to mediate binding to Sec23 (COP II) and this
cotamer complex was needed for exit from the ER (Bar-
lowe et al., 1994), this motif was mutated in both α2 and δ1
to AA. Upon cotransfection, the mutated p24s (Fig. 9 C,
shown is α2) were now found to distribute to the ER to-
gether with the other three nonmutated members (Fig. 9 D,
γ34 proteins). Hence, the proteins may show heterotypic
interactions in vivo and this was further examined in vitro.

Highly purified rat liver Golgi membranes were sub-
jected to detergent solubilization followed by velocity gra-
dient centrifugation and fractionation. Precipitated pro-
teins were then subjected to SDS-PAGE and Western
blotting to reveal the different p24s. As can be seen in Fig.
10, the p24s appear to cosediment as large oligomeric com-
plexes (peaking near 35S as evaluated using the McEwen
method [1966]), well away from the membrane protein,
calnexin (a resident ER protein contaminant of the frac-
tion), and the soluble cargo molecule, serum albumin.

Discussion

The observation that p24 family members redistribute as a
consequence of the ER retrieval motif K(X)KXX(X) was
demonstrated by immunofluorescence upon coexpression
of all five members. This redistribution also affected non-
mutated family members, suggesting heterotypic inter-
actions among the p24 family members. Such interactions
were further demonstrated in vitro by subjecting deter-
gent-solubilized material to velocity gradient centrifuga-
tion. The α3, β1, δ1, and γ3 members cosedimented as large
complexes (evaluated to peak at a sedimentation value of
~35S by the method of McEwen [1966]). This corresponds
to very large complexes of ~1–2 million D, consistent with
values obtained for these proteins upon gel filtration (data
not shown). In yeast, the homologous proteins of β and δ
(emp24 and erv25, respectively) have been demonstrated
by chemical cross-linking to be in a complex when present
in COP II vesicles (Belden and Barlowe, 1996).

KK to SS mutations resulting in a redistribution of the
p24 family members to the cell surface can be readily ex-
plained by the importance of these residues for retrieval
(Nilsson et al., 1989; Jackson et al., 1990) and recruitment
of COP I coatomer (Cosson and Letourneur, 1994; Le-
tourneur et al., 1994). The latter was shown to be recruited
in vitro. Both α2 and δ1 showed clear COP I binding in
what appeared to be a KK-restricted manner, and in com-
parable amounts. Furthermore, mutation of the KK motif of the
α2 (compare α2 and δ1, Fig. 8 A). However, binding of
COP I to δ1 was not completely abolished upon changing
its KK motif to SS, suggesting that upstream sequences
would mediate binding as well. However, this could only be
shown indirectly. When both KK and FF were substitu-
ted to SS and AA, respectively, COP I binding was reduced
even further or effectively eliminated. However, when
substituted alone, such upstream sequences (including the
FF motif) showed very little effect on COP I binding. This
is in stark contrast to that observed by Sohn and co-work-
ers (1996), who showed a complete elimination of COP I
binding when changing the FF motif to AA. At present,
we have no explanation for this discrepancy. What is ap-
parent though, both from this study and that of Sohn and co-workers (1996), is the lack of subcomplexes of COP I
coatomer binding to cytoplasmic domains of p24s.

The β1 and γ chains failed to bind COP I in appreciable
amounts. Furthermore, mutation of the KK motif of the δ1
member would have been expected to only reduce binding
of some of the COP I subunits but not all. Fielder et al.
(1996) found clear binding of COP I subunits to the cyto-
plasmic domains of all p24 family members tested. As

Figure 7. Analytical fractional of all family members in ho-
genomates from rat liver and HeLa cells. Total membranes
from rat liver (A) or HeLa cells (B) were centrifuged on
linear sucrose (rat) or Nycodenz (HeLa) gradients as de-
scribed in Materials and Methods. Equal volumes of each
fraction were determined for their content of calnexin, Man II
(Rat) or GalT (HeLa), syn-
taxin 5, p58 (rat), p53 (HeLa), and different p24 family
members, using specific antibodies generated to the peptides de-
scribed in Fig. 2 A and with antibodies specific to the Golgi markers (Man II, Gal T), the intermediate compartment markers (p58, p53)
or ER marker (calnexin). The high density end of the gradient is on the right.
well, they observed that only a subset of COP I subunits (α, β', and ε) bound in a KK-restricted manner, whereas the other subunits (β, γ, and δ) bound in a FF-dependent manner. It has been shown that the COP I complex can be split into either separate subunits or apparent subcomplexes when using relatively high salt concentrations (0.3–0.5 M). This was observed by Cosson and Letourneur (1994) who compared the binding of COP I under either low or high ionic strength to peptides displaying the K(X)KXX motif. At high ionic strength, binding was only observed for α, β', and possibly ε, whereas at lower ionic strength, binding of β and γ was also observed. Evidence that the COP I complex is sensitive to high ionic strength was also shown by Lowe and Kreis (1995), who subjected the COP I complex to either 1 M NaCl, 0.5 M Tris-HCl, or 0.25 M MgCl₂. This resulted in a breakdown of the COP I complex into either subcomplexes or individual subunits in vitro. However, under the conditions used in this study (0.4 M salt), we were unable to split the COP I complex. Rather, it appeared as a single complex and this persisted upon altering buffer conditions including salt concentra-

Figure 8. Binding of COP I and II proteins to cytoplasmic domains. Peptides corresponding to different cytoplasmic domains were coupled to thiopropyl Sepharose beads as described in Materials and Methods, and then assayed for their ability to bind COP I and II. (A) Binding of coat proteins to the cytoplasmic domains of α₂, β₁, β₂, γ₁, γ₂, ε, δ, E3/19k, E3/19k kk/ss (KK mutated to SS), UDP-GT, GalNac-T1, Man II, and control peptides C1-4. Binding of COP I components (tested for were α, β, β', γ, and δ-COP) to α₂ and δ₁ was comparable to E3/19k and UDP-GT. The human homologue of Sec23 (hSec23) of the COP II coatomer showed specific binding to all cytoplasmic tails, but not to the four control peptides C1-4. Note the additional binding of the Sec23 to β₁ and δ₁ as well as to GalNac-T1. (B) Binding of coat proteins as in (A) but using the cytoplasmic domain of δ₁ where pair-wise amino acid substitutions reveal a decrease of COP I binding upon KK-to-SS substitution, whereas Sec23 of COP II is reduced upon FF-to-AA substitution. (C) Introduction of aromatic residues into the mutated E3/19k peptide increases binding of Sec23.
to hp24δ₁ where its KK motif had been mutated to SS. Clearly, only by revealing the presence of COP I complexes in vivo, can we address the significance of subcomplexes, and examine to what extent such subcomplexes correlate with function (e.g., anterograde and retrograde COP I vesicle-mediated transport).

Other resident proteins of the exocytic pathway reveal motifs similar to the KK motif, although in different configurations and topologies. For example, the GalNAcT-1, which showed a weak binding of COP I, has in its cytoplasmic domain residues that in this type II protein, could be expected to serve as a retrieval signal (Schutze et al., 1994). Future work will reveal if there exists a correlation among resident proteins of the exocytic pathway in their extent of retrieval and their relative ability to bind COP I.

Whereas the KK-to-SS mutation led to a more distal distribution (including the cell surface) of the p24 family members, mutation of FF-to-AA redistributed the same proteins to a more ER-like location. As for the KK-to-SS mutations, the FF-to-AA mutations were only on the α₂ and δ₁ family members. Yet, all family members redistributed to an ER-like location, consistent with their suggested association as heterooligomers in vivo. FF motifs in the cytosolic domains of this family of membrane proteins have previously been implicated in exit from the ER (Fiedler et al., 1996). However, rather than binding COP I

Figure 9. Altered distribution of family members after mutation of K(X)KK(X) on FF motifs of the cytosolic domains of α₂ and δ₁ proteins. Cotransfection of all five p24s in where either the KK (A and B) or the FF (C and D) motif has been altered to SS or AA, respectively, in both α₂ and δ₁. Altered redistribution and apparent cell surface staining can be seen with the KK/SS mutants (shown is α₂)(A). Remarkably, the unmutated members (shown is γ3/4) (B) also reveals redistribution to the cell surface. Similarly, an apparent ER staining can be seen with the FF/AA mutants (shown is α₂)(C). This also leads to the apparent redistribution of the unmutated members to the ER (shown is γ3/4)(D). Bar, 5 µm.
First, we show the FF motif is involved in binding of the COP II coatomer subunit sec23, as mutation of this motif in δ₁ greatly reduced the binding of Sec23. This helps to explain the shift of their steady-state distributions to the ER upon expression, as decreased binding of COP II would reduce their ability to be exported from the ER (Barlowe et al., 1994; Campbell and Schekman, 1997).

Binding of Sec23 was observed in all cytoplasmic domains tested. This suggested a nonlinear motif, since there was no apparent similarity between these domains, except for the presence of one or more aromatic residues. However, the binding to Sec23 is unlikely to be as the result of nonspecific association to an aromatic residue because no binding was detected to the four control peptides used in the study, which all contained at least one aromatic residue. We conclude that Sec23 binding involves aromatic residues, but that these must be presented in a favorable way. Sequences upstream of the FF motif but not downstream showed significant influence on the binding of Sec23 to δ₁. These show a high degree of conservation in all p24s, suggesting that FF is part of a larger motif. For example, the strong endocytosis and putative AP2 binding motif, F/YXXXxF/Y, can be modeled onto sequences corresponding to the cytoplasmic domains of β₁, δ₁, and the γ chains. However, the γ chains showed comparable levels of Sec23 binding to α₂, suggesting that other amino acids are influencing Sec23 binding as well (e.g., the two basic amino acids preceding the FF motif: compare KSFF (α₂, γ₁, and γ₃) with RRFF (β₁ and δ₁)). Further mutational studies using E3/19k showed that Sec23 binding could be increased if an additional aromatic residue to mimic the FF motif was introduced. When this F/YXXxF/Y motif was created, Sec23 binding was increased even further. At this stage, the importance of a F/YXXxF/Y motif in some of the p24s can only be speculated upon, but it raises some exciting parallels to the endocytic pathway where this motif appears to mediate or at least greatly influence binding of AP2 to endocytosed membrane proteins (Johnson et al., 1990; Trowbridge, 1991; Kornfeld, 1992; Milgram et al., 1996; Rapoport et al., 1997).

When coexpressed, the five p24 family members colocalized with the medial Golgi marker NAGT I as judged by indirect immunofluorescence. By EM immunolabeling, the α₂ member was largely (60%) located to small tubular networks approaching one side of the Golgi stack with the stacked saccules accounting for only a minimum (6%). By analytical fractionation, the majority of this family was found in compartments whose density was distinct to that of Golgi markers. Taken together, these observations suggest that at steady state, all family members are found to their greatest extent in the CGN. Since the α₁ family member was terminally glycosylated in rat liver Golgi fractions (the γ₁ member has also been found to be N-glycosylated and endo H resistant in HeLa cells [Füllekrug, J., and T. Nilsson, unpublished data]), then this predicts they are subjected to retrograde transport from compartments as late as the trans-Golgi. The slightly different distributions of the p24s in compartment(s) between the ER and stacked saccules of the Golgi apparatus as revealed by subcellular fractionation and indirect immunofluorescence likely reflects the sum of the opposing anterograde and retrograde signals to which they are subjected and their relative affinities for each other (see above).

The p24s have been suggested to serve as cargo receptors bringing cargo out of the ER. This is supported by the observation that β (emp24) and δ (erv25) are found enriched in COP II vesicles in yeast. Furthermore, the deletion of these membrane proteins decreased the rate of transport of selected ER-derived cargo (Schimmöller et al., 1995; Stamnes et al., 1995; Staines et al., 1995; Elrod and Kaiser, 1996). Consistent with β (emp24) and δ (erv25) being concentrated in COP II vesicles, in yeast we observed a higher binding of Sec23 to their human counterparts. Despite the generation of polyclonal antibodies to nine different peptides of the five p24s examined in this study, none were immunoprecipitating. Future coimmunoprecipitation studies using appropriate antibodies during pulse-chase protocols will clarify the roles of these family members in cargo delivery, recycling, and their oligomerization in mammalian cells. The steady-state concentration of the p24s in the CGN suggests additional roles. For example, their high abundance in Golgi fractions along with the intermediate compartment marker p53/58 that has also been shown to associate with COP I coatomer predicts these three proteins (α₂, δ₁, and p53/58) represent the major COP I coatomer binding proteins of the cell. Indeed, the high proportion of all integral membrane proteins accounted for by the p24 family members in the hepatic Golgi fraction (Fig. 1) suggests that along with p53/58, they represent the major integral membrane proteins of the secretory pathway between the ER and the Golgi apparatus. An explanation for their relative abundance, oligomeric behavior, and steady-state distribution in the CGN could, therefore, be that these serve to form and maintain membrane structures between the ER and Golgi apparatus, and that their relative abilities to bind COP I and II serves to position them in this part of the pathway as well as to perhaps stabilize the membranes in which they reside by binding directly to
COP I (Kreis and Pepperkok, 1994). Such a structural role is testable.

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