Tmp21 and p24A, Two Type I Proteins Enriched in Pancreatic Microsomal Membranes, Are Members of a Protein Family Involved in Vesicular Trafficking*

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We report here on the isolation, cloning, and expression of two M, 21,000 proteins from rat pancreatic acinar cells, the rat-Tmp21 (transmembrane protein, M, 21,000) and the rat-p24A. Both proteins are transmembrane proteins with type I topology and share weak but significant homology to one another (23% identity). We further show the cloning and characterization of the human homologs, hum-Tmp21, which is expressed in two variants (Tmp21-I and Tmp21-II), and hum-p24A. Tmp21 proteins and p24A have highly conserved COOH-terminal tails, which contain motifs related to the endoplasmic reticulum retention and retrieval consensus sequence KXX. The rat-p24 sequence is identical to the hamster CHOp24, a recently characterized component of coatomer-coated transport vesicles, which defines a family of proteins (called the p24 family) proposed to be involved in vesicular transport processes (Stamnes, M. A., Craighead, M. W., Hoe, M. H., Lampen, N., Geromanos, S., Tempst, P., and Rothman, J. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8011–8015). Sequence alignment and structural features identify the Tmp21 protein as a new member of this p24 family. Northern analysis of various tissues indicates that the Tmp21 proteins and the p24A protein are ubiquitously expressed. The integral membrane components Tmp21 and p24A are localized in microsomal membranes, zymogen granule membranes, and the plasma membrane and are absent from the cytosol. Both p24A and Tmp21 show weak homology to the yeast protein Emp24p, which recently has been shown to be involved in secretory protein transport from the endoplasmic reticulum to the Golgi apparatus. This leads us to conclude that the receptor-like Tmp21 and p24A are involved in vesicular targeting and protein transport.

The current knowledge of vesicular transport mechanisms concerns mainly the budding, docking, and fusion of transport vesicles (reviewed by Rothman, 1994). Less is known about the sorting and accumulation of specific proteins in transport vesicles and their delivery from the ER to the Golgi apparatus. Characterization of transmembrane proteins that act like receptors could give new insights into the mechanisms underlying transduction of luminal information to the cytosol.

Several transmembrane proteins of the ER and the Golgi apparatus carry a short cytoplasmically exposed COOH-terminal peptide sequence with the dilysine (KKXX) motif. This motif serves as a retention and retrieval signal that brings proteins back from a sorting compartment such as the Golgi complex to the ER (Jackson et al., 1990). The mechanisms responsible for ER retrieval and retention are not well understood, but it is known that yeast and mammalian dilysine-tagged ER-resident transmembrane proteins interact with the coatomer in cell lysates (Cosson and Letourneur, 1994; Lowe and Kreis, 1995). Mutations that affect the ER retention capacity of the motifs abolish binding of the coatomer (Cosson and Letourneur, 1994). A defect in retrieval was also observed in mutants with a defect in the genes coding for the coatomer proteins α-COP (RET1), β-COP (SEC27), and γ-COP (SEC21) (Letourneur et al., 1994). From these results, it has been suggested that coatomer plays an essential role in retrograde Golgi-to-ER transport and retrieval of dilysine-tagged proteins back to the ER.

Recently, an integral membrane component of coatomer (COPI)-coated vesicles, termed p24, was characterized (Stamnes et al., 1995). This protein defines a family of proteins from plants, yeast, and mammals with integral membrane character. One of these p24 proteins, the yeast Emp24p protein (endomembrane protein precursor of M, 24,000) was shown to be involved in the sorting and/or concentration of a subset of secretory proteins in ER-derived COPII transport vesicles (Schimmüller et al., 1995) that mediate ER-to-Golgi trafficking in yeast (Salama et al., 1993).

Here we report on the identification of two M, 21,000 proteins from rat pancreatic microsomal membranes and the molecular cloning of their homologs. These M, 21,000 proteins with receptor-like structure belong to two subfamilies of the evolutionarily conserved p24 protein family. One of these proteins, p24A, is identical to the CHOp24 that defines the p24 family. The second protein, Tmp21, shows 23% identity to p24A and represents a new subfamily, to which belong two variants (Tmp21-I and Tmp21-II). The Tmp21 protein carries a KKLIE sequence at the COOH terminus, which might be recognized by the coatomer.

**EXPERIMENTAL PROCEDURES**

Preparation of Pancreatic Acinar Cells and Subcellular Fractions—Acinar cells were isolated from rat pancreas by collagenase digestion (Streb and Schulz, 1983). Zymogen granules were prepared from isolated acinar cells on a Percoll gradient as described (Fuller et al., 1989).
Plasma membranes were purified using the MgCl₂ precipitation method (Bayerdorffer et al., 1985). Microsomal membranes and the cytosol were prepared by differential centrifugation, as described previously (Zimmermann et al., 1992).

**Protein Purification and Sealing—**Preparative isoelectric focusing was performed using a Rodocel Bio Rad. Protein samples were suspended in a buffer containing Triton X-100. The whole microsomal fraction (Tmp21) or Triton X-100 were added to the sample. After centrifugation, the supernatant was collected and used for SDS-PAGE (Laemmli, 1970) and stained with Coomassie Blue; then the bands of interest were excised. Protein sequencing was performed using Edman degradation of the whole proteome and tryptic fragments.

**Antibody Production and Western Blot Analysis—**Antibodies against rat-Tmp21-I and rat-p24A were produced by immunizing rabbits with synthetic peptides coupled to hemocyanin containing the amino acid sequence of the NH₂-terminus for rat-Tmp21-I (ISFHLPVNSRKC) and for rat-p24A (YFV5IDAHEEC). For Western blot analysis, proteins were electrophoretically separated on SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose blots were blocked with 3% milk powder in Tris-buffered saline (TBS: 10 mM Tris- HCl, pH 8.0, 150 mM NaCl) for 60 min, followed by a 90-min incubation with various antibodies diluted in TBS plus 0.2% Tween 20 as follows: 1:3000, anti-Tmp21 and anti-SSR; 1:2000, anti-p24A and anti-calnexin; and 1:500, anti-Rab3A/B and anti-Ras. Bound antibodies were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG using the ECL detection kit (Amersham Corp.) or with alkaline phosphatase coupled to goat anti-mouse or goat anti-rabbit IgG, according to the instructions of the manufacturer (Bio-Rad).

**Amplification and Cloning of cDNA Probes—**Degenerated oligonucleotides encoding Tmp21 and p24A peptide sequences were synthesized and used for the reverse transcription-polymerase chain reaction. Reverse transcription-polymerase chain reaction was carried out using 25 pmol of degenerated primers, 66 mM Tris-HCl, pH 8.8, 2 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 200 μM deoxynucleotide triphosphates, 170 μg/ml bovine serum albumin, 10% dimethyl sulfoxide, and 2.5 units of Pwo-polymerase (Boehringer), the rat-Tmp21-I and the hum-p24A were amplified using the T7 Sequencing™ kit (Pharmacia), and the plasmids were recovered according to the method of Del Sal et al. (1988). DNA inserts were sequenced using the T7 Sequencing™ kit (Pharmacia), and inserts encoding Tmp21 and p24A peptide sequences were separated from pUC18 by digestion with KpnI and BamHI, followed by DNA separation. The resulting DNA inserts were isolated from the agarose gels and used for DNA screening.

**cDNA Screening and DNA Sequencing—**We have prepared a random and an oligo(dT)-primed cDNA library from rat pancreatic tissues in ZAPII and ZAP-Express vectors (Stratagene). In addition, we used premade ZAPII cDNA libraries (Stratagene) from human and rat brain. About 10⁶ plaques from different cDNA libraries were screened by hybridization with [³²P]random-labeled partial Tmp21-cDNA and p24A probes (see “Northern Analysis”) under high stringency hybridization conditions (Sambrook et al., 1989). DNA sequencing was performed with a T7 Sequencing™ kit (Pharmacia).

**Data Base Searches and Sequence Analysis—**Protein and nucleic acid sequences were analyzed using the TBLASTN and the FASTA algorithm of the European Molecular Biology network service (GENIUS Network Service; Deutsches Krebsforschungszentrum, Heidelberg, Germany). DNA fragment assembly, protein hydropathy analysis, identification of heptad repeats, alignments, and all other sequence-dependent analyses were performed using the Wisconsin Sequence Analysis Package Genetics Computer Group.

**Northern Analysis—**Total RNA was prepared by the method of Chomczynski and Sacchi (1987) from different rat tissues, from the rat pancreatic acinar tumor cell line AR4-2, and from the human pancreatic duct cell line CAPAN. The RNA was separated by formaldehydeagarose gel electrophoresis and transferred to Hybond-N⁺ membrane (Amersham Corp.). Tmp21- and p24A-specific transcripts were visualized by hybridization with [³²P]-labeled DNA fragments (p24A transcripts with a 265-bp probe (bases 324–589 in accession no. X90297) and Tmp21 transcripts with a 485-bp probe (bases 30–515 in accession no. X97442)). In addition, a 900-bp probe (Xhol fragment of rat-Tmp21-I) was used containing 200 bp of the coding region and about 700 bp from the complete 3'-untranslated region.

**Expression of rat-Tmp21-I and hum-p24A in Escherichia coli—**Using Pwo-polymerase (Boehringer), the rat-Tmp21-I and the hum-p24A were amplified from NotI-linearized DNA screening clones with primers that span the open reading frames (Tmp21 for rat-Tmp21-I: 5'-ATTCCTTCCATCTACCC, and reverse, 5'-TATCTTCTAACTTCTTGG; hum-p24A: primer: forward, 5'-TATCTTGGTAGACGACG; and reverse, 5'-TTAACACACTCCGCCG). The amplification products were blunt-inserted into the Xmal restriction site of the expression vector pMal-C2 (New England Biolabs) and expressed in the E. coli strain TB1. The resulting maltose-binding fusion proteins were purified by binding to maltose-binding affinity resin. The maltose-binding fusion proteins were cleaved from the maltose-binding protein by factor Xa and characterized by Western blot analysis. The expressed proteins were used to confirm the identity of the cloned Tmp21 and p24A cDNAs to the microsomal proteins and for affinity purification of the antibodies described above.

**Triton X-114 Extraction of Microsomal Membranes—**Isolated pancreatic microsomes (see above) were extracted three times with Triton X-114 as described by Bordier (1981). The resulting aqueous and Triton X-114 protein phases were analyzed by immunoblotting with the Tmp21- and p24A-specific antibodies. To determine whether Triton X-114 extraction was complete, we monitored the distribution of the secretory protein amylase, which was found preferentially in the aqueous phase.

**Trypsin Digestion Assay of Microsomes—**Intact microsomes were resuspended in 280 mM mannitol, 5 mM Hepes, 10 mM KCl, and 1 mM MgCl₂, pH 8.0. The microsomes were divided into three aliquots: the "undigested input" aliquot A, the "trypsin-Triton" aliquot B, and the "trypsin, non-detergent" aliquot C. Each aliquot contained 1 mg of microsomal protein. Aliquot B was treated with 0.1% Triton X-100 to lyse the microsomes. Then, 1/100 part of trypsin (110 units/mg; Boehringer Mannheim) was added to each 1/100 part of aliquots B and C and incubated for 10, 20, 30, 60, and 120 min at 37 °C. Samples containing 20 μg of protein taken at different time points were analyzed by Western blot analysis using the anti-Tmp21 and anti-p24A antibodies.

**RESULTS**

Cloning of the Human and Rat cDNAs Encoding Tmp21 and p24A—In our previous studies on intracellular vesicle transport in rat pancreatic acinar cells, we had separated M, 21,000 proteins from the microsomal membrane fraction (Zeuzem et al., 1991, 1992). We have now isolated two of these proteins by preparative isoelectric focusing in the pH range 5.8–6.1, followed by SDS-PAGE and microsequence analysis. Two NH₂-terminal and nine internal peptide sequences were obtained (see underlined sequences in Fig. 1). Using degenerated primers deduced from peptide sequences, we were able to amplify, clone, and sequence cDNAs encoding for the tryptic and the NH₂-terminal protein sequences of both M, 21,000 proteins. Using [³²P]-labeled cDNA fragments as probes, positive λ clones were isolated from rat pancreatic, rat brain, and human brain cDNA libraries. The amino acid sequence information obtained from the screening procedure is summarized in Fig. 1 (hum-p24A, accession no. X92098; rat-p24A, accession no. X92097; hum-Tmp21-I, accession no. X97442; and rat-Tmp21-I, accession no. X97443). All peptide sequences identified by microsequence of the M, 21,000/22,000 protein bands were encoded within the identified open reading frames (ORF) of the rat-Tmp21-I and rat-p24A cDNA screening clones. No tissue-specific differences could be detected between the rat pancreas and the rat brain Tmp21-I and p24A.

**Amplification of a Tmp21-Variant Using Reverse Transcription-Polymerase Chain Reaction Technique—**Using reverse transcription-polymerase chain reaction technique with degenerated primers, we have probed different human tissues with respect to tissue-specific expression of p24A and Tmp21 cDNAs. With this approach, we were able to characterize a human isoform of Tmp21, which we called Tmp21-II (Fig. 1;
The deduced primary sequences of hum- and rat-p24A (which define the p24 family), the yeast proteins Emp24p and YHR110w, hum-Tmp21-I and II, rat-Tmp21-I, the Xenopus laevis (X.lae) protein X1262, dog gp25L, and the human gp25L2, identical amino acid residues among the p24 proteins are shown in black boxes. The predicted transmembrane region is indicated in the figure. The COOH-terminal KKXX consensus motif is marked by ++. Residue +1 represents the first amino acid of the mature proteins; the preceding amino acid residues represent the signal peptides. Underlined sequences correspond to those determined by sequencing the NH2-terminal and tryptic fragments. Sequences which have not yet been determined are indicated by (n.d.). The mammalian p24 family can now be classified into the p24, the Tmp21, and the gp25L subfamilies. Emp24p is the yeast homolog to p24A, whereas X1262 represents the Xenopus homolog to Tmp21-I.

Description of the Tmp21 and p24A Structure—The Tmp21 and p24A proteins display features typical for type I transmembrane proteins localized in intracellular membranes. We termed one of these proteins Tmp21, because it is a typical transmembrane protein and has a molecular weight of Mr 21,000 in SDS-PAGE. The p24A is termed in analogy to the identical hamster homolog CHOp24, which has recently been cloned by Stamnes et al. (1995). Both proteins carry a NH2-terminal signal peptide, essential for the translocation to the ER. The NH2 terminus of the mature rat-Tmp21-I starts with ISFH... whereas the rat-p24A starts with YFVS... (Fig. 1).

Hydropathy plots indicate that both the Tmp21 and the p24A are anchored in the membrane by a hydrophobic domain of about 21 residues with a-a-helical character localized close to the COOH terminus (Fig. 2, A and B). The major portion of the mature Tmp21 and p24A, therefore, seems to be located in the lumen of the ER.

Both Tmp21 and p24A show highly conserved protein motifs, assumed to represent important functional sequence positions. The Tmp21-I has a dilysine ER retrieval signal at its short cytoplasmically exposed COOH-terminal tail (Jackson et al., 1990; Fig. 1). The diarginine motif of the protein p24A is homolog to the KKXX motif and has been described to maintain membrane proteins in the ER (Schutze et al., 1994). In the luminaly exposed part, both proteins contain a short but conserved heptad repeat motif that supports the formation of an amphipathic coiled coil structure with high probability (Lupas et al., 1991; Fig. 2, C and D).

Comparison of the Tmp21s with Homologous Proteins—The rat-Tmp21-I (accession no. X97443) and the rat/hum-p24A (ac-
Comparison of the Tmp21 and p24A with sequences in the SWISS-Prot and the EMBL databank, using the FASTA and TBLASTN algorithm, revealed homology to some known proteins (Fig. 1). Weak but significant homologies (24–36% identity) were found with the gp25L protein (accession no. X53592), a mammalian protein of rough microsomes (Wada et al., 1991), the human gp25L-variant gp25L2 (accession no. X90872) and the Emp24p-protein, a yeast type I transmembrane component of ER-derived COPII-coated vesicles (Schimmo¨ller et al., 1993), 1995). This cDNA sequence shows several

For Northern analysis of the Tmp21 and p24A transcripts, we used total RNA isolated from different rat tissues and labeled them as described under “Experimental Procedures.” Furthermore, we isolated RNA from the rat pancreatic acinar tumor cell line AR4–2J (essep and Hay, 1980) and from the Ki-ras-mutated human pancreatic duct cell line CAPAN (Kyriazia et al., 1982). Duct cells and acinar cells represent the main cellular portion of the pancreas. As shown in Fig. 3, p24A is represented by one transcript of about 1.6 kb, which is ubiquitously expressed in rat tissues and in the investigated pancreatic tumor cell lines.

The situation for the Tmp21 transcripts is different. Hybridization with a hum-Tmp21-I probe (bp 30–550) identifies two transcripts of about 1.4 and 3.5 kb. When reprobing the same blot and a control blot with a probe including 200 bases of the coding region and 700 bases of the 3′-untranslated region of rat-Tmp21-I, we obtained the same pattern. From our oligo(dT)-primed rat pancreatic cDNA library, we know that rat-Tmp21-I has a transcript length of 1.4 kb. The nature of the 3.5-kb transcript is not yet known. Since Tmp21 is expressed in two variants (see Fig. 1), the two Tmp21 isoforms are expressed from two different genetic loci. It is possible that the 3.5-kb transcript could be due to Tmp21-II.

Distribution of Tmp21 and p24A Proteins in Different Tissues—To determine the content of Tmp21 and p24A proteins in different rat tissues, protein homogenate and microsomes were prepared and immunologically analyzed. As compared to other tissues, both proteins are present mostly in pancreatic acinar cells (Fig. 4) and are enriched in the microsomal fraction.

Subcellular Distribution of Tmp21 and p24A Proteins in Rat Pancreatic Acinar Cells—For immunological characterization of the rat pancreatic Tmp21 and p24A, antibodies were raised in rabbits using synthetic peptides that consist of the 12 NH₂-terminal amino acids in both Tmp21-I and p24A. To test the specificity of the antibodies and to confirm the identity of the cDNAs encoding for the microsomal proteins, we expressed the ORF for rat-Tmp21-I and the ORF encoding hum-p24A in the E. coli pMal expression system (New England Biolabs).

As shown in Fig. 5A, lanes 1 and 3, the antibodies raised against the NH₂-terminus of rat-Tmp21 and rat-p24A reacted
Figure 3. Northern blot analysis of Tmp21 and p24A transcripts. The RNA was isolated by the method of Chomczynski and Sacchi (1987) from various rat tissues, the rat pancreatic acinar cell line AR4–2J, and the human pancreatic duct cell line CAPAN. Each lane contained 10 μg of total RNA. The positions of the 18 S and 28 S ribosomal RNAs on the Northern transfer are indicated at the right in each blot.

Figure 4. Immunoblot analysis of recombinant and native Tmp21 and p24A expressed in rat organs. Homogenates (H) and fractions enriched in microsomal membranes (M) from different rat organs were prepared as described under “Experimental Procedures.” Proteins (20 μg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose was probed with antibodies raised against Tmp21 (upper row) and p24A (lower row).

Specifically with the corresponding 65-kDa fusion proteins. The recombinant Tmp21 and p24A were cleaved from the fusion proteins by treatment with factor Xa and were specifically recognized by the corresponding antibodies (Fig. 5A, lanes 2 and 4). Both the recombinant Tmp21 and p24A proteins and the Tmp21/p24A from microsomal membranes showed the same mobility in SDS-PAGE, indicating that the native proteins were not likely to be glycosylated.

To study the cellular distribution of Tmp21 and p24A, different subcellular membrane fractions and cytosol of pancreatic acinar cells were prepared as described under “Experimental Procedures.” As shown in Fig. 5B (left panel, lane a), antibodies raised against Tmp21 reacted specifically with a Mr 21,000 protein in the homogenate. Tmp21 was enriched by a factor of 2.3 in the ER/Golgi microsomal fraction, by 1.6 in the plasma membrane, and by 1.8 in the zymogen granule membrane fraction as compared to the homogenate (Fig. 5B, left panel, lanes a–d). The antibodies did not react, however, with any proteins from the cytosol (Fig. 5B, left panel, lane e). The antibodies raised against p24A recognized a Mr 21,400 protein in the homogenate and showed about 2-fold enrichment in microsomal membrane fractions, about 1.3-fold enrichment in plasma membrane fractions, and about 1.5-fold enrichment in zymogen granule membrane fractions (Fig. 5B, right panel, lanes a–d). No reaction was observed in the cytosol (Fig. 5B, right panel, lane e).

To prove the purity of the different membrane fractions, we tested for the presence of marker proteins using specific antibodies. As shown in Fig. 5C (upper row, left), the ER protein SSRα is about 3-fold enriched in microsomal membranes as compared to homogenate, but its content is 4- and 2.5-fold reduced in plasma membranes and zymogen granule membranes, respectively. The second ER protein tested, calnexin, is only slightly enriched in microsomal membranes, but it is reduced in plasma membranes and has not been detected in zymogen granule membranes and in the cytosol (Fig. 5C, upper row, right).
Western blot using anti-p24A antibodies. The marker proteins for plasma membranes and zymogen granule membranes, Ras and Rab3A/B, are highly enriched in their corresponding membranes (Fig. 5C, lower row). This indicates that plasma membranes and zymogen granule membranes, respectively, do not significantly contaminate other membrane fractions. The presence of the ER proteins SSRα and calnexin in membrane fractions other than the microsomal membranes is due to a contamination of these other membrane fractions with microsomes. However, this contamination is very low, as shown by the reduction of the ER proteins as compared to the homogenate. Comparing the enrichment factors for Tmp21 and p24A mentioned above in plasma membranes and in zymogen granule membranes and the concomitant decrease of anti-SSRα and anti-calnexin in these fractions make it unlikely that the presence of both anti-Tmp21 and anti-p24A in plasma and zymogen granule membrane fractions is due to a contamination of the latter by microsomal membranes.

Topo logy of the Tmp21 and p24A Proteins in Microsomal Membranes—Hydropathy analysis of Tmp21 and p24A revealed a stretch of about 21 amino acids close to the COOH terminus that is sufficiently long to span the lipid bilayer. This suggests that Tmp21 and p24A are type I transmembrane proteins with a large luminal domain and a short COOH-terminal stretch of about 1 kDa, which is exposed to the cytosol. Consistent with this prediction, Tmp21 and p24A partitioned into the Triton X-114 phase, whereas the secretory protein amylase was found in the aqueous phase (Fig. 6). These data support the contention that Tmp21 and p24A are integral membrane proteins.

To confirm that the short COOH-terminal stretch of both Tmp21 and p24A is exposed to the cytosolic side, intact and solubilized pancreatic microsomal vesicles were treated with trypsin. If the NH2-terminal part of our protein were localized in the lumen of intact microsomes and the COOH-terminal part were exposed to the cytosol, the NH2-terminal recognition site of our antibodies should be protected against trypsin digestion, whereas the COOH-terminal stretch of 1 kDa should be trypsinized.

Fig. 7 (left panels) shows that in intact microsomes treated with trypsin, a loss of about 1 kDa from Tmp21 and p24A was observed, as visualized by Western blot analysis. However, in Triton X-100-solubilized microsomes treated with trypsin, the NH2-terminal antibody recognition sites were also lost (Fig. 7, right panels).

DISCUSSION
Here we describe the molecular characterization of two ubiquitously expressed rat type I transmembrane proteins with a molecular weight of M, 21,000 (rat-Tmp21 and rat-p24A) and the molecular cloning of the human homologs (hum-Tmp21-I, hum-Tmp21-II, and hum-p24A) (Fig. 1). Both the Tmp21 and p24A proteins show weak but significant homology to one another (23% identity). This homology is mainly mediated by conserved structural features, the NH2-terminal signal peptide, conserved cysteines and coiled coils in the luminal domain, a transmembrane region with a-helical character, and a highly conserved COOH-terminal tail. The proteins were immunologically localized in microsomal membranes, isolated zymogen granule membranes, and in the plasma membrane.

The Tmp21/p24A are homologous to proteins found in mammals, yeast and plants. Stamnes et al. (1995) recently published a sequence for a protein called CHOp24, which is the hamster homolog to the here shown rat- and hum-p24A. The authors defined the p24 homologous proteins as members of the p24 family. By analogy, we classified the Tmp21-variants as a p24 subfamily. The sequence alignment (Fig. 1) of the p24 homologs indicates an old but evolutionarily conserved family of intracellular transmembrane proteins, consisting of subfamilies and variants. The mammalian p24 family can now be classified into the p24A subfamily, the Tmp21 subfamily, and the gp25L subfamily. Emp24p represents the yeast homolog of the p24 subfamily.

The p24 family has highly conserved structural elements. With respect to the neutral theory of molecular evolution (Kimura, 1983), we conclude that these conserved structural elements represent important functional sequence positions. Besides Emp24p, all p24-like proteins carry a short but highly conserved heptad repeat signal in the luminal part of the protein with a predicted propensity to form amphipathic coiled coils (Lupas et al., 1991; Oas and Endow, 1994). This heptad repeat is followed by a COOH-terminal membrane anchor. Such structures are also observed in receptor-like proteins participating in vesicle targeting processes like the soluble N-ethylmaleimide-sensitive fusion (NSF) attachment protein receptor (v-SNARE; Dascher et al., 1991) and the SNARE-like protein Sft1p (Banfield et al., 1995), as well as the lectin-like protein Emp47p (Schröder et al., 1995). The function of the
heptad repeat structure in the receptor-like p24 family is not known.

The Tmp21 and some of the homologs (Fig. 1) have a dilysine ER-localization signal at their short cytoplasmically exposed COOH-terminal tail. This motif has been identified to be a retrieval motif that brings proteins from an unknown sorting compartment back to the ER (Jackson et al., 1990). It is shown that the KXXX-mediated retrieval can occur at several sites between the intermediate compartment and the trans-Golgi network (Jackson et al., 1993; Martire et al., 1996). COOH-terminal dilysine-motifs bind specifically to the protein complex coatamer (Cosson and Letourneur, 1994; Letourneur et al., 1994; Lowe and Kreis, 1995), which is essential for the retrieval of dilysine-tagged proteins to the ER. We have co-isolated the dilysine-tagged Tmp21 and p24A with ER and Golgi-enriched microsomal membranes but surprisingly also with zymogen granule membranes and PMs. In the case of the dilysine-tagged ERGIC-53, a substantial portion of ERGIC-53 is localized outside the ER (Schweizer et al., 1988). Overexpression of ERGIC-53 results in its appearance in PMs (Kappeler et al., 1994).

Furthermore, it has been shown by substitutional mutagenesis that the motif KKFF at the COOH-terminal, cytosolic tail of ERGIC-53 acts as a signal for endocytosis (Itin et al., 1995). The high expression rate of Tmp21 and p24A in rat pancreas, as compared to other tissues (Fig. 4), is remarkable and might indicate that Tmp21 and p24A are involved in the special function of this organ to synthesize, transport, and exocytose digestive enzymes at high rates. This could lead to a passive flow of Tmp21 and p24A through the ER and Golgi compartments to the zymogen granule membrane and the PM. The appearance of Tmp21 and p24A in zymogen granule membranes and PMs does not presuppose a function of these proteins in granule and plasma membranes, but it makes a sorting mechanism for KXXX-containing proteins at the PM necessary. The proteins ERGIC-53 and VIP36, which carry dilysine motifs, have been, in part, localized to coated pits (Fiedler et al., 1994), supporting the idea that the KXXX-sorting at the PM is mediated by the dathrin adapter complex. The p24A proteins carry a COOH-terminal diarginine motif. Diarginine motifs are similar to dilysine motifs and have been shown to maintain type II membrane proteins in the ER (Schutze et al., 1994).

Until now, the function of the mammalian p24 members is unclear. In yeast it was shown that knocking out the p24 deletion mutant (Schimmo¨ller et al., 1988). Overexpression of ER-GIC-53, a substantial portion of ERGIC-53 is localized out-

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REFERENCES
Banfield, D. K., Lewis, M. J., and Pelham, H. R. B. (1995) Nature 375, 806–809
Bayerdorffer, E., Eckhardt, L., Haase, W., and Schulz I. (1985). J. Membr. Biol. 84, 17189.
Banfield, D. K., Lewis, M. J., and Pelham, H. R. B. (1995) Nature 375, 806–809
Bayerdorffer, E., Eckhardt, L., Haase, W., and Schulz I. (1985). J. Membr. Biol. 84, 17189.