# Targeting of the 22 kDa integral peroxisomal membrane protein

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**Abstract** **Investigating targeting of the 22 kDa peroxisomal membrane protein (Pmp22p) to the peroxisomal membrane we have confined the targeting signal to amino acid residues 16–37 located in the N-terminal cytoplasmic tail. Comparison of Pmp22p orthologous sequences revealed a conserved motif Y3xL3xP3x(KQN) which might represent the core of this targeting signal not found so far in other Pmps. Fusion of the Pmp22p N-terminal tail to the C-terminal portion of Pmp22p which per se is not targeted to peroxisomes, conveys peroxisomal targeting. These data suggest that Pmp22p is targeted to peroxisomes by a new membrane targeting signal which is necessary and sufficient to target a polypeptide containing two transmembrane spans to peroxisomes.**
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*Key words:* Peroxisome; Peroxisomal membrane protein;
Pmp22p; Peroxisomal membrane targeting;
Membrane targeting signal

## 1. Introduction

The cytoplasmic steps of the import pathway of peroxisomal matrix proteins include their synthesis on free polyribosomes and their posttranslational import into peroxisomes (for review see [1]). Peroxisomal targeting is mediated by two cytoplasmic receptors, Pex5p and Pex7p, recognizing targeting signals on the newly synthesized polypeptides [2–4]. Whereas Pex5p specifically interacts with a peroxisomal targeting signal 1 (PTS1) located at the C-terminal end and consisting of the amino acid residues SKL or variants thereof [5], Pex7p recognizes an intrinsic N-terminal PTS2 consisting of the consensus sequence (RK)(LVI)5x(QH)(LA) and found so far in only a few proteins, e.g. peroxisomal thiolase or alkyldihydroxyacetonephosphate synthase [6–8].

The fate of peroxisomal membrane proteins (Pmps) after their synthesis in the cytoplasm is much less understood and currently intensely discussed. Two models have been proposed so far, both of which have strong implications in the biogenesis of peroxisomes. Whereas the one model favors synthesis of Pmps on free polyribosomes and posttranslational insertion into the peroxisomal membrane similar to the pathway of matrix proteins [1,9], the other proposes a yet poorly defined interaction of Pmps with membranes of the endoplasmic reticulum (ER) prior to transport to peroxisomes [10–12]. Shuttling of vesicles between peroxisomes and ER might involve ATP ribosylation factor (ARF) and coatomer [13].

Targeting of Pmps seems to be mediated by a peroxisomal membrane targeting signal (mPTS). Such a signal has first been identified in *Candida boidinii* Pmp47p residing in the hydrophilic loop between transmembrane spans (TMSs) 4 and 5. Sequence comparison with other Pmps revealed the consensus motif (KR)(KR)3–7x(TS)2x(DE) [14]. This signal was recently modified by aligning the CbPmp47p loop sequence with sequences of *Saccharomyces cerevisiae* Pex15p as well as Pex3p of different yeast strains resulting in the consensus motif (RK)x(RK)x(RK)x(IL)9–10x(FY) [11]. Comparison of human and yeast Pex3p sequences indicated a rather identical motif (LIV)x(R)x(KR)x(K)x(IL) which might also be present in Pmp24p of rat liver [15,16].

In uninduced rat liver peroxisomes the 22 kDa peroxisomal membrane protein (Pmp22p) is the major membrane component [17]. Due to its four TMSs it is deeply embedded into the peroxisomal bilayer. Studies on the in vitro insertion of Pmp22p revealed that nascent Pmp22p in the reticulocyte lysate becomes associated with the t-complex polypeptide ring complex (TRiC), the eukaryotic homolog of GroEL, as well as with an about 40 kDa yet unidentified polypeptide which seems to be involved in the insertion process [18]. Insertion itself was found to be dependent on time and temperature, to require proteinaceous membrane components but was otherwise independent on the presence and hydrolysis of ATP [19,20]. Thus, Pmp22p seems to be inserted into the peroxisomal membrane directly from the cytoplasm and formation of complexes with cytoplasmic factors mediate solubility of this very hydrophobic molecule and facilitate membrane insertion.

Based on studies exploring the posttranslational route of ScPex15p, two types of mPTSs were hypothesized [11]. One, an mPTS1, for peroxisomal targeting directly from the cytoplasm, and the other, an mPTS2, carrying information for peroxisomal targeting from the ER to peroxisomes. To test this hypothesis and to extend our current knowledge on targeting of Pmps, we initiated studies on the characterization of the targeting signal of Pmp22p. Thus far, we localized the signal to the cytoplasmic N-terminal tail region between amino acid residues 16 and 37. Comparison of the corresponding peptide sequence with other known peroxisomal membrane targeting sequences suggests that Pmp22p is targeted to peroxisomes by a new targeting signal not found so far in other Pmps.

## 2. Materials and methods

### 2.1. Construction of plasmids

Fragments of PMP22-cDNA were generated by expression cassette PCR [21] with primers containing a *Kpn*I and/or *Eco*RI restriction site

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using PMP22-cDNA in pBluescript as a template [19]. The obtained fragments were cloned into the pEGFP-N1 vector (Clontech, Heidelberg, Germany) for expression as C-terminal fusions with the enhanced green fluorescent protein (EGFP). Total PMP22-cDNA containing *Kpn*I restriction sites on both ends was also obtained by PCR and cloned into the pEGFP-N1 vector resulting in the construct p1–194-EGFP. Point mutations were constructed using the Quik-Change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). PCR using p1–194-EGFP as template and complementary primers containing the appropriate mutations was performed according to the instructions of the manufacturer. Internal deletion mutants were created by PCR using p1–194-EGFP as template and primers starting immediately upstream (antisense) and downstream (sense) of the deletion. The obtained PCR products were ligated using T4-ligase (Roche Diagnostics, Mannheim, Germany) and transformed into *Escherichia coli*. Correctness of all PCR-generated constructs was verified by sequencing as reported previously [22].

### 2.2. Culturing and fractionation of cells

CHO-K1 cells (ATCC, Rockville, USA) were grown in α-MEM containing 7.5% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Biochrom KG, Berlin, Germany).

Transfection of CHO cells with 0.8 μg of plasmid DNA was performed using the Effectene transfection kit (Qiagen, Hilden, Germany) according to the instructions of the supplier. Cells were split 24 h prior to transfection and grown on 13 mm glass coverslips. Medium was first changed 3 h after transfection.

For cell fractionation studies, CHO wild-type cells were transfected with the appropriate plasmids and kept on G418 (Life Technologies, Karlsruhe, Germany) for about 2 weeks. Cells were washed twice with homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM glycylglycine pH 7.4) at 4°C, scraped off from the plastic dishes (about 600 μl) and homogenized using a 1 ml glass-teflon homogenizer. Efficiency of homogenization was microscopically controlled. Unbroken cells and nuclei were pelleted at $1000 \times g$ for 5 min and the organelles of the postnuclear supernatant recovered by centrifugation at $25\,000 \times g$ for 20 min. After resuspension in homogenization buffer the organelles were fractionated in a 5 ml 14.5–36% w/v Nycodenz density gradient, as described elsewhere [23]. Eleven fractions were collected, treated with 100 mM sodium carbonate [24] and membranes pelleted by high speed centrifugation. Washed membranes were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and polypeptides identified by immunoblotting using reagents for enhanced chemiluminescence (Amersham, Braunschweig, Germany). EGFP fusion proteins were detected with a polyclonal affinity-purified anti-EGFP peptide antibody (Clontech, Heidelberg, Germany). Monospecific antibodies directed against peroxisomal markers were raised in rabbits by injecting the electrophoretically separated antigens [13,19,23].

### 2.3. Immunofluorescence

After transfection (18–24 h) cells were washed with phosphate buffered saline (PBS: 100 mM Na$_2$HPO$_4$, 150 mM NaCl, 2.5 mM KCl, pH 7.4) and fixed for 10 min in 3.7% formaldehyde in PBS. Permeabilization was carried out by incubating the cells with 25 μg/ml digitonin for 10 min (Pmp69p) or 1% (v/v) Triton X-100 for 5 min (calreticulin). Subsequently, cells were washed again with PBS and incubated with the primary antibody for 30 min at 37°C. TRITC-conjugated goat anti-rabbit antibodies (Dianova, Hamburg, Germany) were used in order to decorate the first antibody. Fluorescence microscopy was performed on a Zeiss Axiovert 10 inverted microscope (Zeiss, Jena, Germany). Images were acquired with a Photometrics CH250 ccd camera using a 63-fold magnification objective (Zeiss Plan-Achromat, ×63, NA 1.4). Superposition of EGFP- and TRITC-stained pictures was performed using the KHOROS software package as reported previously [25].

### 2.4. Alignment of Pmp22p sequences

Alignment of Pmp22p from rat (Q07066), mouse (P42925), *S. cerevisiae* (S59397) and *Arabidopsis thaliana* (CAA06834) as well as the Pmp22p homolog Mpv17p from human (P39210) and mouse (P19258) was performed using the ClustalW multiple sequence alignment software which can be used on http://mbshortcuts.com/mbsalign.

## 3. Results

### 3.1. Peroxisomal targeting of the Pmp22p-EGFP fusion protein

First, we tested the usefulness of EGFP-tagged Pmp22p for investigating its peroxisomal targeting. The putative topology of Pmp22p as derived from its primary structure predicts four TMSs with N- and C-terminus facing the cytoplasm [26]. The C-terminal cytoplasmic tail consists of a single lysine residue to which we fused the EGFP tag to obtain p1–194-EGFP (Fig. 1A). When this cDNA was expressed in CHO cells, the expression product perfectly localized to peroxisomes, as revealed by colocalization with the peroxisomal membrane marker Pmp69p (Fig. 1B).

### 3.2. The targeting signal is located at the N-terminus of Pmp22p

The loop between TMS1 and 2 contains a motif rather similar to the mPTS consensus motif proposed for CbPmp47p
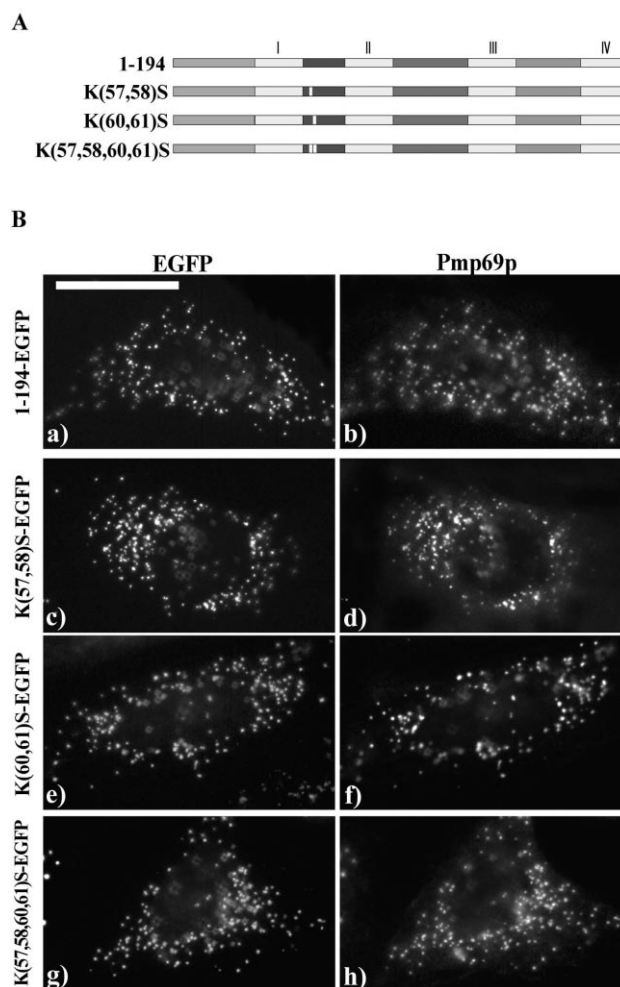


Fig. 1. Subcellular localization in CHO cells of EGFP-tagged wild-type Pmp22p (1–194-EGFP) and mutated versions of it having replaced essential lysine residues by serine. A: Schematic drawing of constructs all fused to the N-terminus of EGFP. B: The EGFP fluorescence of wild-type Pmp22p is shown in a, that of the mutants, K(57,58)S-EGFP, K(60,61)S-EGFP and K(57,58,60,61)S-EGFP in c, e and g, respectively. Peroxisomal colocalization of the EGFP signals was verified by immunofluorescence using anti-Pmp69p and TRITC-labeled goat anti-rabbit IgG antibodies and is shown in b, d, f and h. The bars represent 25 μm.
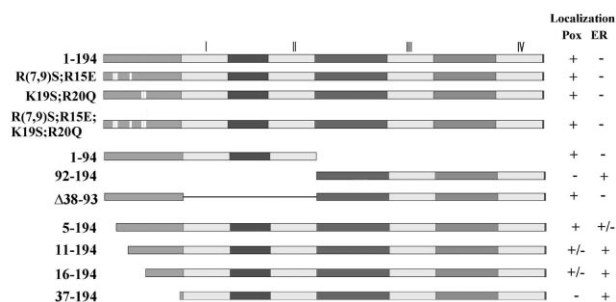
Fig. 2. Overview of mutated versions of wild-type Pmp22p (1–194) prepared for the identification of the Pmp22p mPTS. All constructs were expressed in CHO cells as C-terminal EGFP fusion proteins. Subcellular localization of the proteins is indicated at the right. Roman numerals designate the four putative TMSs.

[14]. Since essential lysine residues are present in this motif and a cluster of lysine residues also is found in the Pmp22p loop region at positions 57, 58, 60 and 61, we checked the functionality of these lysine residues. To this end three EGFP fusion constructs were prepared in which by site-directed mutagenesis the lysine residues were replaced by serine (Fig. 1A). As shown in Fig. 1B, following transfection into CHO cells all the expressed polypeptides perfectly colocalized with the peroxisomal marker Pmp69p. This unexpected result suggested localization of the Pmp22p targeting signal to another part of the molecule.

In our strategy to localize the mPTS we first divided the molecule into two halves of approximately equal size. The first half, p1–94-EGFP, contained the entire N-terminal portion including TMS1 and 2 and the second, p92–194-EGFP, the cytoplasmic loop region between TMS2 and 3 and the entire C-terminus including TMS3 and 4 (Fig. 2). While the N-terminal half revealed the typical punctate peroxisomal fluorescence pattern (Fig. 3a–c), the C-terminal portion showed diffuse fluorescence with some labeled structures resembling ER (Fig. 3d–f). These results demonstrate that the targeting signal resides in the N-terminal half of Pmp22p. Since the loop region between TMS1 and 2 does not contain the signal and the TMSs themselves first were excluded as candidate regions, we assumed the signal to be localized within the cytoplasmic N-terminal tail. We tested this assumption by preparing a construct, pΔ38–93-EGFP (Fig. 1), in which residues 1–37 were directly fused to the N-terminus of p94–194-EGFP which alone was not targeted to peroxisomes (Fig. 3d–f). Upon expression in CHO cells we observed colocalization of the expression product with the peroxisomal marker (Fig. 3g–i).

This construct was also used to demonstrate membrane integration of the expressed polypeptide. After expression of the protein in CHO cells, we fractionated the cells by isopycnic centrifugation [23], prepared membranes of each of the fractions [24] and analyzed the distribution of the EGFP fusion protein by SDS–PAGE and Western blotting (Fig. 4). Peroxisomal localization is demonstrated by comparing the gradient distribution of the EGFP fusion protein with that of the peroxisomal membrane marker Pmp69p. Both polypeptides show identical profiles. These data suggest that (i) the targeting signal is localized to the first 37 N-terminal residues, (ii) it is not only necessary but also sufficient to target a polypeptide containing two TMSs to peroxisomes and (iii) targeting results in the carbonate resistant integration of the polypeptide into the peroxisomal membrane.

As already mentioned, the consensus sequences for targeting Pmps to peroxisomes derived so far are dominated by positively charged residues. Since there are several positively charged residues present in the Pmp22p N-terminal tail we exchanged some of them by site-directed mutagenesis. Three mutants of the wild-type molecule were generated which had mutated (i) R7 and 9/S and R15/E, (ii) K19/S and R20/E and (iii) all the residues changed in (i) and (ii) (Fig. 2). When transfected into CHO cells all the expression products revealed specific peroxisomal staining suggesting that K19 and R20 are not essential for peroxisomal targeting (data not shown).

### 3.3. The N-terminus of Pmp22p also contains a potential ER targeting signal

In order to further characterize the mPTS, we prepared several constructs in which Pmp22p was successively shortened from the N-terminus (Fig. 2). The intracellular localization of the expressed EGFP fusion proteins revealed that up to deletion of the first 15 residues peroxisomal localization is still visible (Fig. 5a–f). However, deletion of the entire cytoplasmic N-terminal tail, p37–194-EGFP, results in localization of the expression product to the ER with no fluorescence staining of peroxisomes (Fig. 5g–i). ER localization was clearly demonstrated by colocalizing the obtained pattern with that of the ER marker calreticulin. With progressive N-terminal shortening of Pmp22p peroxisomal localization vanished in favor of ER localization. These data show that the mPTS is localized between residues 16 and 37 and that a hidden ER targeting signal resides within the N-terminus beyond residue 16.

### 3.4. In addition to the mPTS more than one TMS is required for peroxisomal membrane insertion

The mPTS sequence of CbPmp47p when fused to soluble proteins such as chloramphenicol acetyltransferase or green fluorescent protein results in targeting of the reporter proteins to the peroxisomal matrix [14]. This is surprising because the mPTS completely differs from the known peroxisomal matrix targeting signals (see above). In contrast to the CbPmp47p, the EGFP chimera of the N-terminal tail peptide of Pmp22p, p1–38-EGFP, was clearly targeted to the cytoplasm (Fig. 6a). Even extending the tail peptide until the end of TMS1, p1–56-EGFP (not shown), or TMS1 plus the subsequent loop region, p1–74-EGFP, resulted in localization of the expression products to the cytoplasm (Fig. 6b). However, deletion of residues 38–122 resulting in fusion of the N-terminal tail to TMS4 and about one half of TMS3, directed the polypeptide to peroxisomes (Fig. 6c and d), although less efficiently than p1–94-EGFP (Fig. 3a–c). These results suggest that in addition to the mPTS more than one TMS is required for correct peroxisomal insertion.

## 4. Discussion

### 4.1. Pmp22p is targeted to peroxisomes by a new mPTS

In the present study we used various modifications of Pmp22p in order to identify that region of the molecule which contains the targeting signal for direction of Pmp22p to peroxisomes. All constructs encoded polypeptides having fused EGFP to their C-terminus. The EGFP tag per se seems not to influence subcellular localization of the polypeptides. This is
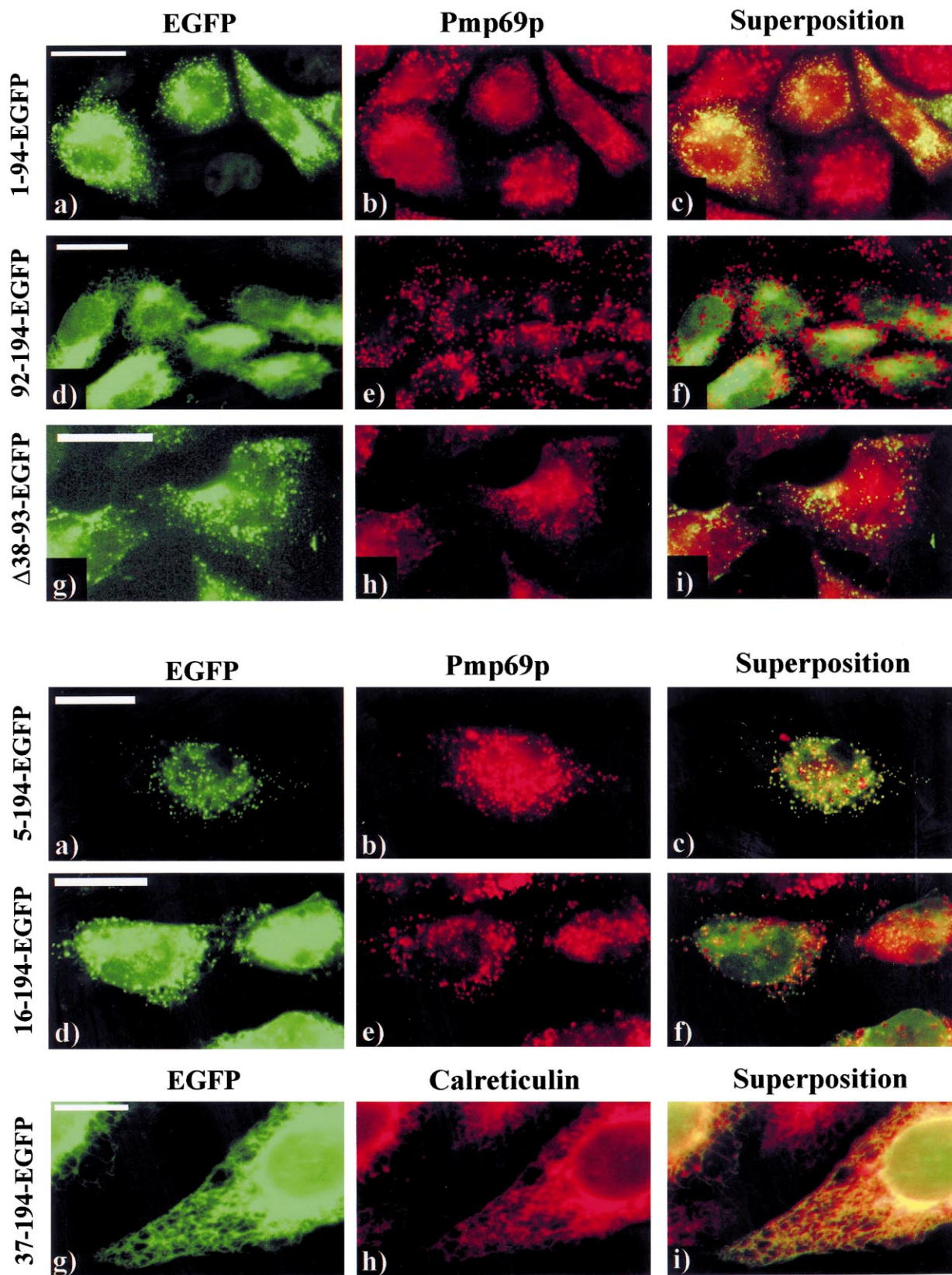
Fig. 3. Subcellular localization of mutated versions of Pmp22p indicated in Fig. 2. EGFP fluorescence is shown in a, d and g, peroxisomal localization (anti-Pmp69p antibodies) in b, e and h and superposition of both stainings in c, f and i. Note that the C-terminal half of Pmp22p, 92–194-EGFP, does not localize to peroxisomes (d–f), but regains peroxisomal localization by fusion to the N-terminal cytoplasmic tail of Pmp22p (Δ38–93-EGFP) (g–i). The bars represent 25 μm.

Fig. 5. Subcellular localization of N-terminally truncated versions of Pmp22p indicated in Fig. 2. EGFP fluorescence is shown in a, d and g, peroxisomal localization (anti-Pmp69p antibodies) in b and e, ER localization (anti-calreticulin antibodies) in h and superposition of EGFP fluorescence and immunostaining in c, f and i. The bars represent 25 μm.
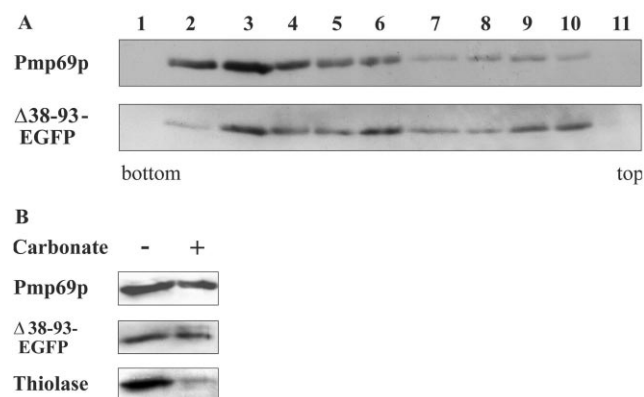
Fig. 4. Carbonate resistant integration of Δ38–93-EGFP into peroxisomes of CHO cells. Following transfection of CHO wild-type cells, the postnuclear supernatant was fractionated by Nycodenz density gradient centrifugation. Membranes were prepared from each fraction and the membrane polypeptides subjected to SDS–PAGE and Western blotting. A shows the distribution of Pmp69p, a peroxisomal membrane marker, and Δ38–93-EGFP. Both polypeptides show nearly identical distribution with maximal concentrations in fraction 3. B: Peroxisomes from fractions 2–4 were pooled and recovered. One aliquot remained untreated (−), the other was carbonate treated (+). Corresponding amounts of membranes were analyzed by immunoblotting. Note that peroxisomal thiolase but not Pmp69p and Δ38–93-EGFP are extracted by carbonate.

indicated by the fact that the C-terminally tagged version of wild-type Pmp22p as well as both C- and N-terminally EGFP-tagged p1–94 are clearly targeted to peroxisomes, although the C-terminally tagged molecule inserted with higher efficiency (not shown). This might be explained by less perturbation of the N-terminal mPTS caused by the EGFP tag.

By site-directed mutagenesis it rapidly turned out that a motif in Pmp22p resembling the CbPmp47p mPTS and residing in the loop region of Pmp22p between TMS1 and 2 might not be functional. In subsequent studies with N- and C-terminal truncations of Pmp22p, localization of the mPTS was confined to the N-terminal cytoplasmic tail between amino acid residues 16 and 37 close to the first TMS. Since this part of the polypeptide chain does not contain sequences related to known mPTSs, Pmp22p must be targeted to peroxisomes by a new mPTS.

Orthologs of Pmp22p have been characterized in various species [27–29]. Alignment of their N-terminal tail sequences showed several common features (Fig. 7). (i) There is a conserved motif Y3xL3xP3x(KQN) found in most of them. In the putative peroxisome organization and biogenesis protein of *Schizosaccharomyces pombe* the motif is slightly changed to Y2xL3xP3xM. (ii) The three-dimensional structure of the Pmp22p N-terminal tail is largely α-helical, as predicted by the secondary structure prediction program GOR IV [30]. (iii) The conserved residues of the motifs Y, L, P and (KQN) are



Fig. 6. Intracellular localization of Pmp22p mutants C-terminally tagged with EGFP. The mutants analyzed are a: 1–37-EGFP, b: 1–74-EGFP, c: Δ38–122-EGFP. In a–c EGFP fluorescence is shown. Peroxisomal localization of Δ38–122-EGFP is demonstrated by colocalizing the EGFP signal (c) with the immunofluorescence signal of Pmp69p (d). The bar represents 12.5 μm.

oriented to the same side of the α-helix. We assume that this motif represents the core of the Pmp22p mPTS.

The new mPTS N-terminally fused to a soluble protein, such as EGFP, might not be able to mediate import of this protein into peroxisomes, since fusion of the N-terminal tail of Pmp22p to the N-terminus of the green fluorescent protein resulted in localization of the polypeptide to the cytoplasm (Fig. 6a). Hydrophobic sequences, most likely more than one TMS, in addition to the mPTS are necessary for targeting and/or insertion.

### 4.2. Does Pmp22p contain a functional ER signal sequence?

Recent work by various groups suggested that selected Pmps might be targeted to peroxisomes via the ER [10–12]. The polypeptides investigated include ScPex15p, YlPex16p and YlPex2p as well as a chimeric protein made of the first 15 N-terminal residues of *Hansenula polymorpha* Pex3p and catalase lacking its PTS1. In light of these suggestions the question as to the presence of a functional signal sequence in Pmp22p becomes important for understanding peroxisome biogenesis. So far we do not have solid indications that wild-type Pmp22p enters the ER membrane. In fact, Pmp22p mRNA is predominantly found on free polyribosomes [9]. Thus, if ER insertion may occur, it must occur posttranslationally. However, the direct posttranslational insertion of both Pmp22p and rat Pex2p into isolated peroxisomes has been demonstrated [19,20] and pulse labeling experiments with isolated primary hepatocytes have shown that Pmp22p is rapidly, within less than 2 min, inserted into the membrane of mature peroxisomes (Just, W.W., unpublished observation) making insertion via the ER rather unlikely.

ER localization of Pmp22p only was observed when the

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Pmp22p rat         1 MAPAASRLRVESELRSLPKRALAQYLLFLKFYPVVTKAVSSGILSALGNLLAQM 54
Pmp22p mouse       1 MAPAASRLRVESELGSLPKRALAQYLLLLKLYPVLTKAVSSGILSALGNLLAQT 54
Pmp22p S.cerevisiae 1 ------------------MKLLHLYEASLKRRPKTTNAIMTGALFGIGDVSAQL 36
Pmp22p A.thaliana  1 -----------MGSSPPKKTTLQRYLSQLQQHPLRTKAITAGVLSGVSDVVSQK 43
mpv17 human        1 ------------------MALWRAYQRALAAHPWKVQVLTAGSLMGLGDIISQQ 36
mpv17 mouse        1 ------------------MALWRAYQRALAAHPWKVQVLTAGSLMGLGDIISQQ 36
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Fig. 7. Alignment of the N-termini of various Pmp22p homologs. Identical amino acid residues are marked in gray. Residues belonging to the first TMS are printed in italics. The peptide sequence of rnPmp22p containing essential parts of the mPTS is printed in bold.
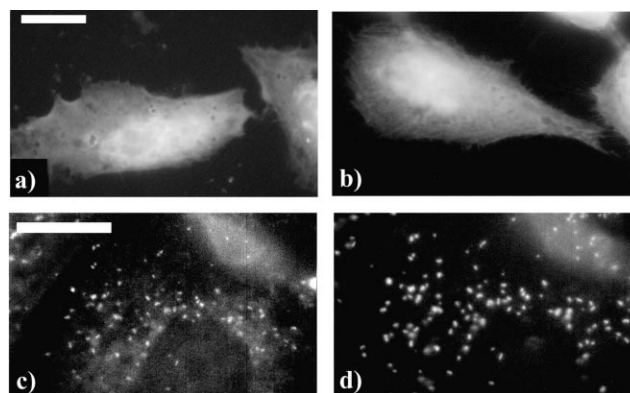
N-terminus was truncated. Like many other integral membrane proteins [31], Pmp22p contains positively charged residues which are located N-terminally to the TMSs, e.g. K37 in front of TMS1. The consensus requirements for ER targeting identified thus far include three distinct domains, an N-terminally positively charged n-region of 1–5 residues, a central hydrophobic h-region of 7–15 residues and a more polar c-region of 3–7 residues [32]. Thus, truncation of the Pmp22p N-terminus might well result in the artifactual generation of an ER signal sequence which otherwise is masked by the truncated N-terminal residues.

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