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Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in *S.cerevisiae*, causes proliferation of the endoplasmic reticulum membrane

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We have cloned *PEX15* which is required for peroxisome biogenesis in *Saccharomyces cerevisiae*. *pex15*Δ cells are characterized by the cytosolic accumulation of peroxisomal matrix proteins containing a PTS1 or PTS2 import signal, whereas peroxisomal membrane proteins are present in peroxisomal remnants. *PEX15* encodes a phosphorylated, integral peroxisomal membrane protein (Pex15p). Using multiple *in vivo* methods to determine the topology, Pex15p was found to be a tail-anchored type II (N_{cyt}–C_{lumen}) peroxisomal membrane protein with a single transmembrane domain near its carboxy-terminus. Overexpression of Pex15p resulted in impaired peroxisome assembly, and caused profound proliferation of the endoplasmic reticulum (ER) membrane. The lumenal carboxy-terminal tail of Pex15p protrudes into the lumen of these ER membranes, as demonstrated by its *O*-glycosylation. Accumulation in the ER was also observed at an endogenous expression level when Pex15p was fused to the N-terminus of mature invertase. This resulted in core *N*-glycosylation of the hybrid protein. The lumenal C-terminal tail of Pex15p is essential for targeting to the peroxisomal membrane. Furthermore, the peroxisomal membrane targeting signal of Pex15p overlaps with an ER targeting signal on this protein. These results indicate that Pex15p may be targeted to peroxisomes via the ER, or to both organelles.

Keywords: endoplasmic reticulum/membrane proliferation/peroxisome biogenesis/protein targeting

Introduction

The peroxisome is a nearly ubiquitous organelle of the eukaryotic cell and is bounded by a single membrane. The proteins [recently renamed as peroxins (Pex) (Distel *et al.*, 1996)] and targeting signals involved in peroxisomal protein import are highly conserved between most eukaryotes. Peroxisomal matrix proteins are synthesized on free polysomes, and are imported post-translationally into the organelle (reviewed by Lazarow and Fujiki, 1985). Translocation across the peroxisomal membrane does not require complete unfolding of the protein (Walton *et al.*, 1995), and some proteins can oligomerize before the actual translocation takes place (Glover *et al.*, 1994; McNew and Goodman, 1994; Elgersma *et al.*, 1996b; Häusler *et al.*, 1996).

Sorting to the peroxisome requires the presence of a peroxisomal targeting signal (PTS). Most peroxisomal matrix proteins possess a PTS1 signal, which is present at the extreme C-terminus and requires a basic amino acid at the penultimate position and a hydrophobic amino acid at the ultimate position (Gould *et al.*, 1987; Elgersma *et al.*, 1996b). The PTS1 is recognized by the cytosolic PTS1 receptor, Pex5p, which docks at the SH3 domain of the peroxisomal integral membrane protein, Pex13p (Elgersma *et al.*, 1996a; Erdmann and Blobel, 1996; Gould *et al.*, 1996). A smaller subset of peroxisomal matrix proteins uses the N-terminal PTS2 signal (Swinkels *et al.*, 1991), which is recognized by Pex7p, the PTS2 receptor (Rehling *et al.*, 1996; Zhang and Lazarow, 1996). Some proteins lacking a PTS1 or PTS2 sequence are still able to enter the peroxisomes in a Pex5p-dependent manner, indicating that additional topogenic sequences may exist (Kragler *et al.*, 1993; Elgersma *et al.*, 1995).

Since the mechanism of import seems to be very conserved between higher and lower eukaryotes, several laboratories have exploited yeasts as model organisms for peroxisomal protein import and biogenesis. The isolation of yeast mutants involved in peroxisome assembly (now collectively called *pex* mutants) has resulted in the identification of several proteins (peroxins) involved in peroxisome biogenesis (reviewed in Distel *et al.*, 1996; Elgersma and Tabak, 1996). This has not only resulted in a substantial increase in our knowledge of how peroxisomes are assembled, but has also facilitated the cloning of the corresponding human genes, many of which are defective in peroxisome-related disorders (reviewed in Subramani, 1997).

Despite our increasing knowledge regarding aspects of peroxisomal import of matrix proteins, little is known about the targeting of peroxisomal membrane proteins. Although it has been demonstrated that several peroxisomal membrane proteins are synthesized on free polysomes (Fujiki *et al.*, 1984; Bodnar and Rachubinski, 1991) and that at least two of them (Pmp70 and Pmp22) are inserted directly into the peroxisomal membrane (Diestelkötter and Just, 1993; Imanaka *et al.*, 1996), these data are insufficient rigorously to rule out the involvement of the endoplasmic reticulum (ER). In this study, we report the cloning of a novel gene (*PEX15*) which encodes an integral peroxisomal membrane protein involved in peroxisome biogenesis. We have identified its peroxisomal targeting signal (mPTS) and found that Pex15p accumulates in the ER under various conditions (overexpression,

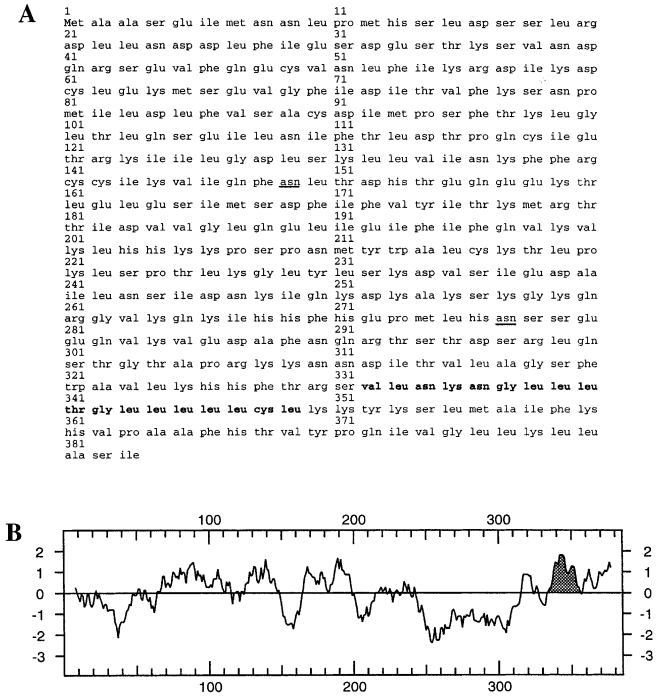


Fig. 1. (**A**) Protein sequence of Pex15p. The predicted transmembrane domain is in bold, putative *N*-glycosylation sites are underlined. (**B**) Hydrophobicity plot of Pex15p using the algorithm described by Kyte and Doolittle (1982). The window size used is 15 amino acids. The predicted transmembrane domain is highlighted.

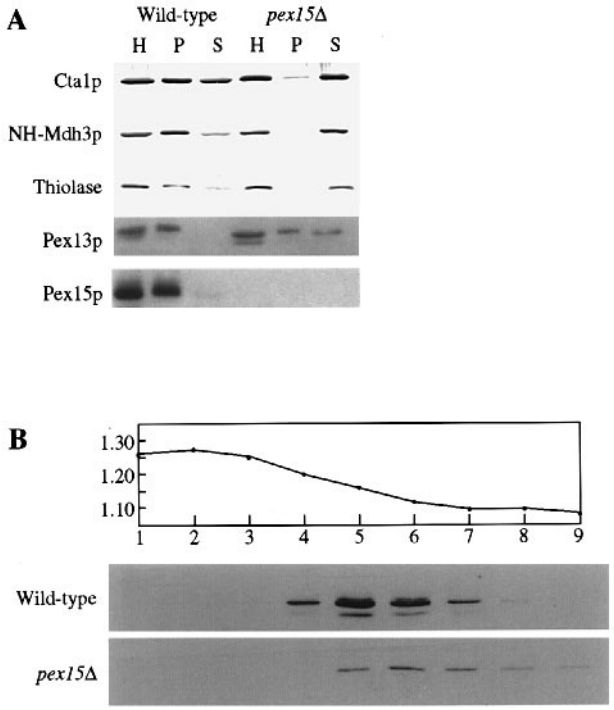


Fig. 2. Characterization of the *pex15*Δ mutant. (**A**) Subcellular distribution of peroxisomal enzymes in oleate-induced wild-type and *pex15*Δ cells. After subcellular fractionation, equivalent volumes of the 1000 *g* post-nuclear supernatant [homogenate (H)], 27 000 *g* pellet (P) and 27 000 *g* supernatant (S) were analyzed by Western blotting. For NH-Mdh3p detection, we used the NH antibody; otherwise we used the antibodies directed against the proteins as indicated. (**B**) Pex13p location in oleate-induced wild-type and *pex15*Δ cells. A 27 000 *g* organellar pellet fraction was layered at the bottom of a sucrose flotation gradient and centrifuged for 18 h at 150 000 *g*. Fractions obtained from these gradients were analyzed by Western blotting using αPex13p antibodies. The graph represents the density at 4°C in g/cm^3.

deletion of the mPTS or C-terminal fusion proteins). These results suggest that Pex15p may be targeted to the peroxisomes via the ER, which is of great importance in the understanding of peroxisome biogenesis.

Results

Cloning of the PEX15 gene

The *pex15.1* mutant (previously called *pas21.1* mutant) was obtained using a positive selection procedure for the isolation of *pex* mutants in *Saccharomyces cerevisiae* (Elgersma *et al.*, 1993). It was used to clone the corresponding *PEX15* wild-type gene by functional complementation with a genomic library. Nucleotide sequencing of the smallest complementing insert (1641 bp) revealed an open reading frame (ORF) of 1152 bp, encoding a 383 amino acid protein with a predicted mass of 43.6 kDa (Figure 1A). More recently, this gene has also been sequenced by the Genome Sequencing Project of *S.cerevisiae* (accession No. Z74786/YOLO44W). Despite the fact that most peroxins are relatively well conserved in evolution (Distel *et al.*, 1996), we have been unable to identify a putative human ortholog of Pex15p in the databases. In addition, database searches did not reveal any significant homology to other proteins. A Kyte–Doolittle hydrophobicity plot of Pex15p revealed several putative hydrophobic regions, but only one region reached the threshold value of 1.6, which is generally used as a criterion for prediction of transmembrane domains (TMDs) (Figure 1B). Programs which predict putative TMDs according to the algorithms of Klein *et al.* (1985) or Kyte and Doolitttle (1982) indeed classified Pex15p as an integral membrane protein with a single TMD at its carboxy-terminus (amino acids 332–349).

pex15Δ cells are disturbed in the import of peroxisomal matrix proteins

To study the function of Pex15p, most of the chromosomal *PEX15* gene (corresponding to amino acids 13–301 of the 383 amino acid Pex15p) was replaced by the *LEU2* gene, resulting in the *pex15*Δ strain. The *pex15*Δ mutant was crossed with the *pex15.1* strain. The resulting diploids were unable to grow on oleate, indicating that the authentic *PEX15* gene had been cloned and deleted. Furthermore, *pex15*Δ regained its ability to grow on oleate when it was transformed with the *PEX15* gene, indicating that the growth defect of *pex15*Δ on oleate is reversible.

We next analyzed the localization of peroxisomal enzymes in the *pex15*Δ mutant. Oleate-induced wild-type and *pex15*Δ cells were fractionated, and the organellar pellet and the cytosolic supernatant were analyzed. Thiolase, peroxisomal catalase (Cta1p) and peroxisomal malate dehydrogenase (Mdh3p) were present in the organellar (27 000 *g*) pellet fraction of wild-type cells, whereas they were present in the cytosolic (27 000 *g*) supernatant fraction of *pex15*Δ cells (Figure 2A). This demonstrates that the import of enzymes containing a PTS1 (catalase, Mdh3p) or PTS2 (thiolase) is disturbed in *pex15*Δ.

In contrast to the peroxisomal matrix proteins, we

observed that on average 40–80% of the peroxisomal membrane proteins Pex13p (Elgersma *et al.*, 1996a) and Pat1p (Hettema *et al.*, 1996) was present in the organellar (27 000 *g*) pellet fraction obtained from *pex15*Δ cells (shown for Pex13p in Figure 2A). This indicates either that membrane proteins aggregate to large cytosolic protein complexes in *pex15*Δ, or that they are targeted to peroxisomal remnants ('ghosts'). To distinguish between these two possibilities, the crude organellar pellet was layered at the bottom of a two-step sucrose flotation gradient, which was spun for 18 h at 150 000 *g* (Walworth *et al.*, 1989; Heyman *et al.*, 1994). Protein aggregates will remain at the bottom, whereas proteins attached to membranes will rise to the lighter sucrose density. As shown in Figure 2B, Pex13p floated to a density of 1.17 g/cm^3 (peak fraction) when the organellar pellet obtained from wild-type cells was used. Flotation of Pex13p was also observed when using the organellar pellet obtained from *pex15*Δ cells, although Pex13p was now found at a slightly lighter density (peak at 1.11 g/cm^3). This indicates that membrane proteins are still targeted to a membrane. Moreover, expression of a Pex13p–green fluorescent protein (GFP) fusion protein in *pex15*Δ cells showed a punctate fluorescence for GFP, suggesting that *pex15*Δ cells have peroxisomal remnants.

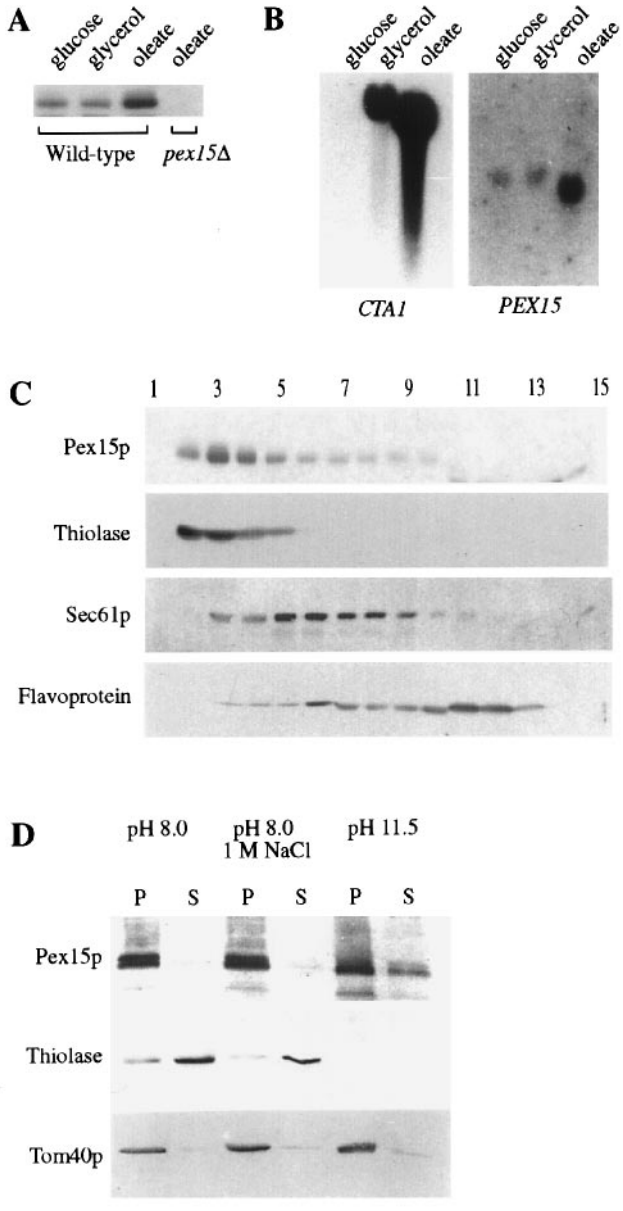


Fig. 3. Expression and subcellular location of Pex15p. (**A**) Induction of Pex15p. TCA lysates obtained from wild-type or *pex15*Δ cells were run on an SDS–polyacrylamide gel, blotted onto nitrocellulose and probed with the antibody directed against Pex15p. The Western blot shows the expression of Pex15p in cells grown on either glucose, glycerol or oleate. (**B**) Northern blot analysis of *PEX15* and *CTA1* expression. RNA was isolated from cells grown on glucose, glycerol or oleate; 10 µg of total RNA was used for each lane of the agarose gel. After blotting onto nitrocellulose, the filters were probed with radiolabeled *PEX15* and *CTA1* probes of comparable specific activity. The *PEX15* autoradiograph was exposed 15 times longer than the *CTA1* autoradiograph. An actin probe used as control showed the same amount of mRNA in every lane (not shown). (**C**) Subcellular location of Pex15p. Oleate-grown wild-type cells were fractionated and the post-nuclear supernatant was analyzed on a Nycodenz gradient. Fractions were subjected to SDS–PAGE, blotted and probed with the antibodies listed. Thiolase is a marker for peroxisomes, Sec61p for the ER and flavoprotein for the mitochondria. Fraction 1 corresponds to the bottom fraction, whereas fraction 15 corresponds to the top fraction. (**D**) Resistance of Pex15p to carbonate extraction. A portion of the organellar pellet was extracted with Tris buffer pH 8.0. Following centrifugation, the pellet was extracted with Tris buffer pH 8.0, 1 M NaCl and, after further centrifugation, this pellet was finally extracted with 100 mM sodium carbonate pH 11.5. Following a final centrifugation step, equivalent portions of pellet (P) and supernatant (S) were used for Western blotting with the antibodies as indicated.

Pex15p is a low abundance integral peroxisomal membrane protein

Antibodies to Pex15p recognized a protein of the expected size in wild-type cells, but not in *pex15*Δ cells (Figures 2A and 3A). Western blot analysis of cell lysates prepared from cells grown on either glucose, glycerol or oleate generally showed a 5- to 10-fold induction of Pex15p in cells grown on oleate, compared with cells grown on glucose or glycerol (Figure 3A). Northern blot analysis confirmed this result, and in addition it revealed that Pex15p was expressed at a low level (Figure 3B).

To determine the location of the Pex15p, we fractionated oleate-grown wild-type cells. Pex15p was present exclusively in the organellar pellet fraction (Figure 2A). Its location was analyzed further by subjecting a post-nuclear (1000 *g*) supernatant (PNS) to Nycodenz density gradient centrifugation, which separates peroxisomes from the mitochondria and ER, leaving cytosolic proteins on top of the gradient. Fractions of this gradient were used for Western blotting using thiolase as a peroxisomal marker, Sec61p as an ER marker and the flavoprotein subunit of succinate dehydrogenase as a mitochondrial marker. Using the Pex15p antibody, we observed that the bulk of Pex15p co-migrated with the peroxisomal marker enzyme, indicating that Pex15p is a peroxisomal protein (Figure 3C). Nevertheless, we frequently observed that the most dense peroxisomal fractions contained relatively low amounts of Pex15p. In addition, Pex15p was often in lighter fractions which contain small amounts of peroxisomal matrix protein (Figure 3C). The relevance of this finding is unclear.

The subcellular localization of Pex15p was analyzed further using electron microscopy. Since the Pex15p anti-

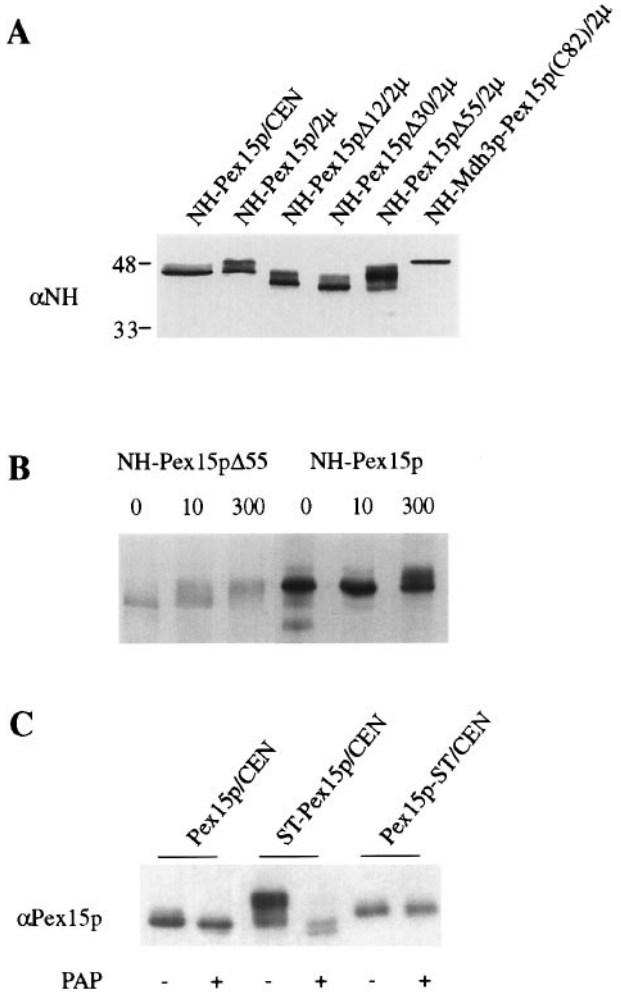


Fig. 4. Phosphorylation of Pex15p. (**A**) Western blot of TCA lysates made from oleate-grown cells expressing Pex15p or Pex15p deletion proteins under the control of the *CTA1* promoter. The amount of protein layered was 20-fold less for the 2μ transformants, relative to the amount layered for the CEN transformant. Proteins were detected using the αNH antibody. (**B**) Pulse–chase experiment showing the modification of Pex15pΔ55 and Pex15p during a time course. Glycerol-grown cells were labeled with [^{35}S]methionine for 7 min at 22°C, and chased by addition of cold methionine and yeast extract for the indicated time (in minutes) at 28°C. After immunoprecipitation using the Pex15p antibody, samples were subjected to SDS–PAGE using a 10% gel. After fixation, the gel was subjected to fluorography. (**C**) Treatment of cell lysates with (+) or without (–) potato acid phosphatase (PAP). Lysates were obtained from oleate-grown cells expressing Pex15p and ST-tagged Pex15p under the control of the *CTA1* promoter.

bodies did not yield a signal using immunoelectron microscopy, Pex15p was epitope tagged with the NH tag (Elgersma *et al.*, 1996b) at the amino-terminus. This protein could restore the formation of normal peroxisomes in the *pex15*Δ mutant when expressed under the control of its own promoter or the peroxisomal catalase (*CTA1*) promoter on a single-copy plasmid (NH-Pex15p/CEN), indicating that the tag did not impair the functioning of Pex15p (not shown). We observed a clear labeling of the peroxisomal membrane in oleate-grown wild-type cells expressing NH-Pex15p/CEN (see below, Figure 5A), whereas no labeling was observed in untransformed control cells (not shown). These results demonstrate that Pex15p is a peroxisomal membrane protein.

Since the primary amino acid sequence suggests that Pex15p has a TMD near its C-terminus, we used a crude organellar pellet fraction for carbonate extraction analysis, a treatment that removes peripheral but not integral proteins from the membrane (Fujiki *et al.*, 1982). As expected, most of the thiolase was already removed after extraction with Tris–HCl at pH 8.0, whereas the mitochondrial integral membrane protein Tom40p could not be extracted either by 1 M NaCl or by carbonate at pH 11.5 (Figure 3D). We found that Pex15p was not extractable with 1 M NaCl, and it was largely (60–80%) resistant to carbonate extraction, suggesting that Pex15p is indeed an integral peroxisomal membrane protein (Figure 3D).

Pex15p is a tail-anchored type II membrane protein

To determine the topology of Pex15p, we used cells lacking either cytosolic malate dehydrogenase (*mdh2*Δ) or peroxisomal malate dehydrogenase (*mdh3*Δ). We have shown previously that *mdh3*Δ cells are unable to grow on plates containing oleate as sole carbon source and that *mdh2*Δ cells can not grow on ethanol (Van Roermund *et al.*, 1995a). By making mutations in the PTS1 of Mdh3p, we could demonstrate further that 5% or less of Mdh3p import is already sufficient to complement the *mdh3*Δ mutant (Elgersma *et al.*, 1996b). In addition, cytosolically mislocalized Mdh3pΔPTS1 is able to rescue the growth defect of *mdh2*Δ cells on ethanol. We made use of this observation by expressing various hybrid proteins of Pex15p fused to Mdh3pΔPTS1 in *mdh2*Δ and *mdh3*Δ cells. Mdh3p–Pex15p was targeted to peroxisomes, but did not complement the *mdh3*Δ mutant. Mdh3p–Pex15p (partially) complemented *mdh2*Δ cells, indicating that the N-terminus is facing the cytosol. Conversely, Pex15p fused to the N-terminus of Mdh3pΔPTS1 (Pex15p–Mdh3p) was unable to complement the *mdh2*Δ mutant whereas (partial) complementation of *mdh3*Δ cells was observed. This suggests that the C-terminus of Pex15p is lumenal.

Additional evidence for a type II topology (N-terminus in the cytosol, C-terminus in the lumen) was obtained by immunoelectron microscopy analysis of cells expressing NH-Pex15p. Most gold particles were present at the cytosolic side of the peroxisomal membrane when using the NH antibodies, suggesting that the N-terminus is facing the cytosol (see below, Figure 5A).

Taken together with the sequence analysis of Pex15p, which predicts one TMD at the C-terminus, the results suggest that Pex15p is a tail-anchored type II membrane protein. This finding is confirmed by several additional experiments described later.

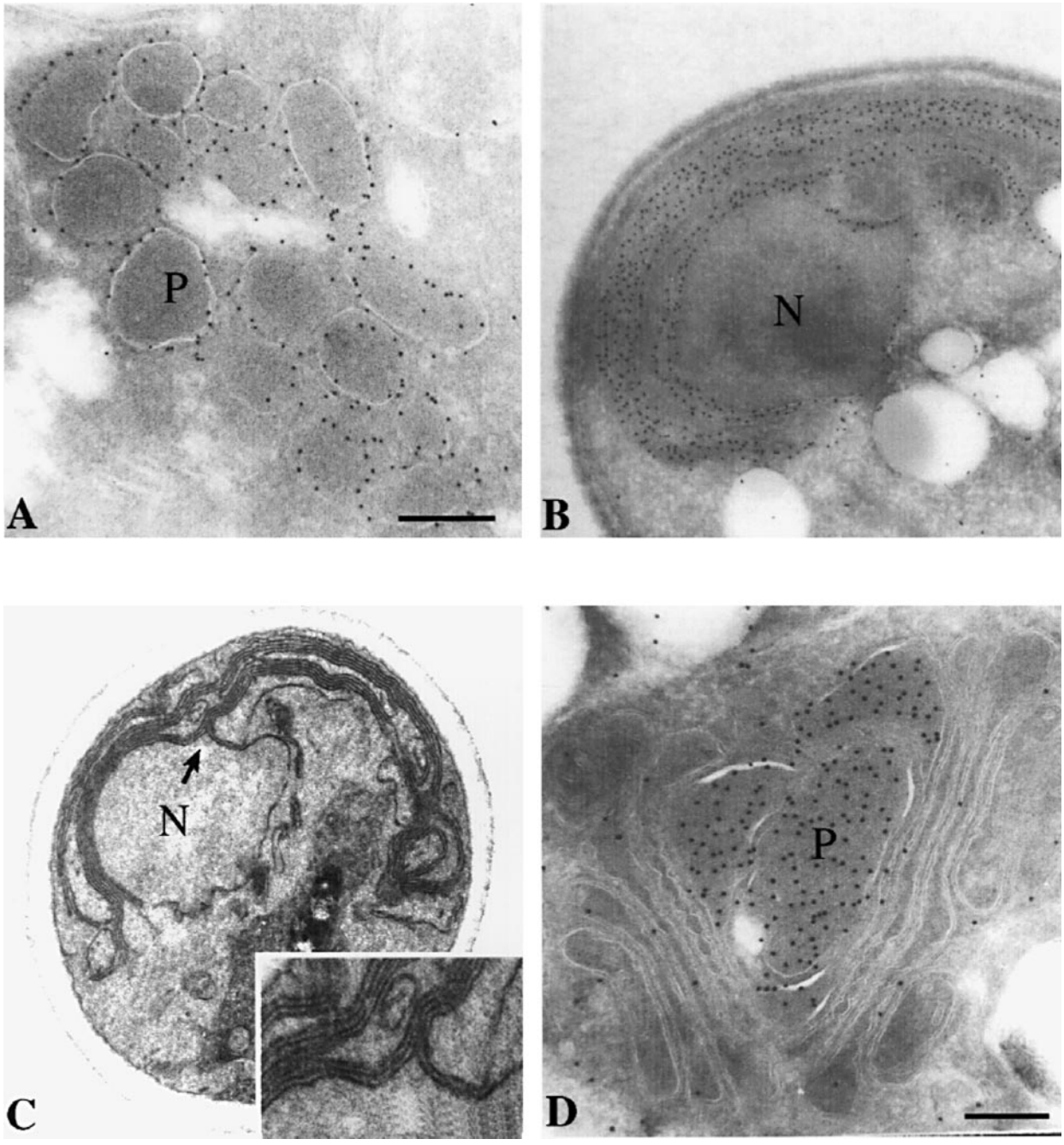
Pex15p is phosphorylated

Interestingly, we noted that Pex15p never migrates as a single sharp band upon being subjected to SDS–PAGE (see Figures 2A, 3A and C). This heterogeneity in the observed molecular weight was more pronounced when Pex15p was overexpressed (Figure 4A). The molecular weight heterogeneity was also observed when the NH antibody was used to detect NH-Pex15p, which rules out alternative initiation of translation or N-terminal breakdown (Figure 4A). Furthermore, the size of the αNH-reactive protein bands changed as expected when truncated

forms of NH-Pex15p (Δ12 and Δ30) were expressed, ruling out the possibillity of C-terminal breakdown (Figure 4A). The largest increase of the expected molecular weight of the protein was observed when the last 55 amino acids were removed (NH-Pex15pΔ55) (Figure 4A). The modification was lost when only the C-terminal 82 amino acids of Pex15p were fused to NH-Mdh3p lacking its PTS1 signal [NH-Mdh3p–Pex15p(C82)]. These experiments suggest that the modification was in the cytosolic domain of Pex15p (Figure 4A).

To test whether Pex15p is post-translationally modified, cells expressing NH-Pex15p/2μ or a C-terminal truncated form of the protein (NH-Pex15pΔ55/2μ) were labeled with [^{35}S]methionine for 7 min, followed by a chase with excess cold methionine (Figure 4B). Slower migrating forms of Pex15p were observed during the chase time, indicating that Pex15p is indeed post-translationally modified. Since Pex15p has several Thr and Ser residues which might serve as substrates for phosphorylation, we made lysates of cells which expressed Pex15p from the *CTA1* promoter (Pex15p/CEN) and treated them with potato acid phosphatase (PAP). The higher molecular weight species could be removed by this treatment, suggesting that Pex15p is indeed phosphorylated (Figure 4C). The same result was obtained for NH-Pex15pΔ55/2μ (not shown).

Although phosphorylation can occur in the ER of mammals, to our knowledge phosphorylation does not occur in the ER or peroxisome lumen in *S.cerevisiae*. We wanted to exploit this to derive additional (*in vivo*) evidence for a type II topology. Therefore, we fused a small Ser/Thr-rich epitope at either the N- or C-terminus of Pex15p, resulting in ST-Pex15p and Pex15p-ST respect-



ively (see Materials and methods). When expressed from a CEN plasmid under the control of either the *PEX15* or *CTA1* promoter, both fusion proteins could restore normal peroxisome assembly in the *pex15*Δ strain and were targeted to the peroxisomes (data not shown). We observed a drastic increase in the phosphorylation of Pex15p when cells expressed ST-Pex15p at both expression levels (shown for *CTA1*-ST-Pex15p in Figure 4C). No increased phosphorylation was observed when cells expressed Pex15p-ST (Figure 4C). These data provide additional evidence for a type II topology of Pex15p.

Overexpression of Pex15p leads to impaired peroxisome assembly and profound ER membrane proliferation

The effect of overexpression of Pex15p was analyzed by expressing it under the control of the *CTA1* promoter on a multicopy plasmid (NH-Pex15p/*2*μ). Remarkably, when oleate-grown wild-type cells expressing NH-Pex15p/2μ were subjected to subcellular fractionation, we found that <50% of catalase and thiolase were present in the crude (27 000 *g*) organellar pellet fraction, and gradient analysis showed a shift of peroxisomes to a lighter density (not

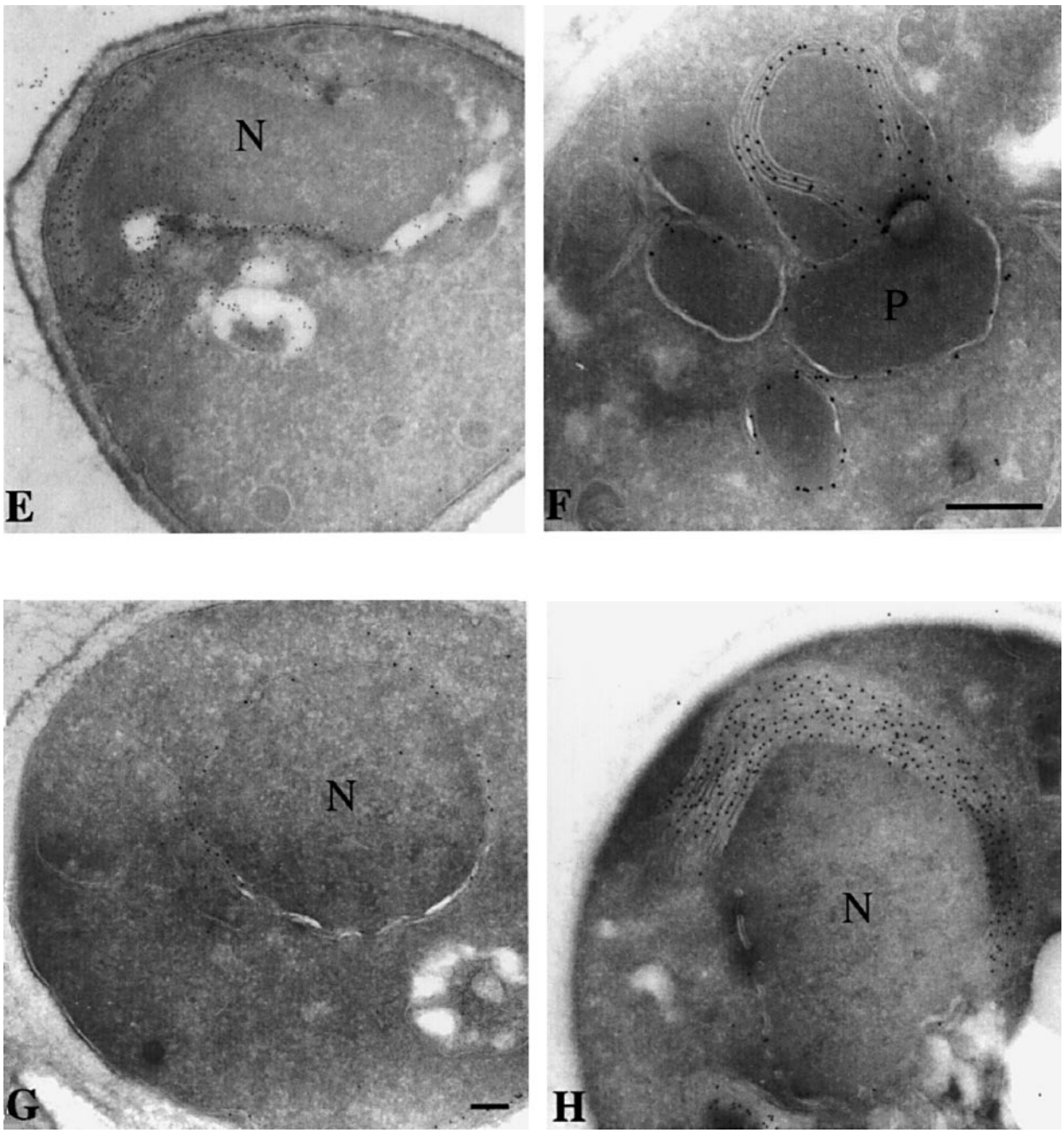


Fig. 5. Electron microscopic analyses of the targeting of Pex15p. The oleate-grown wild-type cells expressed the following proteins: (**A**) NH-Pex15p/CEN (α-NH); (**B**) NH-Pex15p/2μ (α-NH); (**C**) NH-Pex15p/2μ (permanganate-fixed cells; the arrow points to the enlarged region as shown in the inset); (**D**) NH-Pex15p/2μ (α-thiolase); (**E**) Pex15p/2μ + NH-Pex3p/2μ (α-NH); (**F**) NH-Pex15pΔ55 (α-NH); (**G**) NH-Mdh3p–Pex15p(C82)/CEN (α-NH); and (**H**) NH-Mdh3p–Pex15p(C82)/2μ (α-NH). Antibodies used for immunolabeling are shown in parentheses. P = peroxisome; N = nucleus. Bar = 0.2 μm.

shown). We therefore examined the peroxisome morphology of wild-type cells overexpressing NH-Pex15p by electron microscopy. In most cells, we found a strong induction of membranes containing Pex15p (Figure 5B). These membranes resembled karmellae: a morphological arrangement of membranes around the nucleus caused by the overexpression of resident ER proteins (Wright *et al.*, 1988). We found that the membranes containing Pex15p were mostly wrapped around the nucleus, and we often observed a continuity between the membranes and the nuclear envelope in potassium permanganate-fixed cells (Figure 5C and inset). In cells where membrane proliferation was reduced or absent, we often observed the presence of Pex15p in the nuclear envelope. These results demonstrate that overexpressed Pex15p accumulates in the ER, where it causes membrane proliferation.

Using immunoelectron microscopy, we could hardly detect normal looking peroxisomes in wild-type cells overexpressing Pex15p/2μ, which is consistent with the abnormal position of these peroxisomes in gradients. Sometimes we observed thiolase-containing peroxisomes from which several membranes protruded (Figure 5D). Additional evidence that overexpression of Pex15p was obstructing the formation of normal peroxisomes was obtained from overexpressing the peroxisomal membrane proteins NH-Pex3p or NH-Pex13p together with overexpressed Pex15p. Both proteins were found in the Pex15p-induced membranes (shown for NH-Pex3p in Figure 5E). Significant membrane proliferation did not occur when these proteins were expressed with normal levels of Pex15p, although we occasionally observed some ER labeling in the case of Pex3p.

Taken together, these EM analyses indicate that both the peroxisomal and the ER membranes strongly proliferate upon overexpression of Pex15p and that the peroxisomal membrane proteins NH-Pex3p and NH-Pex13p are present in the Pex15p-induced membranes. These observations suggest that Pex15p overexpression interfered with a maturation step in peroxisome formation and that Pex15p might be targeted to peroxisomes via the ER. To test the important possibility that the ER is directly involved in peroxisome biogenesis, we analyzed the targeting of Pex15p in more detail.

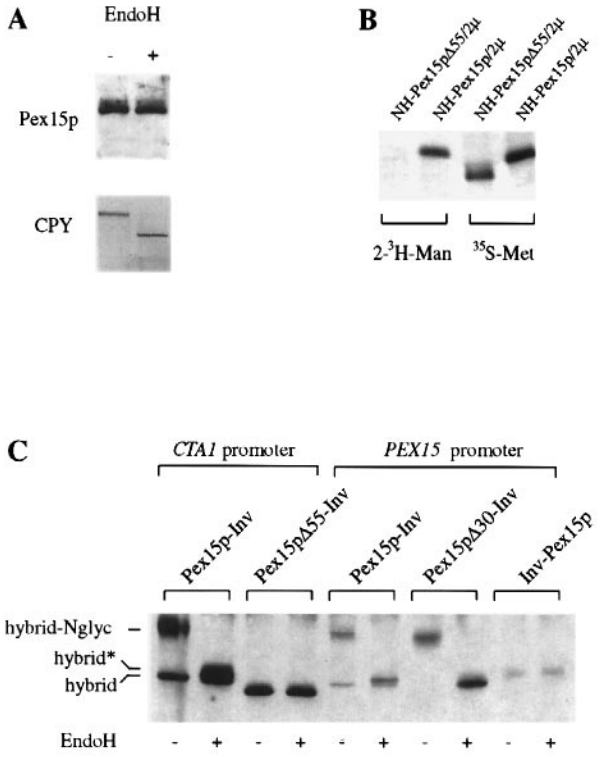


Fig. 6. Glycosylation of Pex15p and Pex15p fusion proteins. (**A**) Western blot of cell lysates after treatment with EndoH. Lysates were obtained from oleate-grown cells expressing Pex15p/2μ under the control of the *CTA1* promoter, treated with EndoH and subjected to Western blotting using the antibodies as indicated. (**B**) Labeling of Pex15p with 2-[^{3}H]mannose or [^{35}S]methionine. Glycerol-grown cells were labeled with 2-[^{3}H]mannose or [^{35}S]methionine for 1 h at 28°C. After immunoprecipitation using the Pex15p antibody, samples were subjected to SDS–PAGE using a 10% gel. After fixation, the gel was subjected to fluorography for 8 weeks. The ^{35}S-labeled samples were 50-fold diluted to get the same intensity as the 2-[^{3}H]mannose-labeled samples. (**C**) *N*-glycosylation of Pex15–invertase hybrid proteins. Lysates were obtained from oleate-grown cells expressing Pex15p–invertase fusion proteins under the control of the *CTA1* or *PEX15* promoter, treated with (+) or without (–) EndoH and subjected to Western blotting using the αPex15p antibody. 'Hybrid-Nglyc' indicates the core-glycosylated hybrid protein; 'Hybrid*' indicates the core-glycosylated hybrid protein after EndoH treatment (does not remove the first GlcNAc); 'Hybrid' indicates the unglycosylated form.

Overexpressed Pex15p carries an ER-specific modification

Many proteins which are destined for the secretory pathway undergo modifications in the ER or in ER-derived compartments. For instance, *N*-linked glycosylation has been of great value in monitoring the trafficking of proteins through the secretory pathway. In *S.cerevisiae*, *N*- and *O*-linked glycosylation occur exclusively in the ER or ER-derived compartments. Therefore, finding such a modification on a protein is firm evidence for targeting to, and temporal residence in, the secretory pathway. Pex15p has putative *N*-linked glycosylation sites on the predicted cytosolic domain (Figure 1A). Endoglycosidase H (EndoH) or *N*-glycosidase F (not shown) treatment of lysates from cells expressing NH-Pex15p/2μ caused no molecular weight shift for Pex15p, whereas carboxypeptidase Y (CPY) was entirely shifted to the deglycosylated species (Figure 6A). This result suggests that Pex15p is not *N*-glycosylated.

The lumenal tail of Pex15p contains several Ser/Thr residues which might be a target for *O*-mannosylation. In contrast to higher eukaryotes, *O*-glycosylation in *S.cerevisiae* is confined strictly to the secretory pathway (reviewed by Herscovics and Orlean, 1993). *O*-Glycosylation in *S.cerevisiae*, often referred to as *O*-mannosylation, is initiated by the integral ER membrane protein Dol-P-mannose synthase, for which *S.cerevisiae* has only one gene. The first mannose is added by an integral ER membrane protein, Dol-P mannose:(Ser/Thr) mannosyltranferase, for which *S.cerevisiae* possesses seven genes (Gentzsch and Tanner, 1996). Subsequent mannose chain elongation involves α1,2 and α1,3 linkages and takes place in the Golgi, resulting in a total of five mannoses per Ser or Thr residue. In contrast to *N*-glycosylation, no consensus site has been elucidated. Since *O*-mannosylation of a few Ser/Thr residues would result in only a minor shift in molecular weight (<0.2 kDa per mannose), we

could not easily use this criterion to test whether Pex15p is indeed a target for *O*-mannosylation. Therefore, we tested whether Pex15p was able to bind to concanavalin A (ConA). We did observe binding to ConA of overexpressed as well as endogenous Pex15p, in a manner competable by the addition of α-methyl mannoside. However, we obtained similar results with *in vitro* translated Pex15p, which made this method unreliable. We then tested *O*-mannosylation of Pex15p by incubating cells expressing NH-Pex15p/2μ with [^{3}H]mannose. After immunoprecipitation with αPex15p antibody, we could detect ^{3}H-labeled Pex15p, suggesting that at least some NH-Pex15p/2μ is indeed *O*-mannosylated (Figure 6B). As a control, we used cells expressing a truncated form of Pexp15p which lacked the TMD and lumenal tail (NH-Pex15pΔ55/2μ). As expected, we did not observe [^{3}H]mannose labeling of this protein (Figure 6B). These experiments provide firm biochemical evidence, and corroborate the morphological evidence presented earlier, that (overexpressed) Pex15p is targeted to the ER. Although we do not have a clear picture of the carbohydrate moiety on Pex15p, it most probably does not have mannose chain elongation (which occurs in the Golgi) since it was not recognized by an antibody that detects mannose in α1,2 and α1,3 linkages (data not shown). This suggests that residence of Pex15p remains limited to the ER.

Pex15p–invertase accumulates in the ER

Although *O*-mannosylation provided evidence for trafficking to the ER, the labeling with [^{3}H]mannose was too insensitive to determine whether endogenous Pex15p had traveled through the ER. We therefore tried to generate a substrate for *N*-glycosylation by fusing Pex15p to mature invertase, which lacks its ER signal sequence. Invertase is *N*-glycosylated at 10–12 of its 13 potential Asn residues. Mature (lacking its signal sequence) invertase fused to the N-terminus of Pex15p and expressed from the *PEX15* promoter (Inv–Pex15p/CEN) was normally targeted to peroxisomes. In agreement with our finding that the N-terminus of Pex15p is facing the cytosol, we did not observe *N*-glycosylation (Figure 6C). In contrast, when invertase was fused to the C-terminus of Pex15p and expressed from the *CTA1* promoter (Pex15p–Inv/CEN), we found a shift of the molecular weight corresponding to core *N*-glycosylation of the invertase moiety. Treatment with EndoH showed that the shift is indeed caused by *N*-glycosylation (Figure 6C). Not all of the Pex15p–Inv is *N*-glycosylated. This might indicate that the unmodified protein has not been in the ER, or that some Pex15p–Inv is not inserted correctly in the membrane. The first possibility was ruled out because gradient analysis revealed that both the modified and the unmodified Pex15p–Inv co-migrated with the ER marker, and we failed to detect any Pex15p–Inv in the peroxisomes (not shown). This implies that either the fusion to invertase or the increased expression level interferes with normal peroxisomal targeting of Pex15p. In order to distinguish between these two possibilities, and to see whether the fusion expressed at endogenous Pex15p levels would go to the ER, we expressed Pex15p–Inv also under the control of the *PEX15* promoter from a single-copy plasmid. We found very similar results to those observed with Pex15p–Inv under the control of the *CTA1* promoter; a core *N*-glycosylated form and a non-modified species (Figure 6C). Notably, the ratio between the two had not changed. In addition, we found again that both species remained in the ER, with none of the protein present in peroxisomes (not shown). As expected, *N*-glycosylation was not observed when invertase was fused to the C-terminus of a truncated Pex15p which lacks its TMD (Pex15pΔ55–Inv) (Figure 6C). Complete core *N*-glycosylation was observed if invertase was fused to Pex15p which only lacks its lumenal tail (Pex15Δ30–Inv), indicating that the lumenal tail is not required for the targeting to the ER (Figure 6C). Furthermore, these experiments show that even at endogenous expression levels, Pex15p contains the topogenic signals to direct invertase to the ER.

Since the Pex15p–Inv fusion protein was no longer targeted to the peroxisome, we also tried to make Pex15p a substrate for *N*-glycosylation by fusing only a small epitope to the C-terminus of Pex15p which contained a consensus *N*-glycosylation site (Pex15p–Nglyc, see Materials and methods). Despite accumulation in the ER of the overexpressed Pex15–Nglyc, the protein was not a substrate for *N*-glycosylation and therefore not useful for studying Pex15p trafficking (data not shown).

The lumenal tail is required for targeting to peroxisomes, but not for targeting to the ER

The results described above indicate that Pex15p contains a topogenic signal that can target it to the ER, and a signal that can target it to peroxisomes. These signals may overlap or may be distinct from each other. Like most other peroxisomal membrane proteins, the Pex15p protein does not contain a PTS1-like or PTS2-like targeting signal. Sequences responsible for the targeting of membrane proteins to the peroxisomal membrane have been delineated for the peroxisomal membrane proteins Pex3p and Pmp47 (Höhfeld *et al.*, 1992; Baerends *et al.*, 1996; Dyer *et al.*, 1996; Wiemer *et al.*, 1996). Both mPTSs are close to (but do not include) a transmembrane region and are located on the lumenal side of the membrane. By analogy with these findings, we expected the lumenal tail of Pex15p to contain the mPTS. In addition, we noticed that there is some homology between the tail of Pex15p and the published mPTSs (Figure 7A).

To test whether the tail was indeed required for targeting to the peroxisomes, we made C-terminal deletions of Pex15p and expressed them under the control of the *CTA1* promoter (Figure 7B). To analyze the locations of these proteins, we subjected a PNS derived from oleate-grown cells to Nycodenz density gradient centrifugation. As can be seen in Figure 7C, wild-type NH-Pex15p expressed under the control of the *CTA1* promoter behaved indistinguishably from endogenous Pex15p, showing co-migration with the peroxisomal marker (compare Figure 7C with 3C).

Deletions up to 12 amino acids did not affect the targeting of NH-Pex15p (Figure 7D), nor its ability to complement the *pex15*Δ growth defect on oleate. When the last 30 amino acids were deleted, leaving only four amino acids downstream of the hydrophobic domain, the protein could only complement the *pex15*Δ mutant very weakly. The bulk of NH-Pex15pΔ30 accumulated in a compartment of intermediate density, which co-migrates with most of the ER marker Sec61p (Figure 7E). Immuno-

A

```
Consensus                R.K.K.L..........F
                         K.R.R.I          Y

ScPex15p 349             LKKYKSLMAIFKHVPAAFHTVYP              371
PpPex3p    1   MLEYTAGLIRRNKKKFLISSGIIG VGYVYTKTINNKIQEF      40
ScPex3p    1 MAPNQRSRSLLQRHRGKVLISLTGIA ALFTTGSVVVFFVKRWLYKQ  46
HpPex3p    1   MFQYCRDLVSRHKKKLLFGTGVIA VSYAVSSFVSNKL         37
CbPmp47A 224     EQLKSFIVKIKKRNITPVDALLLGAFGKLIA              254
```

B

	301 325 350 375	Location
Pex15p WT	STGTAPRKKNNDITVLAGSFWAVLKHHFTRS**VLNKNGLLLTGLLLLLCL**KKYKSLMAIFKHVPAAFHTVYPQIVGLLKLLASI	Px
Pex15pΔ12	STGTAPRKKNNDITVLAGSFWAVLKHHFTRS**VLNKNGLLLTGLLLLLCL**KKYKSLMAIFKHVPAAFHTVYP	Px
Pex15pΔ30	STGTAPRKKNNDITVLAGSFWAVLKHHFTRS**VLNKNGLLLTGLLLLLCL**KKYK	ER
Mdh3p-Pex15p(C82)	-TGTAPRKKNNDITVLAGSFWAVLKHHFTRS**VLNKNGLLLTGLLLLLCL**KKYKSLMAIFKHVPAAFHTVYPQIVGLLKLLASI	Px, ER
Mdh3p-Pex15p(C53)	-S**VLNKNGLLLTGLLLLLCL**KKYKSLMAIFKHVPAAFHTVYPQIVGLLKLLASI	Unstable
Mdh3p-Pex15p(C34)	-KKYKSLMAIFKHVPAAFHTVYPQIVGLLKLLASI	Cyt

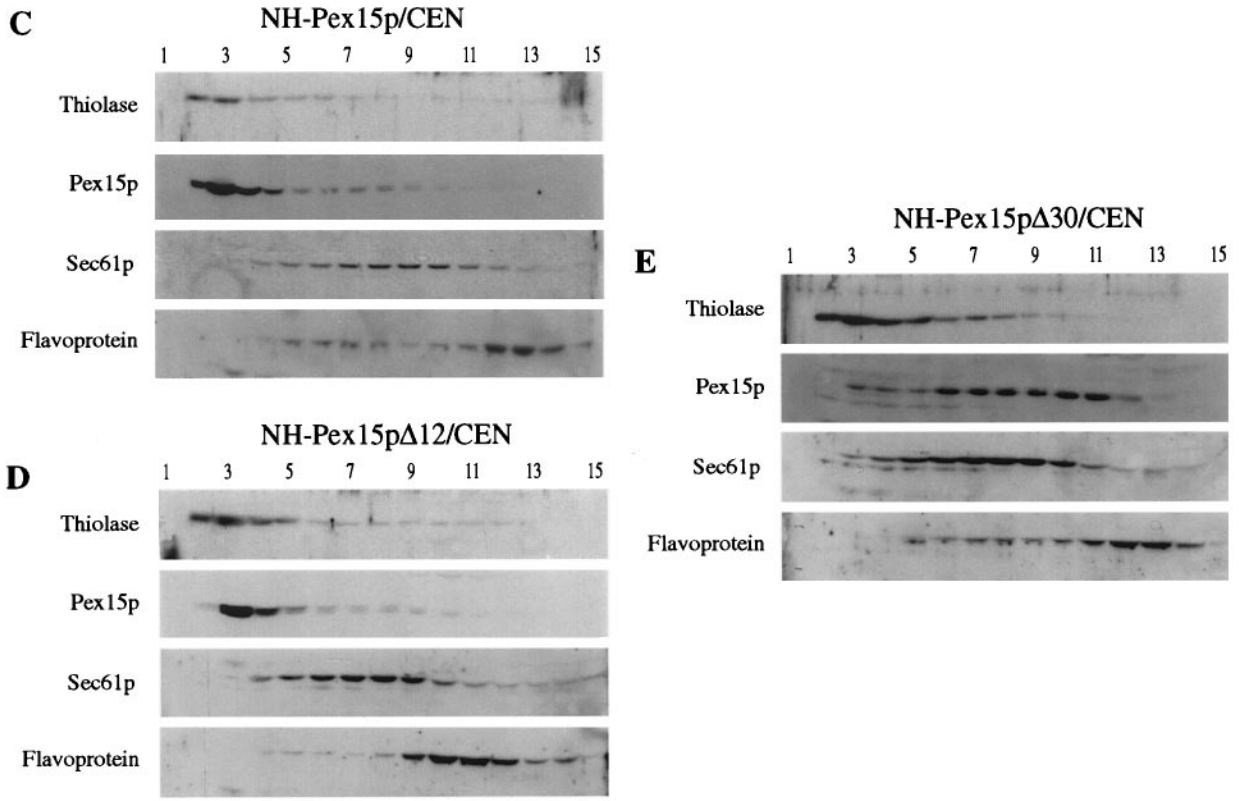


Fig. 7. Targeting analysis of Pex15p and Pex15p deletion proteins. (**A**) Alignment of the lumenal tail of Pex15p with all the mPTS signals elucidated to date. The sequence printed in italics is not part of the delineated mPTS but is given for comparison. (**B**) Carboxy-terminal amino acid sequence of Pex15p-derived constructs used for identification of the mPTS. The transmembrane domain is in bold. Px, peroxisome; ER, endoplasmic reticulum; Cyt, cytosol. (C, D and E) Subcellular locations of Pex15p and Pex15p deletion proteins. Oleate-grown wild-type cells were fractionated and the post-nuclear supernatants were analyzed on a Nycodenz gradient. Fractions were subjected to SDS–PAGE, blotted and probed with antibodies against the proteins as indicated. Thiolase is a marker for peroxisomes, Sec61p for the ER and flavoprotein for the mitochondria. Fractions 1 and 15 correspond to the bottom and top fractions, respectively. Wild-type cells expressing (**C**) NH-Pex15p/CEN; (**D**) NH-Pex15pΔ12/CEN; (**E**) NH-Pex15pΔ30/CEN.

electron microscopy analysis of these cells revealed only very little peroxisomal labeling, but we were unable to detect significant labeling of other compartments at this expression level (not shown). These results indicate that the amino acids 354–371, which reside in the lumenal tail, are required for targeting Pex15p to the peroxisome. Notably, this domain is not responsible for targeting Pex15p to the ER, since NH-Pex15pΔ30 accumulates in the ER which was also found for Pex15pΔ30–Inv as described earlier.

The minimal mPTS of Pex15p overlaps with the ER targeting signal

As shown earlier, deletion of the C-terminal 30 amino acid tail of Pex15p caused the protein to accumulate in the ER. The question arises then whether or not accumulation in the ER is a result of mistargeting of Pex15p caused by the lack of its mPTS. Alternatively, Pex15p is first targeted to the ER and needs its mPTS to be sorted subsequently to peroxisomes. In the latter case, we expected (i) that the C-terminal 34 amino-acids ('tail') would not be sufficient to direct a reporter protein to peroxisomes, and (ii) that the lumenal tail would function only as an mPTS in combination with the ER targeting signal on Pex15p.

To test these predictions, we asked whether the last 34 amino acids of Pex15p could target a reporter enzyme to the peroxisomal membrane. For this, we made use of NH-epitope-tagged Mdh3p from which the five C-terminal amino acids, including its PTS1, had been deleted. We have demonstrated previously that this protein is mistargeted to the cytosol (Elgersma *et al.*, 1996b). Gradient analysis of *mdh3*Δ cells expressing the NH-Mdh3p–Pex15p(C34)/CEN fusion protein under the control of the *CTA1* promoter revealed that NH-Mdh3p–Pex15p(C34) remained on top of the gradient, and no peroxisomal targeting was detected. In addition, NH-Mdh3p–Pex15p(C34) could not complement the *mdh3*Δ mutant, suggesting that <5% of this protein was targeted to peroxisomes. This indicates that, at least in the context of this reporter protein, the last 34 amino acids are not sufficient for targeting Mdh3p to peroxisomes (Figure 8A). This result differs from the observations described for CbPmp47, PpPex3p and HpPex3p for which it has been shown that the lumenal mPTS (without a TMD) was sufficient to direct a reporter protein to the peroxisome (Baerends *et al.*, 1996; Dyer *et al.*, 1996; Wiemer *et al.*, 1996).

We tried to delineate the mPTS further by making a fusion protein with Mdh3p which contained only the TMD and lumenal tail of Pex15p [NH-Mdh3p–Pex15p(C53)]. However, this protein was unstable (data not shown). No instability was observed when we made a fusion protein of NH-Mdh3pΔPTS1 fused to the last 82 amino acids of Pex15p [NH-Mdh3p–Pex15p(C82)/CEN]. Gradient analysis showed that a portion of this protein co-migrated with the peroxisomal marker enzyme, and some of the protein was found at lighter densities (Figure 8B). Immunoelectron microscopy analysis confirmed that the NH-Mdh3p–Pex15p(C82) fusion protein was partially targeted to the peroxisomal membrane (Figure 5F). In addition, some of the protein was present in the ER since we frequently observed labeling of the nuclear envelope (Figure 5G). Overexpression of this protein [NH-Mdh3p–Pex15p(C82)/2μ] resulted in the proliferation of membranes, similar to what was seen with the overexpressed wild-type Pex15p (Figure 5H).

Taken together with the Pex15p deletion experiments described above, the results indicate that the signal for directing Pex15p to the peroxisome is located between amino acids 302 and 371 (see Figure 7B). This overlaps with the ER targeting signal which resides between amino acids 302 and 353.

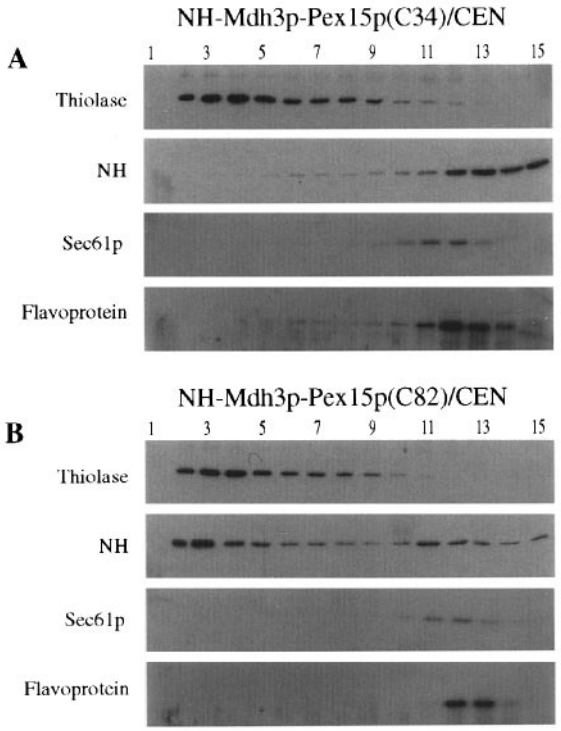


Fig. 8. Targeting of Mdh3p–Pex15p fusion proteins. Oleate-induced *mdh3*Δ cells were fractionated and the post-nuclear supernatants were analyzed on a Nycodenz gradient. Fractions were subjected to SDS–PAGE, blotted and probed with antibodies against the proteins as listed. Thiolase is a marker for peroxisomes, Sec61p for the ER and flavoprotein for the mitochondria. Fractions 1 and 15 correspond to the bottom and top fractions, respectively. *mdh3*Δ cells expressing (**A**) NH-Mdh3p–Pex15p(C34)/CEN; (**B**) NH-Mdh3p–Pex15p(C82)/CEN.

Discussion

Pex15p is a phosphorylated protein required for peroxisome assembly

We have cloned and sequenced the *PEX15* gene by functional complementation of the *pex15.1* mutant (previously called *pas21.1*, Elgersma *et al.*, 1993). Disruption of the gene results in mistargeting to the cytosol of PTS1- and PTS2-containing peroxisomal matrix proteins, indicating that Pex15p plays an important role in peroxisome assembly and that it is therefore a true member of the peroxin family (Distel *et al.*, 1996). Like most *pex* mutants of *S.cerevisiae* (Erdmann and Kunau, 1992), *pex15*Δ cells lack morphologically detectable peroxisomes as judged by electron microscopy. However, this probably reflects a detection problem rather than a true absence of peroxisomal remnants, since further analysis revealed that peroxisomal membrane proteins (Pex13p and Pex13p–GFP) were still targeted to peroxisomal membrane remnants.

Pex15p is the first peroxin shown to be post-translationally modified. Previous studies have identified one other presumed peroxisomal membrane protein which is phosphorylated, but the gene encoding this 63 kDa protein has not been identified yet (Skorin *et al.*, 1986). It will be of particular interest to test whether or not the observed phosphorylation is important for Pex15p function.

Pex15p is a type II tail-anchored membrane protein

We demonstrated that Pex15p is a (tail-anchored) integral peroxisomal membrane protein with a type II topology (N_{cyt}–C_{lumen}). Evidence for the N-terminus being cytosolic was provided by a number of observations: (i) the Mdh3p–Pex15p fusion complements *mdh2*Δ and not *mdh3*Δ; (ii) immunoelectron microscopy showed that the αNH antibodies labeled the cytosolic side of the peroxisomal membrane when cells expressed NH-Pex15p; and (iii) a Ser/Thr-rich epitope was phosphorylated only when fused to the N-terminus of Pex15p. Evidence for a lumenal C-terminus was provided by a Pex15p–Mdh3p fusion (complements *mdh3*Δ and not *mdh2*Δ), and by a fusion to invertase which was only glycosylated when fused to the C-terminus of Pex15p.

The knowledge that Pex15p is a (bitopic) type II tail-anchored membrane protein is important for understanding how it is targeted to the peroxisome. With the possible exception of one membrane protein, it has been shown that most peroxisomal membrane proteins are synthesized on free polyribosomes, and post-translationally inserted into the peroxisomal membrane (Fujiki *et al.*, 1984; Bodnar and Rachubinski, 1991). In contrast, most integral membrane proteins of the ER are synthesized on membrane-bound ribosomes and inserted co-translationally. Consequently, this criterion can be used to investigate whether a peroxisomal membrane protein travels via the ER. However, tail-anchored proteins which insert into the ER are predicted to insert post-translationally since at least part of the transmembrane domain will still be buried in the ribosome before the termination codon is reached. Recently, this prediction has been confirmed by demonstrating that the tail-anchored protein synaptobrevin inserts via a novel pathway into the ER, independently of SRP and Sec61p (Kutay *et al.*, 1995). Therefore, it is very likely that Pex15p will also be inserted post-translationally into membranes.

Overexpression of Pex15p causes ER membrane proliferation

A striking feature of Pex15p is its capacity to proliferate the ER membrane upon overexpression. This phenomenon has been well described for overexpression of several residential ER proteins such as the cytochromes P-450 and *b5*, and HMG-CoA reductase (Jones and Fawcett, 1966; Wright *et al.*, 1988; Vergeres *et al.*, 1993). These proteins induce karmellae, a morphological arrangement of membranes around the nucleus. Interestingly, overexpression of the yeast HMG-CoA reductase Hmg1p, leads to the formation of karmellae whereas overexpression of Hmg2 induces short stacks of membranes. By exchanging segments between Hmg1p and Hmg2p, it was shown that in the context of HMG-CoA reductase, the karmellae formation was caused by a lumenal loop (Parrish *et al.*, 1995). Likewise, we found that the morphology of the induced membranes changed to large vesicle-like structures when the lumenal tail (NH-Pex15pΔ30) was deleted (not shown). For cytochrome *b5*, it was shown that a membrane anchor was required for karmellae production (Vergeres *et al.*, 1993). Our Pex15p analyses support this finding. When the TMD of Pex15p was deleted (Pex15pΔ55), membrane proliferation did not occur (data not shown), whereas the C-terminal 82 amino acids of Pex15p fused to Mdh3p led to membrane proliferation (Figure 5H).

The nature of the mPTS of Pex15p

Sequences responsible for the targeting of membrane proteins to the peroxisomal membrane have been delineated for Pex3p of *S.cerevisiae* (Sc), *Pichia pastoris* (Pp) and *Hansenula polymorpha* (Hp), and for *Candida boidini* (Cb) Pmp47 (Höhfeld *et al.*, 1992; Baerends *et al.*, 1996; Dyer *et al.*, 1996; Wiemer *et al.*, 1996). Both mPTSs are close to, but do not include, a transmembrane region and are located on the lumenal side of the membrane. In agreement with these findings, we found that the lumenal tail of Pex15p is required for peroxisomal targeting. Furthermore, as shown in Figure 7A, there is some homology with the tail of Pex15p and the published mPTSs. This alignment is somewhat different from that used by Dyer *et al.* (1996) and is based on information available since their publication. However, the mPTS of Pex15p differs in two important aspects from that of CbPmp47. Deletion of the mPTS of Pmp47 results mainly in mistargeting to the cytosol whereas deletion of the mPTS of Pex15p results in ER accumulation. Furthermore, in contrast to CbPmp47, the lumenal domain alone of Pex15p is not sufficient to target a protein to the peroxisome. Peroxisomal targeting of Pex15p requires, in addition, a domain (including a TMD) which is also able to direct Pex15p to the ER.

Pex15p and Pex3p have some striking similarities. Overexpression of Pex15p leads to ER proliferation, and overexpressed NH-Pex3p can be found in these same membranes. Furthermore, we showed that the C-terminal 82 amino acids were able to target a reporter protein to both the ER and the peroxisome. Similarly, it has been observed that the first 16 amino acids of the HpPex3p were able to target a reporter protein to both the ER and the peroxisome (Baerends *et al.*, 1996). These results indicate that Pex15p and Pex3p may be targeted to the peroxisomes via the ER, and that there are two distinct classes of mPTS (discussed below).

Pex15p may be targeted to the peroxisome via the ER

Multiple lines of evidence show that overexpressed Pex15p has an effect on the proliferation of ER membranes, and that this protein is targeted to both the ER and the peroxisome. First, in cells overexpresssing Pex15p from a multicopy plasmid, a massive proliferation of membranes was observed. These membranes are ER derived because they are morphologically contiguous with the nuclear envelope and are similar to the membrane proliferation produced by overexpression of other ER membrane proteins such as HMG-CoA reductase and cytochrome *b5*. Under these conditions, growth of the cells on oleate was unaffected, despite the observation that overexpression of Pex15p interfered with normal peroxisome biogenesis. Thus at least some of the overexpressed protein must have been targeted to the peroxisomes in a functional state. Second, the overexpressed Pex15p was labeled with mannose which, according to the information available currently, is added solely in the secretory pathway in *S.cerevisiae*. Third, the accumulation and *N*-glycosylation

of Pex15p–invertase fusions in the ER provides additional support for the conclusion that Pex15p has an ER targeting signal and that the C-terminal lumenal tail of Pex15p does see the ER lumen. Importantly, this fusion behaved identically when expressed from the endogenous *PEX15* promoter, from a single-copy plasmid, showing thereby that the ER targeting and modification of this protein could not be attributed to overexpression alone. Finally, we have provided ample evidence using deletions of Pex15p, as well as fusions with other proteins, that there exist within Pex15p both ER and peroxisomal targeting signals.

We have been unable rigorously to rule out the possibility of mistargeting due to the technical difficulties faced in carrying out pulse–chase experiments with endogenous Pex15p. The low levels of endogenous Pex15p and the long exposure times required for the mannose labeling have greatly hampered our ability to detect an ER-specific modification on the endogenous protein. Without the presence of irrefutable ER modifications on the endogenous protein, pulse–chase experiments would not allow us to distinguish between pro-peroxisomes of lighter density and true ER. If any of the *sec* mutants affect the targeting of Pex15p to peroxisomes, the use of such mutants may be of great help in addressing the targeting of Pex15p and Pex3p. However, these analyses were impeded by the fact that most *sec* mutants are present in a strain background (SEY6210) that cannot utilize oleate.

The origin of peroxisomes

If the ER involvement as described above truly reflects the nature of the trafficking of (some) peroxisomal membrane proteins, this would be in conflict with the current view of the origin of peroxisomes. The cellular and evolutionary origin of peroxisomes has long been a matter of debate, and has recently aroused new interest. Historically, peroxisomes were thought to form by budding from the ER, thereby sharing their origin with other DNA-less organelles bounded by a single membrane. This interpretation was based primarily on many morphological studies, showing that peroxisomes and (smooth) ER are closely associated, and occasionally connected, with each other (Novikoff and Shin, 1964; reviewed by Lazarow *et al.*, 1980). However, the finding that many (if not all) peroxisomal matrix proteins and at least a few peroxisomal membrane proteins are synthesized on free polysomes, followed by post-translational import from the cytosol, strongly suggested that peroxisomes are formed by growth and division of pre-existing (pro-) peroxisomes, and that all peroxisomal membrane and matrix proteins are imported from the cytosol to the peroxisome directly (Lazarow and Fujiki, 1985). In order to explain the morphological and biochemical data in a consistent fashion, the peroxisome reticulum hypothesis was put forward, which implies that peroxisomes may be connected with each other via tubular structures (Lazarow *et al.*, 1980). Additional evidence for the existence of such a network came from histochemical studies which suggested the existence of a peroxisome-forming sheet (Ohno and Fujii, 1990).

Although the growth and division model has hardly been challenged with contradicting data, the model leaves us still with a few unsolved problems. For instance, the origin of the pro-peroxisome or peroxisome-forming sheets (which are very similar to smooth ER) remains unclear. In addition, all the peroxisome biogenesis mutants isolated thus far can be complemented by the corresponding wild-type gene. Since several of these mutants (e.g. *pex3*Δ) do not have any recognizable peroxisomes, it is hard to envisage where the new peroxisomes came from during the complementation of such mutants with the corresponding genes.

Assuming that the ER is involved directly in peroxisome biogenesis, some peroxisomal membrane proteins would be imported directly from the cytosol to the organelle using one type of mPTS (mPTS1). Another class, which would include Pex15p, goes to the peroxisomes via the ER using a related, but distinct mPTS (mPTS2) (Subramani, 1991). The two types of mPTSs are distinguished primarily by the fact that the first is recognized in the cytosol and acts without the requirement for an ER targeting signal, while the second requires an ER targeting signal for its function (as shown here for Pex15p) and acts from the ER lumen. The lumenal domain has to be recognized by a sorting machinery which accumulates proteins destined for the peroxisome on the ER membrane. Thereafter, vesicles containing these proteins would bud off subsequently from the ER. These vesicles can function immediately as a pro-peroxisome by importing matrix and some membrane proteins from the cytosol, or they may be able to fuse with each other or with mature peroxisomes to allow organelle growth.

Our model of a direct involvement of the ER in peroxisome biogenesis provides an alternative explanation for the observation that some peroxisomal membrane protein and peroxisomal matrix proteins first appear in an intermediate or light density fraction after synthesis, and later in a dense fraction (Lüers *et al.*, 1993; Van Roermund *et al.*, 1995b). The observed heterogeneity in protein content between the so-called light and dense peroxisomes may reflect the different stages of peroxisome biogenesis, starting with ER-derived vesicles and ending with mature peroxisomes. Recent findings that two NSF-like ATPases (Pex1p and Pex6p) are required for peroxisome biogenesis, and that these are located on vesicles distinct from mature peroxisomes (Faber *et al*., 1998), provide additional support for our proposal for a role of vesicles in peroxisome biogenesis. This model would also provide an explanation for the mechanism of recruitment of phospholipids during peroxisome proliferation.

Materials and methods

Strains and culture conditions

The *S.cerevisiae* strains used in this study were: BJ1991 (*MAT*α, *leu2, trp1, ura3-251, prb1-1122, pep4-3*); COP161 U7 (*MATa, ade2, ura3, lys*) (used for back-crossing of *pex15.1*); *pex15*Δ (*Pex15::LEU2, MAT*α, *leu2, trp1, ura3-251, prb1-1122, pep4-3*); *pex15.1* (Y84) (*pex15, MAT*α, *leu2, trp1, ura3-251, prb1-1122, pep4-3*); *pex15.1-1d* (*pex15, MAT*α, *leu2, ura3, lys2*) (used for cloning of the wild-type gene); *pex15.1-3a* (*pex15, MAT*α, *ade2, ura3, trp1, lys2*) (used for crossing with *pex15*Δ); and *Escherichia coli* DH5α (*recA, hsdR, supE, endA, gyrA96, thi-1, relA1, lacZ*).

Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO) (Difco), 2% glucose and L-amino acids (20 mg/ml) as needed. Cells transformed with a genomic library were plated on minimal medium containing 0.3% glucose, to allow direct replica-plating onto oleate

plates. Oleic acid plates contained 0.1% oleic acid/0.4% Tween-40, 0.67% YNB-WO, 0.1% yeast extract (Difco) and amino acids (20 mg/ml) as needed. To obtain oleate-induced cells, cells were grown for a minimum of 24 h on 0.3% glucose minimal medium, and subsequently were grown for 15–18 h on oleate medium [0.5% potassium phosphate buffer (pH 6.0), 0.3% yeast extract, 0.5% peptone (Difco) and 0.1% oleic acid/0.2% Tween-40].

For expression studies, cells were grown on 0.3% glucose minimal medium for at least 24 h, and were grown subsequently on medium containing 0.5% potassium phosphate buffer (pH 6.0), 0.3% yeast extract, 0.5% peptone and either 2% glucose, 2% glycerol or 0.1% oleic acid/0.2% Tween-40 as carbon source. The cultures were inoculated at a density such that they reached OD_{600} = 0.7–1.0 after ~15 h.

Cloning and sequencing of the PEX15 gene

The *pex15.1* mutant was back-crossed with strain COP161 U7. After tetrad dissection, the progeny of spore *pex15.1-1d* were used for transformation with a genomic single-copy (YCp50) and multicopy library (YEp13) (Nasmyth and Tatchell, 1980; Rose *et al.*, 1987). Transformed cells were plated onto selective glucose medium and replica-plated onto minimal oleate plates. Among 14 000 Yep13 library transformants, four clones were able to grow on oleate. No complementing clones were obtained among the 12 000 Ycp50 library transformants. The complementing plasmids of the positive clones were rescued and transformed to *E.coli* DH5α. From all four positive clones, we recovered a plasmid containing an 11 kb insert (p21.2). From two clones, we recovered an additional plasmid containing a 4.8 kb insert (p21.1). Both plasmids were able to complement the *pex15.1* growth defect on oleate after re-transformation. Restriction analysis revealed that the 4.8 kb insert of p21.1 was part of the 11 kb insert of p21.2, suggesting that p21.1 is the result of a recombination event of p21.2, either in *E.coli* or in yeast. To localize the *PEX15* gene on the 4.8 kb insert, subclones were made from p21.1 and tested for their ability to complement the mutant. A complementing 2500 bp *Eco*RI fragment (p21.9) was reduced further in size by ExoIII nuclease treatment. The smallest complementing clone resulting from this procedure contained an insert of 1641 bp (p21.9–R2). Both strands of this insert were entirely sequenced with the T7 DNA polymerase sequencing kit (Pharmacia), which is based on the dideoxyribonucleotide chain terminating method (Sanger *et al.*, 1977).

Disruption of the PEX15 gene

The *LEU2* gene was cloned in the *Sal*I site of pUC19, resulting in pUC19-LEU2. An *Nsi*I fragment of the *PEX15* gene was cloned in the *Pst*I site of pUC19-LEU2 (p21.20). This plasmid was then digested with *Sma*I and *Sac*I, and was re-circularized by ligating a *Sca*I (blunted)–*Sac*I fragment of the *PEX15* gene in this plasmid (p21.21). The insert of this plasmid was amplified by PCR using universal sequencing primers, and this DNA was used to transform BJ1991 cells. Leu^+ transformants were analyzed by Southern blotting for correct integration in the *PEX15* gene. The disruption deleted the amino acids 13–301 of Pex15p.

Construction of Pex15p expression plasmids

A *Bam*HI site was introduced just before the start codon by PCR on p21.9 with the 5′ P121* (Table I) primer and a 3′ universal primer. The 5′ end of the gene was sequenced until the *Nsi*I site, and the remainder of the gene was swopped with the *Nsi*I–*Hin*dIII fragment of the authentic *PEX15* gene obtained from p21.9-R2 (resulting in p21.26). The *Bam*HI–*Hin*dIII insert of p21.26 containing the entire *PEX15* gene, was placed downstream of the *CTA1* promoter with or without the NH tag (NH-Pex15p/CEN, p21.27; Pex15p/2μ, 21.31) (*CTA1* expression plasmids are described in Elgersma *et al.*, 1996b). To facilitate additional manipulations, the *Sac*I–*Hin*dIII fragment containing the *NH-PEX15* gene was cloned in the *Sac*I and *Hin*dIII sites of the *CTA1* promoter expression plasmids in which the multiple cloning site was replaced by the P1/P2 adaptor, which resulted in p21.44 (NH-Pex15p/CEN) and 21.45 (NH-Pex15p/2μ). A *PEX15* promoter expression plasmid was made by PCR on P21.9 using a 5′ universal primer and introducing a *Bam*HI site at the 3′ end of the promoter using primer P16. The PCR product was digested with *Eco*RI (831 bp upstream of the *PEX15* ORF and *Bam*HI and cloned in the *Eco*RI and *Bam*HI sites of YCpLAC33 (p21.60). Pex15p and its derivatives were cloned in the *Bam*HI and *Hin*dIII sites of this plasmid to obtain endogenous expression levels.

Pex15pΔ55 was constructed by replacing the *Pst*I–*Hin*dIII fragment of the *PEX15* gene by the *Pst*I–*Hin*dIII fragment of p21.9-R2 (obtained by ExoIII nuclease treatment). This resulted in a replacement of the last 55 amino acids by the tripeptide Lys–Leu–Lys [NH-Pex15pΔ55/CEN (p21.33), NH-Pex15pΔ55/*2*μ (p21.34)]. Construction of Pex15pΔ12 (p21.58) and Pex15pΔ30 (p21.68) was achieved by PCR using a 5′ specific *PEX15* primer and a 3′ primer with the desired truncation (P13, P14). To exclude mutations due to PCR, we sequenced the 3′ end of the constructs until the *Bst*XI site, and replaced the remainder of the gene with the wild-type *PEX15* sequence.

Table I. Oligonucleotides used in this study

Primer	Sequence
P1	5′AGCTAGATCTGAGCTCGGATCCATGGAAGCTTG3′
P2	5′AATTCAAGCTTCCATGGATCCGAGCTCAGATCT3′
P13	5′AAAAAGCTTCAGGGGTATACAGTGTGAAAGGCCGC3′
P14	5′AAAAAGCTTCACTTATATTTTTTCAAACATAAGAG3′
P16	5′AAAGGATCCTATTCACAGAACCCTCTTG3′
P81	5′AAGCTTATACTCGCTAGAAGTTTTAGCAACCC3′
P84	5′CATGTCAACTAGTTCATCTTCCTCTAGTACTG3′
P85	5′GATCCAGTACTAGAGGAAGATGAACTAGTTGACATG AGCT3′
P86	5′AGCTTAACTAGTTCATCTTCCTCTAGTTCATG3′
P87	5′AATTCATGAACTAGAGGAAGATGAACTAGTTA3′
P105	5′AGCTTAGGTGTTAACGCATCAGGAGGACTAGCGAGT ATATGATG3′
P106	5′AATTCATCATATACTCGCTAGTCCTCCTGATGCGTT AACACCTA3′
P111	5′AAATCTAGAGAAAAAATATAAGTCATTGATGGCAA3′
P121*	5′AAAGGATCCATGGCTGCAAGTGAGATAATG3′
PUC19-40	5′GTTTTCCCAGTCACGAC3′
P21FLINV	5′AAGCTTGTATCTCGCTAGAAG3′
P21Δ55INV	5′AAGCTTGGAAATGATGCTTCAGG3′
P21Δ30INV	5′CCCTTGCTTGCTTATATTTTTTCGGGCATAAG3′
BP21INV	5′AAAGGATCCATGGCTACAAACGAAACTAGCG3′
FUJ2	5′CATTATCTCACTTGCAGCTACTTCCCTTACTTGG3′
FUJ1	5′CCAAGTAAGGGAAGTAGCTGCAAGTGAGATAATG3′

The targeting constructs with *MDH3* were made by cloning the *Sca*I–*Hin*dIII fragment of p21.26 in the *Pst*I (blunt)–*Hin*dIII site of pEL149, which contains the *NH-MDH3* gene, described in Elgersma *et al.* (1996b), resulting in NH-Mdh3p–Pex15p(C82)/CEN (p21.38) and NH-Mdh3p–Pex15p(C82)/*2*μ (p21.39). NH-Mdh3p–Pex15p(C34)/CEN was constructed by PCR on p21.44 using the 3′ P111 and a 5′ universal primer. The obtained PCR fragment encoding the last 34 amino acids of Pex15p was digested with *Pst*I–*Hin*dIII and cloned in the *Pst*I and *Hin*dIII sites of pEL 149.

ST-Pex15p was constructed by replacing the *Sac*I–*Bam*HI fragment of p21.44 (containing the NH tag) by the adaptor (P84/P85). The encoded amino acids of this adaptor are: MSTSSSSSSTGS. The resulting *ST-PEX15* gene was cloned as a *Sac*I–*Hin*dIII fragment in the various expression plasmids. To construct Pex15p-ST and Pex15p–Nglyc, we first removed the stop codon of *PEX15* by PCR on p21.44 using a 5′ universal primer and the 3′ primer P81. The PCR fragment obtained was digested with *Bst*XI and *Hin*dIII, and this was used to replace the 5′ end of *PEX15* in p21.45. The resulting plasmid (p21.83) was partially sequenced and digested with *Hin*dIII and *Eco*RI, followed by a ligation with the ST adaptor (P86/87; encoding the amino acids SLTSSSSSS), or with the Nglyc adaptor (P105/106; encoding the amino acids SLGVNASGGLASI). The resulting *PEX15-ST* and *PEX15-Nglyc* genes were cloned as *Bam*HI–*Eco*RI fragments in the appropriate expression plasmids.

All of the invertase (*SUC2*) fusions were constructed by PCR with Vent polymerase (NEB). For the Pex15p–invertase constructs with the *CTA1* promoter, an upstream primer, PUC19-40, was hybridized upstream of the *CTA1* promoter–*PEX15* in plasmid p21.31. A universal primer (T3) was used as an upstream primer for the *PEX15* promoter-containing constructs utilizing pBSPEX15, which contains *PEX15* in a pBluescript vector, as the template. Downstream primers were designed to hybridize just before the stop codon (P21FLINV), or at codons which create the truncations (P21Δ55INV, P21Δ30INV). Downstream primers contained a *Hin*dIII site for in-frame fusion to invertase in the vector pSEYC308 (CEN, *URA3*) (Johnson *et al.*, 1987). Invertase–Pex15p was constucted by PCR using the overlap extension method. A set of complementary primers containing the fusion joint (FUJ1 and FUJ2), encoding the next to last amino acid of invertase followed by the second amino acid of Pex15 and additional flanking sequences on both sides, were used to

create the fusion joint and provide the overlap for the second round of PCR. An upstream primer (BP21INV) contained a *Bam*HI site, followed by the first two codons of *PEX15*, then 16 nuclotides that hybridize to *SUC2* starting at the codon for amino acid 3 of the mature (signal sequence cleaved) protein. The downstream primer was the universal primer T7. Primers FUJ1 and T7 were used to amplify *PEX15* from pBSPEX15. Primers BP21INV and FUJ2 were used to amplifiy invertase from pSEYC308. Both products were gel purified and ~50 ng of each mixed for PCR with BP21INV and T7 primers. The resulting product was purified, digested with *Bam*HI and *Hin*dIII, and cloned into p21.60.

Antibodies

The Pex15p protein was expressed in *E.coli* by cloning an *Nsi*I–*Hin*dIII fragment [obtained from a *PEX15* clone (p21.9-R3) treated by ExoIII nuclease] in the *Pst*I–*Hin*dIII site of plasmid pQE-9 (Qiagen). The resulting 6×His-tagged protein encompassing amino acids 12–329 of Pex15p subsequently was expressed in *E.coli* and purified by Ni-chelating chromatography under denaturing conditions according to the manual provided by Qiagen. The protein was purified further by SDS–PAGE, visualized with 0.25 M KCl, 1 mM dithiothreitol (DTT), and subsequently excised and eluted from the gel in elution buffer [50 mM Tris (pH 8.0), 0.1% SDS, 0.1 mM EDTA, 5 mM DTT, 0.15 M NaCl]. This highly purified protein was used to immunize rabbits. The antibody was used at a 1:5000 dilution for Western blotting, and 1:500 for immunoprecipitations.

The thiolase and NH antibodies are described by Elgersma *et al.* (1996b) and were both used at a 1:5000 dilution. The Pex13p (1:3000) antibody is described by Elgersma *et al.* (1996a). The antibody against Cta1p (1:10 000) will be described elsewhere. Antibodies against Sec61p (1:3000) and flavoprotein subunit of succinate dehydrogenase (1:3000) were a kind gift of R.Schekman and I.Scheffler, respectively.

Subcellular fractionation and Nycodenz gradient analysis

Subcellular fractionations of oleate-grown cells were performed as described by Van der Leij *et al.* (1992), in the presence of protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mg/ml of chymostatin, leupeptin, antipain and pepstatin]. Continuous 16–35% Nycodenz gradients were used (30 ml), with a cushion of 4 ml of 42% Nycodenz dissolved in 5 mM 2[*N*-morpholino]ethane sulfonic acid (MES) (pH 6.0), 1 mM EDTA, 1 mM KCl and 8.5% sucrose. The sealed tubes were centrifuged for 2.5 h in a vertical rotor at 29 000 *g* at 4°C. Afterwards, fractions were collected and analyzed by SDS–PAGE and Western blotting.

Sucrose flotation gradients

A two-step gradient was made by resuspending the organellar pellet in 500 ml of 60% sucrose (w/w) in sucrose gradient buffer [5 mM MES (pH 6.0), 1 mM KCl, 1 mM EDTA]. This was transferred to a Beckman centrifuge tube and overlaid with 2.4 ml of 50% (w/w) sucrose in sucrose gradient buffer, and overlaid with 2.4 ml of 20% (w/w) sucrose in sucrose gradient buffer. The samples were spun for 18 h at 150 000 *g*, and fractions were analyzed by SDS–PAGE and Western blotting.

Carbonate extraction

An organellar pellet (27 000 *g*) prepared from wild-type cells was resuspended in 100 mM Tris buffer (pH 8.0). Following freezing and centrifugation (233 000 *g* for 60 min), the pellet was extracted with 100 mM Tris buffer (pH 8.0), 1 M NaCl. After another centrifugation step, the pellet was extracted with 100 mM sodium carbonate (pH 11.5). This was centrifuged once more, and equivalent portions of pellet (P) and supernatant fractions (S) were layered in each lane of an SDS–polyacrylamide gel, blotted onto nitrocellulose and probed with the antibodies indicated. All extraction steps were performed at 4°C, in the presence of protease inhibitors (1 mM PMSF and 2 mg/ml of chymostatin, leupeptin, antipain and pepstatin).

Alternatively, a freshly prepared organellar pellet was used directly for carbonate extraction, and further treated as described above. This procedure gave the same result with respect to the distribution of the membrane proteins.

EndoH and PAP treatment

Cells from a 50 ml overnight-grown oleate culture (OD_{600} = 0.8) were pelleted, washed and lysed with glass beads. The pellet was resuspended in 500 µl of phosphate-buffered saline (PBS) containing 0.5% SDS and 1% β-mercaptoethanol, and cell debris was removed by centrifugation for 1 min at 8000 *g*. The supernatant was boiled for 5 min. For PAP treatment, 10 µl of cell-free lysate was added to 40 µl of PAP buffer (50 mM MES pH 6.0, 1 mM DTT) containing 1 U of PAP (Boehringer) and protease inhibitors as described above. Samples were incubated for 18 h at 28°C. For EndoH treatment, 10 µl of cell-free lysate were added to 40 µl of EndoH buffer (100 mM NaAc pH 4.5, 1% β-mercaptoethanol, 0.5% SDS) containing 2 mU of EndoH (Boehringer) and protease inhibitors. Samples were incubated for 2 h at 37°C. Mock treatments were treated in a similar manner, but without the addition of enzyme.

Fusion proteins with invertase were expressed in oleate as described above. Two OD_{600} equivalents of each strain were harvested by centrifugation and resuspended in 10% trichloroacetic acid (TCA). Following at least 15 min on ice, the samples were pelleted, washed twice with cold acetone and dried. The pellet was resuspended in 160 µl of 1% β-mercaptoethanol, 0.5% SDS and lysed by glass bead vortexing and boiling. The samples were then cleared by centrifugation and 120 µl of cleared lysate were mixed with 30 µl of 0.25 M Na citrate pH 5.5. The samples were split into two aliquots. To one sample, 1 µl (1 mU) of Endo H was added. All samples were incubated at 37°C for 2 h. Samples were mixed 1:1 with 2× sample buffer and heated to 95°C for 5 min prior to SDS–PAGE and Western blotting as described above.

Miscellaneous

[^{35}S]Methionine or (D)-2-[^{3}H]mannose labeling of cells and the subsequent immunoprecipitation were carried out essentially as previously described (Elgersma *et al.*, 1996b), with the exception that we used 150 µCi of [^{35}S]methionine for pulse–chase experiments and 500 µCi for [^{3}H]mannose labeling. We used minimal glycerol medium for all labelings, and added 0.2 mg/ml methionine and cysteine plus 0.5% yeast extract for chases. When indicated, cells were chased in rich oleate medium, by diluting them 10 times in rich oleate supplemented with 0.2 mg/ml methionine and cysteine. Isotopes were obtained from NEN.

Western blotting was performed essentially as described in Elgersma *et al.* (1996a). Blots were incubated with either IgG-coupled alkaline phosphatase staining (NBT/BCIP; KPL Laboratories) or with goat anti-rabbit peroxidase conjugate (ECL; Amersham) and further treated as recommended by the manufacturers.

For TCA lysates, cells (50 ml of culture OD_{600} = 0.7) were spun, washed and collected in a 2 ml Eppendorf tube. After addition of 500 µl of 10% TCA and ~200 µl of glass beads, the cells were broken by vigorous vortexing for 30 min at 4°C. Cell debris was pelleted by spinning for 30 s at 6000 r.p.m., and the supernatant was centrifuged further for 20 min at 12 000 r.p.m. at 4°C. The pellet was washed once with acetone, dried and resuspended in Laemmli sample buffer for SDS–PAGE analysis.

For immunoelectron microscopy analyis, oleate-induced cells were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. Ultra-thin sections were prepared as described by Gould *et al.* (1990). Potassium permanganate-fixed sections were prepared as described by Wiemer *et al.* (1996).

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References

Baerends,R.J.S. *et al.* (1996) The *Hansenula polymorpha PER9* gene encodes a peroxisomal membrane protein essential for peroxisome assembly and integrity. *J. Biol. Chem.*, **271**, 8887–8894.

Bodnar,A.G. and Rachubinski,R.A. (1991) Characterization of the integral membrane polypeptides of rat liver peroxisomes isolated from untreated and clofibrate-treated rats. *Biochem. Cell. Biol.*, **69**, 499–508.

Diestelkötter,P. and Just,W.W. (1993) *In vitro* insertion of the 22-kD peroxisomal membrane protein into isolated rat liver peroxisomes. *J. Cell Biol.*, **123**, 1717–1725.

Distel,B. *et al.* (1996) A unified nomenclature for peroxisome biogenesis factors. *J. Cell Biol.*, **135**, 1–3.

Dyer,J.M., McNew,J.A. and Goodman,J.M. (1996) The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop. *J. Cell Biol.*, **133**, 269–280.

Elgersma,Y. and Tabak,H.F. (1996) Proteins involved in peroxisome biogenesis and functioning. *Biochim. Biophys. Acta*, **1286**, 269–283.

Elgersma,Y., Van den Berg,M., Tabak,H.F. and Distel,B. (1993) An efficient positive selection procedure for the isolation of peroxisomal import and peroxisome assembly mutants of *Saccharomyces cerevisiae*. *Genetics*, **135**, 731–740.

Elgersma,Y., Van Roermund,C.W.T., Wanders,R.J.A. and Tabak,H.F. (1995) Peroxisomal and mitochondrial carnitine acetyltransferases of *Saccharomyces cerevisiae* are encoded by a single gene. *EMBO J.*, **14**, 3472–3479.

Elgersma,Y., Kwast,L., Klein,A., Voorn-Brouwer,T., Van den Berg,M., Metzig,B., America,T., Tabak,H.F. and Distel,B. (1996a) The SH3 domain of the *Saccharomyces cerevisiae* peroxisomal membrane protein Pex13p functions as a docking site for Pex5p, a mobile receptor for the import of PTS1-containing proteins. *J. Cell Biol.*, **135**, 97–109.

Elgersma,Y., Vos,A., Van den Berg,M., Van Roermund,C.W.T., Van der Sluijs,P., Distel,B. and Tabak,H.F. (1996b) Analysis of the carboxyl-terminal peroxisomal targeting signal 1 in a homologous context in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **271**, 26375–26382.

Erdmann,R. and Blobel,G. (1996) Identification of Pex13p, a peroxisomal membrane receptor for the PTS1 recognition factor. *J. Cell Biol.*, **135**, 111–121.

Erdmann,R. and Kunau,W.H. (1992) A genetic approach to the biogenesis of peroxisomes in the yeast *Saccharomyces cerevisiae*. *Cell Biochem. Funct.*, **10**, 167–174.

Faber,K.N., Heyman,J.A. and Subramani.S. (1998) The AAA-family peroxins, PpPex1p and PpPex6p, interact with each other in an ATP-dependent manner and are associated with different subcellular membranous structures distinct from peroxisomes. *Mol. Cell. Biol.*, in press.

Fujiki,Y., Fowler,S., Shio,H., Hubbard,A.L. and Lazarow,P.B. (1982) Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.*, **93**, 103–110.

Fujiki,Y., Rachubinski,R.A. and Lazarow,P.B. (1984) Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes. *Proc. Natl Acad. Sci. USA*, **81**, 7127–7131.

Gentzsch,M. and Tanner,W. (1996) The *PMT* gene family: protein *O*-glycosylation in *Saccharomyces cerevisiae* is vital. *EMBO J.*, **15**, 5752–5759.

Glover,J.R., Andrews,D.W. and Rachubinski,R.A. (1994) *Saccharomyces cerevisiae* peroxisomal thiolase is imported as a dimer. *Proc. Natl Acad. Sci. USA*, **91**, 10541–10545.

Gould,S.J., Keller,G.A. and Subramani,S. (1987) Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. *J. Cell Biol.*, **105**, 2923–2931.

Gould,S.J., Keller,G.A., Schneider,M., Howell,S.H., Garrard,L.J., Goodman,J.M., Distel,B., Tabak,H.F. and Subramani,S. (1990) Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO J.*, **9**, 85–90.

Gould,S.J., Kalish,J.E., Morrell,J.C., Bjorkman,J., Urquhart,A.J. and Crane,D.I. (1996) Pex13p is an SH3 protein of the peroxisome membrane and a docking factor for the predominantly cytoplasmic PTS1 receptor. *J. Cell Biol.*, **135**, 85–95.

Häusler,T., Stierhof,Y.D., Wirtz,E. and Clayton,C. (1996) Import of a DHFR hybrid protein into glycosomes *in vivo* is not inhibited by the folate-analogue aminopterin. *J. Cell Biol.*, **132**, 311–324.

Herscovics,A. and Orlean,P. (1993) Glycoprotein biosynthesis in yeast. *FASEB J.*, **7**, 540–550.

Hettema,E.H., Van Roermund,C.W.T., Distel,B., Van den Berg,M., Vilela,C., Rodrigues-Pousada,C., Wanders,R.J.A. and Tabak,H.F. (1996) The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. *EMBO J.*, **15**, 3813–3822.

Heyman,J.A., Monosov,E. and Subramani,S. (1994) Role of the PAS1 gene of *Pichia pastoris* in peroxisome biogenesis. *J. Cell Biol.*, **127**, 1259–1273.

Höhfeld,J., Mertens,D., Wiebel,F.F. and Kunau,W.H. (1992) Defining components required for peroxisome assembly in *Saccharomyces cerevisiae*. In Neupert,W. and Lill,R. (eds), *Membrane Biogenesis and Protein Targeting*. Elsevier, New York, pp. 185–207.

Imanaka,T., Shiina,Y., Takano,T., Hashimoto,T. and Osumi,T. (1996) Insertion of the 70-kDa peroxisomal membrane protein into peroxisomal membranes *in vivo* and *in vitro*. *J. Biol. Chem.*, **271**, 3706–3713.

Johnson,L.M., Bankaitis,L.M. and Emr,S.D. (1987) Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. *Cell*, **48**, 875–885.

Jones,A.L. and Fawcett,D.W. (1966) Hypertrophy of the agranular endoplasmic reticulum in hamster liver induced by phenobarbital. *J. Histochem. Cytochem.*, **14**, 215–231.

Klein,P., Kaneshi,M. and DeLisi,C. (1985) The detection and classification of membrane-spanning proteins. *Biochim. Biophys. Acta*, **815**, 468–476.

Kragler,F., Langeder,A., Raupachova,J., Binder,M. and Hartig,A. (1993) Two independent peroxisomal targeting signals in catalase A of *Saccharomyces cerevisiae*. *J. Cell Biol.*, **120**, 665–673.

Kutay,U., Ahnert,H.G., Hartmann,E., Wiedenmann,B. and Rapoport,T.A. (1995) Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane. *EMBO J.*, **14**, 217–223.

Kyte,J. and Doolittle,R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, **157**, 105–132.

Lazarow,P.B. and Fujiki,Y. (1985) Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.*, **1**, 489–530.

Lazarow,P.B., Shio,H. and Robbi,M. (1980) Biogenesis of peroxisomes and the peroxisome reticulum hypothesis. In Bucher,T., Sebal,W. and Weiss,H. (eds), *31st Mosbach Colloquium on Biol. Chem.-organelle Formation*. Springer Verlag, New York, pp. 187–206.

Lüers,G., Hashimoto,T., Fahimi,H.D. and Volkl,A. (1993) Biogenesis of peroxisomes: isolation and characterization of two distinct peroxisomal populations from normal and regenerating rat liver. *J. Cell Biol.*, **121**, 1271–1280.

McNew,J.A. and Goodman,J.M. (1994) An oligomeric protein is imported into peroxisomes *in vivo*. *J. Cell Biol.*, **127**, 1245–1257.

Nasmyth,K.A. and Tatchell,K. (1980) The structure of transposable yeast mating type loci. *Cell*, **19**, 753–764.

Novikoff,A.B. and Shin,W. (1964) The endoplasmic reticulum in the Golgi zone and its relation to microbodies, Golgi apparatus and autophagic vacuoles in rat liver cells. *J. Microscopie*, **3**, 187–206.

Ohno,S. and Fujii,Y. (1990) Three-dimensional and histochemical studies of peroxisomes in cultured hepatocytes by quick-freezing and deep-etching method. *Histochem. J.*, **22**, 143–154.

Parrish,M.L., Sengstag,C., Rine,J.D. and Wright,R.L. (1995) Identification of the sequences in HMG-CoA reductase required for karmellae assembly. *Mol. Biol. Cell*, **6**, 1535–1547.

Rehling,P., Marzioch,M., Niesen,F., Wittke,E., Veenhuis,M. and Kunau,W.H. (1996) The import receptor for the peroxisomal targeting signal 2 (PTS2) in *Saccharomyces cerevisiae* is encoded by the *PAS7* gene. *EMBO J.*, 15, 2901–2913.

Rose,M.D., Novick,P., Thomas,J.H., Botstein,D. and Fink,G.R. (1987) A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-contaning shuttle vector. *Gene*, **60**, 237–243.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Skorin,C., Soto,U., Necochea,C. and Leighton,F. (1986) Protein phosphorylation in peroxisomes. *Biochem. Biophys. Res. Commun.*, **140**, 188–194.

Subramani,S. (1996) Protein translocation into peroxisomes. *J. Biol. Chem.*, **271**, 32483–32486.

Subramani,S. (1997) *PEX* genes on the rise. *Nature Genet.*, **15**, 331–333.

Swinkels,B.W., Gould,S.J., Bodnar,A.G., Rachubinski,R.A. and Subramani,S. (1991) A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO J.*, **10**, 3255–3262.

Van der Leij,I., Van den Berg,M., Boot,R., Franse,M., Distel,B. and Tabale,H. (1992) Isolation of peroxisome assembly mutants from *Saccharomyces cerevisiae* with different morphologies using a novel positive selection procedure. *J. Cell Biol.*, **119**, 153–162.

Van Roermund,C.W.T., Elgersma,Y., Singh,N., Wanders,R.J.A. and Tabak,H.F. (1995a) The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under *in vivo* conditions. *EMBO J.*, **14**, 3480–3486.

Van Roermund,C.W.T., Van den Berg,M. and Wanders,R.J.A. (1995b) Localization of peroxisomal 3-oxoacyl-CoA thiolase in particles of varied density in rat liver: implications for peroxisome biogenesis. *Biochim. Biophys. Acta*, **1245**, 348–358.

Vergeres,G., Yen,T., Aggeler,J., Lausier,J. and Waskell,L. (1993) A model system for studying membrane biogenesis. Overexpression of cytochrome *b5* in yeast results in marked proliferation of the intracellular membrane. *J. Cell Sci.*, **106**, 249–259.

Walton,P.A., Hill,P.E. and Subramani,S. (1995) Import of stably folded proteins into peroxisomes. *Mol. Biol. Cell*, **6**, 675–683.

Walworth,N.C., Gould,B., Ruohola,H. and Novick,P.J. (1989) Fractionation of yeast organelles. *Methods Cell Biol.*, **31**, 335–356.

Wiemer,E.A.C., Lüers,G.H., Faber,K.N., Wenzel,T., Veenhuis,M. and Subramani,S. (1996) Isolation and characterization of Pas2p, a peroxisomal membrane protein essential for peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris*. *J. Biol. Chem.*, **271**, 18973–189780.

Wright,R., Basson,M., D'Ari,L. and Rine,J. (1988) Increased amounts of HMG-CoA reductase induce 'Karmellae': a proliferation of stacked membrane pairs surrounding the yeast nucleus. *J. Cell Biol.*, **107**, 101–114.

Zhang,J.W. and Lazarow,P.B. (1996) Peb1p (Pas7p) is an intraperoxisomal receptor for the NH_2-terminal, type 2, peroxisomal targeting sequence of thiolase: Peb1p itself is targeted to peroxisomes by an NH2-terminal peptide. *J. Cell Biol.*, **132**, 325–334.

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