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Pex12p of *Saccharomyces cerevisiae* is a component of a multi-protein complex essential for peroxisomal matrix protein import

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We have isolated the *Saccharomyces cerevisiae pex12 – 1* mutant from a screen to identify mutants defective in peroxisome biogenesis. The *pex12Δ* deletion strain fails to import peroxisomal matrix proteins through both the PTS1 and PTS2 pathway. The *PEX12* gene was cloned by functional complementation of the *pex12 – 1* mutant strain and encodes a polypeptide of 399 amino acids. *Sc*Pex12p is orthologous to Pex12 proteins from other species and like its orthologues, *S. cerevisiae* Pex12p contains a degenerate RING finger domain of the C3HC4 type in its essential carboxy-terminus. Localization studies demonstrate that Pex12p is an integral peroxisomal membrane protein, with its NH_2-terminus facing the peroxisomal lumen and with its COOH-terminus facing the cytosol. Pex12p – deficient cells retain particular structures that contain peroxisomal membrane proteins consistent with the existence of peroxisomal membrane remnants ("ghosts") in *pex12Δ* null mutant cells. This finding indicates that *pex12Δ* cells are not impaired in peroxisomal membrane biogenesis. In immunoisolation experiments Pex12p was co-purified with the RING finger protein Pex10p, the PTS1 receptor Pex5p and the docking proteins for the PTS1 and the PTS2 receptor at the peroxisomal membrane, Pex13p and Pex14p. Furthermore, two-hybrid experiments suggest that the two RING finger domains are sufficient for the Pex10p-Pex12p interaction. Our results suggest that Pex12p is a component of the peroxisomal translocation machinery for matrix proteins.

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Abbreviations. AD GAL4 activation domain. – CHO Chinese hamster ovary. – DB GAL4 DNA-binding domain. – ER Endoplasmic reticulum. – FOX Fatty acid oxidation. – HA Influenza virus hemagglutinin epitope tag. – onu Oleic acid non-utilizing. – ORF Open reading frame. – PAGE Polyacrylamide gel electrophoresis. – PEX Peroxisomal assembly. – PrA Protein A from *S. aureus*. – PTS Peroxisomal targeting signal.

Introduction

Peroxisomal protein targeting can be separated into two different processes; protein translocation across and insertion into the peroxisomal membrane. Genetic evidence for independent mechanisms in protein topology of membrane and matrix proteins was first observed in mutants lacking components of the translocation machinery for peroxisomal matrix proteins (for review see (Kunau and Erdmann, 1998; Subramani, 1998)). These mutants are blocked in matrix protein import but still insert membrane proteins into residual peroxisomal membranes ("ghosts"). Two distinct pathways for protein translocation into the peroxisomal lumen have already been identified in yeast and in higher eukaryotes (for review see (Subramani, 1998; Tabak et al., 1999)). Distinguishing features of these pathways are specific signal sequences (termed peroxisomal targeting signals (PTSs)) and their cognate signal sequence receptors.

The PTS1 represents the major targeting signal for polypeptides on their way to the peroxisome. It is defined by the carboxy-terminal tripeptide Ser-Lys-Leu (SKL) and its species-specific variations (Gould et al., 1987) (for review see (McNew and Goodman, 1996)). A much smaller subset of peroxisomal matrix proteins is targeted to the peroxisome by an NH_2-terminal nonapeptide (RLX5(H/QL) termed PTS2 (Osumi et al., 1991; Swinkels et al., 1991) (for review see (De Hoop and Ab, 1992; Subramani, 1993)).

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Peroxisomal matrix protein import is thought to occur in a posttranslational manner (Lazarow and Fujiki, 1985). Consequently, signal recognition takes place in the cytosol, performed by soluble PTS-specific receptors. The receptor for the PTS1, Pex5p, is a member of the tetratricopeptide repeat family whereas the PTS2 is recognized by the WD40 protein Pex7p (for review see (Erdmann et al., 1997; Subramani, 1996)). In parallel with their distinct binding capacities, cells deficient in one of the two PTS receptors exhibit a selective import defect for either PTS1 or PTS2 populations of peroxisomal matrix proteins (for review see (Elgersma and Tabak, 1996)). There are conflicting findings regarding the localization of these receptors in the cell. Both receptors have been found in the cytosol, associated with the peroxisomal membrane or in the peroxisomal matrix (for review see (Rachubinski and Subramani, 1995)). A possibility to reconcile this controversy is proposed by the shuttle (cycling receptor) model of both receptors (Dodt and Gould, 1996; Marzioch et al., 1994)

Two potential docking sides for Pex5p and Pex7p at the peroxisomal membrane have been defined by means of protein-protein interaction. First, Pex13p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Girzalsky et al., 1999; Gould et al., 1996; Shimozawa et al., 1999) was characterized as an integral membrane protein that specifically binds the PTS1 receptor through a cytosolic exposed SH3 domain. New reports suggest that Pex13p is also involved in the import of the PTS2 receptor, Pex7p (Girzalsky et al., 1999). Second, Pex14p (Albertini et al., 1997; Brocard et al., 1997; Fransen et al., 1998; Schliebs et al., 1999; Will et al., 1999) is located at the outer peroxisomal periphery of the peroxisome and physically interacts with both receptors. Therefore, docking of the import complex at the peroxisomal membrane appears to be the point of convergence of the two import pathways (Albertini et al., 1997). A third component of the peroxisomal import machinery is Pex17p (Huhse et al., 1998; Snyder et al., 1999). Like Pex14p, Pex17p is located at the outer surface of the peroxisome and binds to Pex14p (Huhse et al., 1998). Recent data using human fibroblasts also indicate the involvement of two RING finger proteins, Pex10p and Pex12p, in protein translocation (Chang et al., 1999). Even though several components that are likely to be relevant for the protein translocation process have been identified, little is understood about the actual translocation process.

Materials and methods

Strains and growth conditions

Except for PCY2 (*MATα, gal4Δ, gal80Δ, URA3::GAL1-lacZ, lys2–801^{amber}, his3-Δ200, trp1-Δ63, leu2 ade2–101^{ochre}* (Chevray and Nathans, 1992)) all yeast strains used in this study were *S. cerevisiae* wild-type *UTL-7A* (*MATα, ura3–52, trp1, leu2–3,112* (W. Duntze, Bochum)) and its derivates *pex5Δ(MATα, ura3–52, trp1, pex5::kanMX4* (Girzalsky et al., 1999)), and *pex12Δ* (*MATα, ura3–52, trp1, pex12:: LEU2* (this study)).

Complete and minimal media used for yeast culturing have been described previously (Erdmann et al., 1989). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. Manipulation of yeast cells was performed according to standard methods (Rose et al., 1990).

Isolation of the *pex12* mutant strain

The *pex12–1* strain was isolated after mutagenesis of wild-type *S. cerevisiae UTL-7A* cells using ethyl methanesulfonate (Sherman et al., 1979). The screening protocol included replica plating on YNO agar plates, fractionation of yeast cells, and electron microscopy, all performed as described (Erdmann et al., 1989). Genetic analysis was performed by standard yeast techniques for *S. cerevisiae* (Ausubel et al., 1992).

Cloning and DNA sequencing of the *PEX12* gene

The *PEX12* gene was isolated by functional complementation of the *pex12–1* mutation using a yeast genomic library of *S. cerevisiae* in *E. coli*-yeast shuttle vector YCp50 (Rose et al., 1987). Transformation of *S. cerevisiae* was carried out by a modified lithium acetate method (Gietz and Woods, 1994). LEU2^{+} transformants were replica-plated on YNO agar plates and screened for restoration of the ability to use oleic acid as the sole carbon source. Complementing plasmids were recovered as described (Ausubel et al., 1992).

The smallest complementing plasmid contained a 6.3-kb insert and was designated YCp/K1/*PEX12*. To further analyze the *PEX12* gene, defined restriction fragments were subcloned into the low copy *CEN4-URA3* plasmid, pRS316 (Sikorski and Hieter, 1989). The resulting plasmids were tested for complementation by transformation of the *pex12–1* mutant, selection for URA3^{+} and subsequent screening on YNO agar plates for oleic acid utilization. pRS/*PEX12* contained a 3.5-kb genomic EcoRI/HincII fragment which comprised the full complementing activity.

Sequence analysis was carried out according to the dideoxy chain termination method (Sanger et al., 1977). The deduced *PEX12* amino acid sequence was compared to other known protein sequences using the BLAST and FASTA programs of the Heidelberg UNIX Sequence Analysis Resource (HUSAR 4.0: Deutsches Krebsforschungszentrum, Heidelberg, Germany).

Gene replacement and protein A fusion

Strains in which the genomic copies of genes bear epitope tags were produced by transforming haploid yeast cells with the products of assembly PCR reactions. Transformants were selected for the appropriate marker and proper integration was assessed by PCR. The Pex12p-PrA in wild-type background and Pex12p-PrA/*pex5*Δ were generated as described by (Aitchison et al., 1995). Primers KU571 (5′-ACATACAAC AAACACACGAATAAATGGGAAGTTGTGACAGGTATTAGG AAGCTACTAATCGGTG AAGCTCAAAAACTTATT-3′) and KU 572 (5′-TGCTTGTAACACAACTGCAAAGAGGTGGAACAAGT GACATGAATTACCTGCTGGATAACATTTACTTATAATACAG TTTTTTAG-3′) were used for PCR reactions.

To delete the *PEX12* gene in wild-type strain UTL-7A, a complementing 3.5-kb EcoRI/HincII fragment of pRS*PEX12* was subcloned into pBluescript vector (Stratagene, USA) resulting in pSK/*PEX12*/SE. The *S. cerevisiae LEU2* gene of plasmid pJJ252 (Jones and Prakash, 1990) was isolated by digestion with PstI and SmaI and used to substitute the internal 935-bp PstI/StuI fragment of the *PEX12* open reading frame leading to pSK/*PEX12*/DEL. A SacI/HindIII deletion cassette containing the *LEU2* gene and 5′ and 3′ flanking regions of *PEX12* was subsequently introduced into wild-type strain UTL-7A. Resultant leucin-prototrophic transformants were mated with wild-type JKR101, the diploid was induced to sporulate, and the meiotic progeny were examined by standard tetrad analysis. Crossing of the original mutant with the resultant leucine-prototrophic transformant led to diploid cells that were unable to grow on YNO agar. In addition, integration was confirmed by Southern blot analysis (Sambrook et al., 1989).

Plasmid constructions

The pYPGE15-derived plasmid pYPGE15/*PEX12*, for expressing *PEX12* under control of the strong constitutive phosphoglycerate kinase promotor (Brunelli and Pall, 1993), was obtained as follows: The *PEX12* open reading frame was amplified by PCR using oligonucleotides KU 11 (5′-GGAATTCTCCCGGGAATGAGCTTTTATTCAA

AC-3′) and KU 13 (5′-GGACTAGTGGATCCTCATCAGATTAGTA GCTTC-3′) as well as pRS*PEX12* as template. The 1.2-kb PCR product was subcloned into EcoRI/BamHI-digested pATH1 (Körner et al., 1991) and subsequently cloned into EcoRI/SalI-digested pYPEG15 (Brunelli and Pall, 1993) resulting in the plasmid pYPGE15/*PEX12*. For expression of truncated *PEX12*, a 1-kb EcoRI/StuI *PEX12* fragment corresponding to amino acids 1 to 332 was subcloned into EcoRI/StuI-digested pBluescript vector (Stratagene, USA) and finally cloned into EcoRI/XhoI-digested pYADE4 (Brunelli and Pall, 1993) resulting in the plasmid pYADE4/*PEX12*/DEL1.

Fractionation of yeast lysates and purification of peroxisomes

Organelle preparation by differential centrifugation of yeast lysates was performed as described (Erdmann et al., 1989). For separation of cell organelles by density gradient centrifugation, cell lysates of wild-type and mutant strains were loaded onto continuous 20 – 53% (w/w) sucrose density gradients (24 ml). Centrifugation, fractionation of the gradient and preparation of samples for SDS-PAGE were carried out as described (Höhfeld et al., 1991). Organellar pellets of oleate-induced wild-type and mutant strains were prepared according to (Erdmann et al., 1989).

Antibodies, immunoblots and co-immunoprecipitation

Anti-thiolase (Fox3p), anti-Pcs60p, anti-Pex3p, anti-Pex5p, anti-Pex14p, anti-Pex13p, and anti-Pex17p antibodies have been described previously (Albertini et al., 1997; Blobel and Erdmann, 1996; Erdmann and Kunau, 1994; Girzalsky et al., 1999; Höhfeld et al., 1991). Rabbit polyclonal antibodies against the N-terminal amino acids 10 – 125 of Pex12p were raised against anthranilate synthase (trpE)-Pex12p/10 – 125 (D94345) and glutathione-S-transferase (GST)-Pex12p/10 – 125 (D94346) fusion proteins using pATH (Körner et al., 1991) and pGEX (Pharmacia Biotech, Piscataway, NJ) *E. coli* expression systems. Rabbit polyclonal antibodies against C-terminal amino acids 279 – 337 of Pex10p were raised against glutathione-S-transferase (GST)-Pex10p fusion proteins. The rabbit polyclonal antibodies against the Pex12p C-terminus were raised against a synthetic peptide (YKRVNDLDEDPRP PF) corresponding to amino acids 302 – 317 of Pex12p. All antibodies were produced by Eurogentec (Seraing, Belgium). Electrophoresis and electroblotting onto nitrocellulose was carried out according to standard protocols (Harlow and Lane, 1988). Anti-rabbit IgG-coupled HRP (Amersham Corp., Illinois) was used as the secondary antibody and blots were developed using the ECL system (Amersham Corp., Illinois). Immunoprecipitation of Pex12p-PrA was performed as described (Götte et al., 1998) with the exception of using 50 mM HEPES, pH 7.4, instead of Tris buffer.

Two-hybrid analysis

The two-hybrid assay was based on the method of Fields and Song (1989). The tested genes were fused to the DNA-binding domain or trans-activating domain of *GAL4* in the vectors pPC86 and pPC97 (Chevray and Nathans, 1992). To generate a *PEX10* construct in pPC86, a *PEX10* fragment was amplified by PCR using primer set KU672 (5′-CCTATAGTCGACCATGGCCAATTTTAGAGAAGAGGG-3′) and KU673 (5′-CCTATAGAGCTCGCGGCCGCTATTGCCGCAGGAC CAG-3′) as well as genomic DNA as a template. The PCR fragment was cloned into SalI/NotI-digested pPC97. This construct (pBA17) encoded a Gal4-Pex10p fusion protein consisting of aa 238 – 337 of Pex10p. A *PEX12* fragment encoding aa 293 – 399 was amplified by PCR with primer set KU 13 (see above) and KU 238 (5′-CGAATTCGCCCGGG GATGACCACACAAGACATGACT-3′) and cloned into SmaI/Bam-HI-digested pPC86 resulting in the plasmid pUP12/1.

Co-transformation of two-hybrid vectors into strain PCY2 was performed according to Gietz and Woods (1994). Transformed yeast cells were plated onto SD synthetic medium without tryptophane and leucine. β-Galactosidase filter assays were performed according to Rehling et al. (1996).

Immunofluorescence and electron microscopy

Immunofluorescence microscopy was performed essentially according to Rout and Kilmartin (1990) with modifications described by Erdmann (1994). CY3-conjugated donkey anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as 6 μg/ml solutions for detection. Potassium permanganate fixation and preparation of intact yeast cells for electron microscopy were performed as described (Erdmann et al., 1989).

Membrane preparation and protease protection assay

Membrane preparation from an organelle pellet enriched for peroxisomes and mitochondria has been described before (Crane et al., 1994). For protease protection assays, an organelle pellet was resuspended in homogenization buffer (Erdmann et al., 1989) lacking protease inhibitors. Equal amounts were incubated for 30 min on ice with increasing amounts of trypsin. After the proteinase was inhibited by the addition of trypsin inhibitor, samples were immediately precipitated with TCA and subsequently processed for SDS-PAGE.

Miscellaneous methods

Acetyl-CoA acyltransferase (3-oxoacyl-CoA thiolase; EC 2.3.1.16), catalase (EC 1.11.1.6), and fumarate hydratase (fumarase; EC 4.2.1.2) were assayed by established procedures (Moreno de la Garza et al., 1985; Veenhuis et al., 1987).

Results

Isolation, characterization and deletion of the *PEX12* gene

We identified the *pex12 – 1* mutant strain by its inability to grow on agar plates containing oleic acid as sole carbon source. The meiotic segregation behavior revealed the defect to be caused by a single gene. The diploids resulting from backcrossing the mutant strain with wild-type cells did not show the mutant phenotype, confirming the *pex12 – 1* mutant to be recessive. The *PEX12* gene was isolated from a library of *S. cerevisiae* genomic DNA by functional complementation of the *pex12 – 1* strain. DNA sequencing of the smallest complementing fragment revealed an open reading frame (ORF) of 1200 nucleotides, encoding a protein of a calculated molecular mass of 45.8 kDa. This gene was later also identified in the genome sequencing project of *S. cerevisiae* (YMRO26c). Hydrophobicity plots according to Kyte and Doolittle (1982) revealed several hydrophobic regions (data not shown). Two of them were predicted to fulfill the criteria for transmembrane segments (amino acids 191 to 207, 270 to 286) (Klein et al., 1985). Another striking feature of the deduced amino acid sequence of Pex12p is a cysteine-rich region contained within the COOH-terminal amino acids 334 to 379; this region shares a certain similarity to the C3HC4 zinc finger motif. This protein module is thought to mediate protein-protein interactions (for review see (Borden, 2000; Klug, 1999)). A search of protein data bases revealed a significant overall amino acid sequence identity between our protein and other proteins (Fig. 1) from *Pichia pastoris* (*Pp*Pex12p; (Kalish et al., 1996)) human (*Hs*Pex12p, (Chang et al., 1997)) and *Rattus norvegicus* (*Rn*Pex12p, (Okumoto and Fujiki, 1997)). Therefore, the newly identified gene was designated *Sc*Pex12p.

A *PEX12* deletion strain (*pex12*Δ) was generated by replacing the majority of the *PEX12* gene with the *LEU2* gene. Functional complementation studies of backcrosses of the

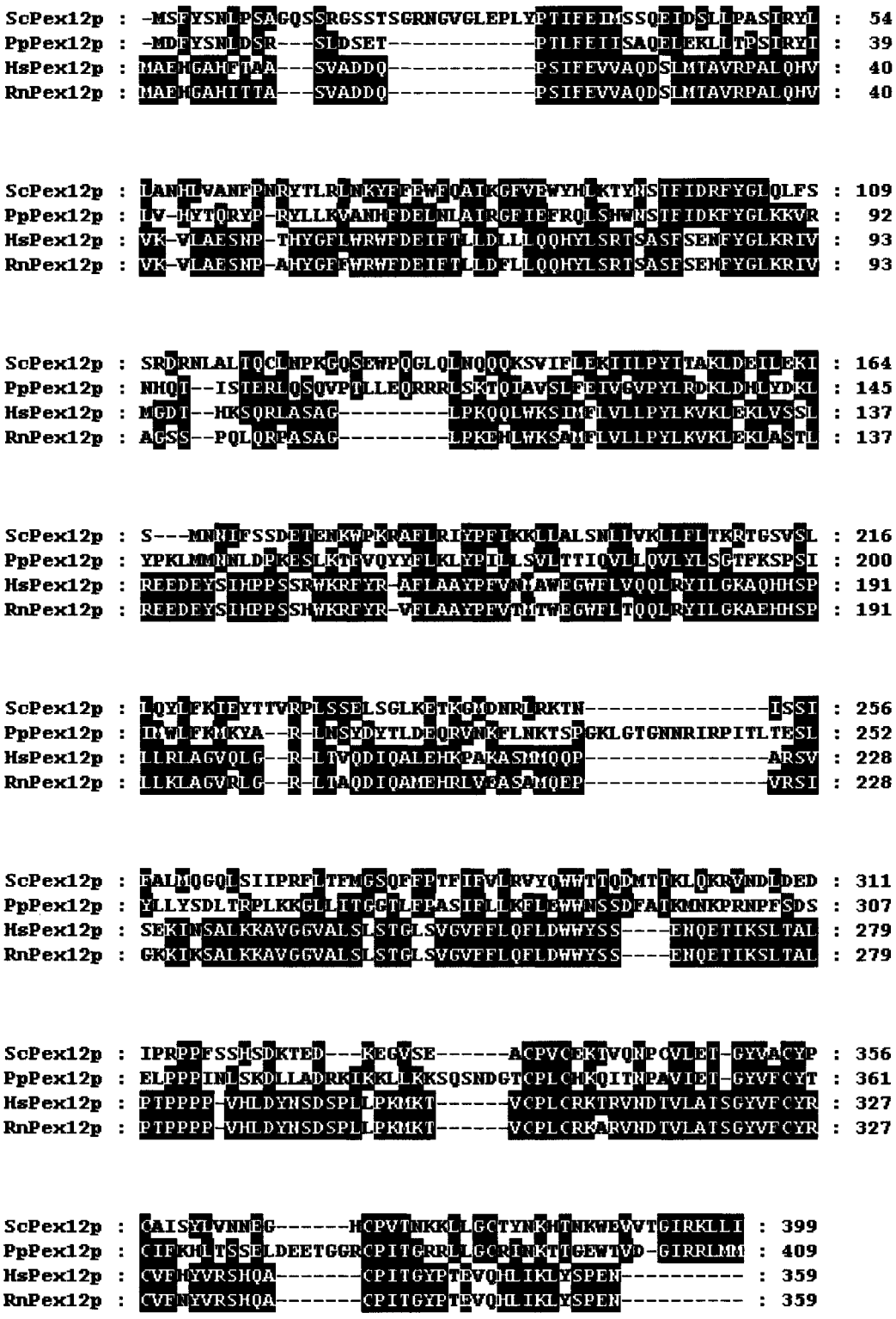

Fig. 1. Sequence comparison of Pex12p from *Saccharomyces cerevisiae* (*Sc*Pex12p), *Pichia pastoris* (*Pp*Pex12p; Kalish et al., 1995) *Homo sapiens* (*Hs*Pex12p, Chang et al., 1997) and *Rattus norvegicus* (*Rn*Pex12p, Okumoto and Fujiki, 1997). Conserved amino acids that are present in at least two of the four proteins are indicated. Identity of *Sc*Pex12p to *Pp*Pex12p, *Hs*Pex12p and *Rn*Pex12p is 32%, 23% and 22%, respectively.

*pex12*Δ strain with the original *pex12–1* mutant indicated that both mutants are allelic (data not shown).

*pex12*Δ cells are deficient in peroxisomal matrix protein import

Cells deficient in *PEX12* were viable on YPD, SD, and ethanol media, but were unable to grow on media with oleic acid as the single carbon source (Fig. 2A), typical for *S. cerevisiae* mutant strains that are deficient for either peroxisome metabolism or biogenesis (Erdmann and Kunau, 1992; Erdmann et al., 1989). The impaired growth defect was restored upon transformation with the *PEX12* gene (Fig. 2A).

To establish the basis of the *pex12Δ* defect, we investigated the intracellular localization of peroxisomal proteins in wild-type and mutant cells. By immunofluorescence microscopy analysis we showed that *pex12*Δ cells fail to import peroxisomal matrix proteins (Fig. 2B). Wild-type cells exhibited the peroxisome-characteristic punctate staining for both Pcs60p (PTS1-containing peroxisomal matrix protein) and thiolase (Fox3p, PTS-2 containing peroxisomal matrix protein). In contrast, both of these peroxisomal matrix proteins gave a cytosolic fluorescence pattern in *pex12*Δ cells (Fig. 2B). These data suggest that *pex12*Δ cells are unable to import peroxisomal matrix proteins of the PTS1 and PTS2 classes.

To quantify the import defect, we next analyzed the subcellular distribution of the peroxisomal matrix proteins catalase (PTS1-containing peroxisomal matrix protein) and thiolase by cell fractionation analysis of wild-type, *pex12*Δ and complemented *pex12*Δ cells. The different strains were grown on oleic acid and subjected to subcellular fractionation to give a 25000*g* pellet enriched for peroxisomes and mitochondria and a 25000*g* supernatant enriched for cytosol. As expected, the majority of the peroxisomal matrix proteins catalase and thiolase as well as mitochondrial fumarase were detected in the 25000*g* pellet of wild-type cells (Fig. 2C). In contrast, in *pex12*Δ cells all peroxisomal matrix proteins were preferentially localized to the 25000*g* supernatant consistent with their mislocalization to the cytosol. This result is in agreement with the observed mislocalization of thiolase and Pcs60p in *pex12Δ* cells reported above. Only partial restoration of the wild-type phenotype was observed in *pex12Δ* cells expressing Pex12p from a plasmid. Taken together immunofluorescence microscopy and biochemical data clearly show an import defect for peroxisomal matrix proteins of the PTS1 and PTS2 classes. We therefore conclude that Pex12p is involved in peroxisome biogenesis and not in peroxisomal metabolism.

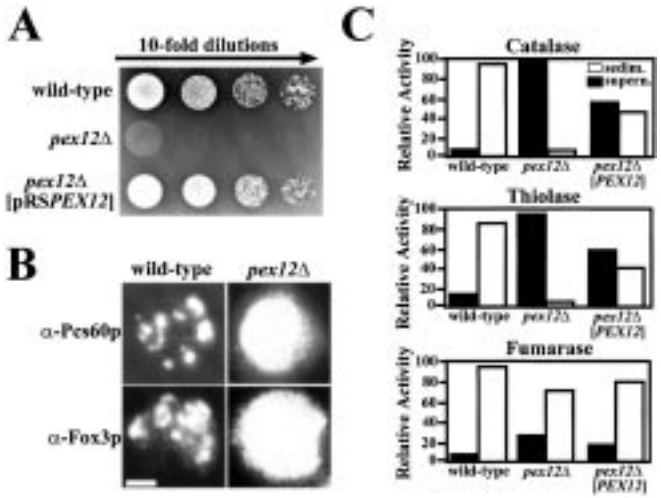

Fig. 2. *pex12*Δ cells are defective in peroxisomal matrix protein import. (**A**) Growth of wild-type cells, *pex12*Δ cells, and *pex12Δ* cells complemented with low-copy plasmid pRS/*PEX12* on oleic acid medium (YNO). Equal amounts of cells were diluted in distilled H_2O, and aliquots were applied onto oleic acid plates (spots on the left correspond to 2×10^4 cells). In contrast to the wild-type strain UTL-7A and *pex12Δ* complemented with plasmid pRS*PEX12*, the *pex12Δ* null mutant was unable to grow on YNO. (**B**) Indirect immunofluorescence microscopy localization of PTS1-containing Pcs60p and PTS2-containing thiolase (Fox3p) in wild-type and *pex12*Δ cells. Oleic acid-induced wild-type and *pex12*Δ cells were processed for indirect immunofluorescence microscopy using rabbit polyclonal antibodies specific for Pcs60p or Fox3p. Secondary antibodies were CY3-conjugated anti-rabbit IgG. One representative cell is shown for each sample. While wild-type cells exhibit a peroxisome-characteristic punctate pattern when stained for the PTS1 protein Pcs60p or the PTS2 protein thiolase, a diffuse staining pattern for both of these peroxisomal matrix proteins was observed in *pex12Δ* cells. Bar, 5 μm. (**C**) Subcellular distribution of peroxisomal and mitochondrial marker enzymes in oleic acid-induced wild-type, *pex12*Δ and complemented *pex12*Δ cells. After centrifugation of cell-free homogenates at 25000*g*, sediments and supernatants were assayed for peroxisomal catalase and thiolase as well as for mitochondrial fumarase activities. In *pex12Δ* cells peroxisomal catalase and thiolase were found exclusively in the soluble fraction, consistent with a general import defect for peroxisomal matrix proteins in *pex12Δ*.

*pex12*Δ cells exhibit peroxisomal remnants

In electron micrographs of *S. cerevisiae* cells, normal peroxisomes appear as round vesicular structures, with a granular electron-dense core and a single unit membrane (Fig. 3A).

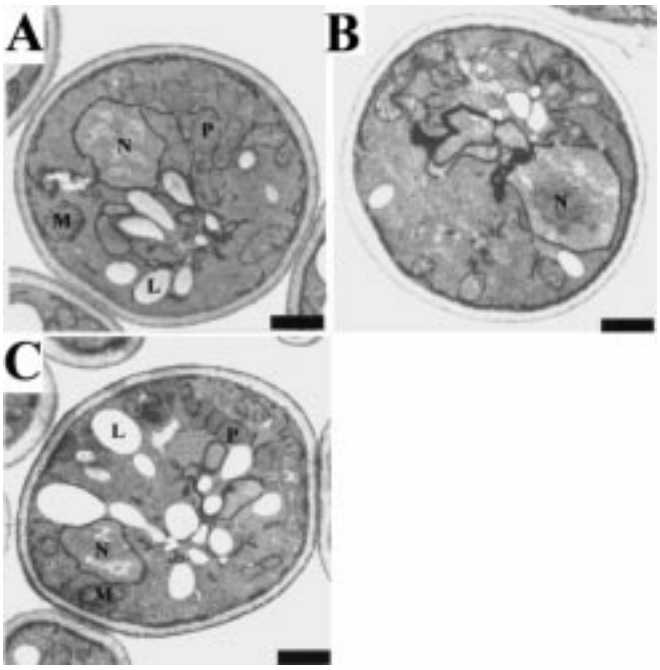

Fig. 3. *pex12Δ* cells lack morphologically detectable peroxisomes. Electron micrographs of oleic acid-induced cells of (**A**) wild-type, (**B**) *pex12Δ* and (**C**) *pex12Δ* complemented with low copy plasmid pRS/*PEX12*. In contrast to wild-type cells and complemented *pex12Δ* cells the *pex12Δ* null mutant was characterized by the absence of morphologically detectable peroxisomes. L, lipid droplet; M, mitochondrion; N, nucleus; P, peroxisome. Bar, 2 μm.

Conversely, *pex12*Δ cells were characterized by the absence of morphologically detectable peroxisomes (Fig. 3B). As expected, peroxisome biogenesis was restored in *pex12Δ* cells upon expression of Pex12p from low copy plasmid pRS/*PEX12* (Fig. 3C). Interestingly, in contrast to the wild-type strain we observed clusters of peroxisomes rather than single organelles in the complemented *pex12Δ* strain. We expect this phenotype to result from slightly different expression profiles for the genomic and the plasmid-coded alleles of *PEX12*, suggesting a tight regulation of intracellular Pex12p dosage. Clusters of peroxisomes might well be more labile to cell fractionation than single organelles.

To determine whether Pex12p effects the topogenesis of peroxisomal proteins in general, we investigated the localization of the peroxisomal membrane proteins Pex11p (Erdmann and Blobel, 1995) and Pex14p (Albertini et al., 1997; Brocard et al., 1997) in *pex12Δ* cells. Double-label immunofluorescence microscopy localization of Pex11p and Pex14p revealed a peroxisome-characteristic punctate pattern for both of these membrane proteins in *pex12Δ* cells (Fig. 4A). These results suggest a membrane-bound localization of Pex11p and Pex14p and thus the existence of peroxisomal membrane structures that are import-incompetent for peroxisomal matrix proteins in *pex12Δ*.

This result was further substantiated by cell fractionation of *pex12*Δ using sucrose density gradient centrifugation (Fig. 4B). Fumarase peaked at a sucrose density of 1.18 g/cm^3, characteristic for mitochondria, whereas peroxisomal catalase was found, in agreement with the *pex12Δ* import defect, at the top of the gradient. The gradient fractions were further analyzed by Western blot using polyclonal antibodies against the integral peroxisomal membrane protein Pex3p (Höhfeld et al., 1991) and the peripheral membrane protein Pex14p. Importantly, both membrane proteins migrated at a density of 1.14 g/cm^3 (Fig. 4B) suggesting that they are particulate rather than cytosolic. Since peroxisomal matrix proteins are mislocalized to the cytoplasmic fraction, our results indicate that Pex12p is involved in the topogenesis of peroxisomal matrix proteins but not in the topogenesis of peroxisomal membrane proteins.

Immunological detection of Pex12p

To detect Pex12p in *S. cerevisiae*, polyclonal antibodies were raised against NH_2-terminal amino acids 10 to 125 and COOH-terminal amino acids 303 to 317 (see Materials and methods). Two polypeptides with apparent molecular masses of 42 and 46 kDa were specifically detected in whole cell extracts derived from *pex12*Δ transformants expressing Pex12p from either the *CEN* plasmid pRS/*PEX12* or the *2μ* plasmid pYPGE/*PEX12*, but neither was detected in wild-type cells (data not shown). We therefore assume that Pex12p is a peroxin expressed at low level.

The 46-kDa band corresponds well to the predicted molecular mass of Pex12p. The appearance of a second Pex12p–specific polypeptide with an apparent molecular mass of 42 kDa was expected to result from either post-translational Pex12p modification or perhaps from insufficient denaturing of the protein prior to SDS-PAGE. In the latter case strong intramolecular disulfide bounds between the seven RING finger-like cysteines characteristic of Pex12p would result in a slightly faster migration of the protein on SDS gels. Indeed after a strong reduction of Pex12p followed by an alkylation reaction by treatment with iodoacetamide most of the 42-kDa Pex12p band was shifted to the expected molecular mass of 46 kDa (Fig. 5A).

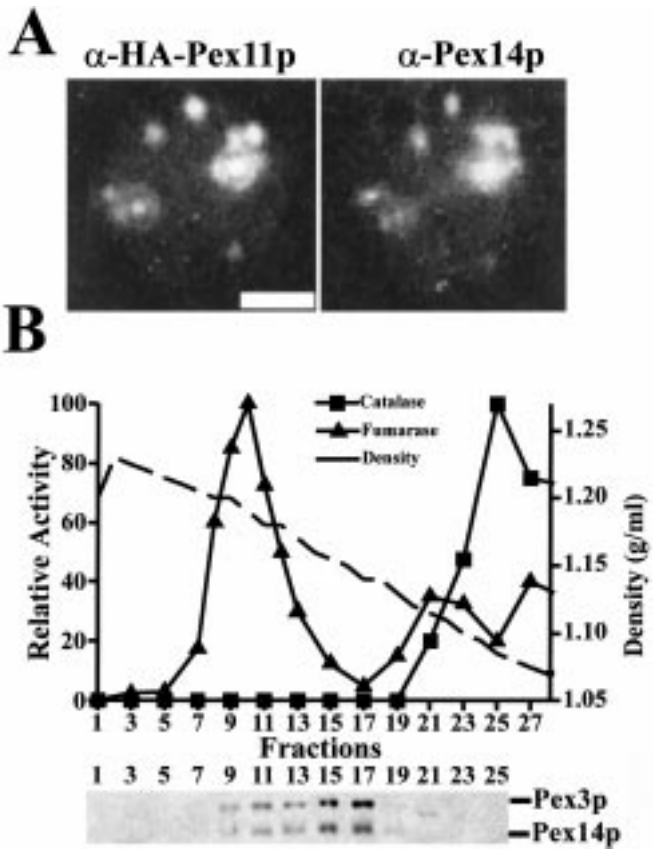

Fig. 4. *pex12*Δ mutant cells contain peroxisomal membrane ghosts. (**A**) Oleic acid-induced *pex12*Δ mutant cells expressing HA-tagged Pex11p were processed for double immunofluorescence microscopy using rabbit polyclonal antibodies specific for peroxisomal Pex14p and mouse monoclonal antibodies specific for the HA epitope. Secondary antibodies were CY3-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG. Both peroxisomal membrane proteins gave rise to a punctate fluorescence pattern indicative of vesicle-like structures in *pex12Δ*. Bar, 5 μm. (**B**) Western blot analysis of the peroxisomal membrane proteins Pex3p and Pex14p in fractions obtained by isopycnic 20% – 54% sucrose density gradient centrifugation of cell-free homogenates from oleic acid-induced *pex12Δ* cells. Peroxisomal marker enzyme catalase as well as mitochondrial fumarase were monitored by activity measurements. Equal volumes of each fraction were analyzed for the presence of Pex3p and Pex14p using rabbit polyclonal antibodies specific for Pex3p or Pex14p. The peroxisomal matrix protein catalase was detected on top of the gradient (fractions 20 – 27), suggesting its cytoplasmic localization. In contrast, the peroxisomal membrane proteins Pex3p and Pex14p did not co-localize with peroxisomal catalase but were localized to gradient fractions 9 – 17, indicating an organelle-associated localization of Pex3p and Pex14p.

Induction of Pex12p by oleic acid

In *S. cerevisae,* growth on oleic acid-containing medium results in a robust proliferation of peroxisomes accompanied by the induction of peroxisomal β-oxidation (Veenhuis et al., 1987). To analyze induction of endogenous Pex12p by oleic acid we made use of a yeast strain in which the *PEX12* gene was replaced by Pex12p-PrA which codes for a chimeric Pex12 protein that is COOH-terminally fused to IgG-binding domains of *Staphylococcus aureus* protein A. The resulting strain enabled us to detect endogenous Pex12p via the highly sensitive PrA-rabbit IgG interaction.

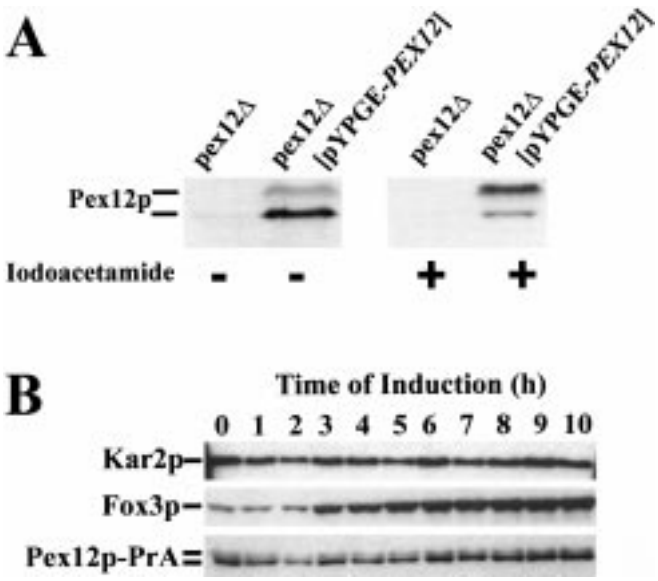

Fig. 5. (**A**) Treatment of Pex12p with iodoacetamide. Whole cell lysates of oleic acid-induced *pex12*Δ mutant cells and *pex12*Δ mutant expressing Pex12p from plasmid pYPGE15/*PEX12* were treated with or without 20 mg/ml iodoacetamide and prepared for immunological detection of Pex12p. The amount loaded per lane corresponds to 0.3 mg cells. (**B**) Time course of Pex12-PrA induction during growth on oleic acid. Wild-type cells expressing Pex12-PrA were precultured in 0.3% SD and subsequently shifted to YNO. At the indicated time points, whole cell extracts were prepared for immunological detection of oleic acid-induced peroxisomal thiolase (Fox3p), constitutively expressed Kar2p, and Pex12-PrA. In contrast to the constitutively expressed Kar2p, Pex12p is slightly induced over the induction period.

A time course of induction of this Pex12p variant and the β-oxidation enzyme thiolase by oleic acid is shown in Fig. 5B. At various time points, cell homogenates were prepared for Western blot analysis and probed for the constitutively expressed Kar2p (Rose et al., 1989), the oleic acid-induced Fox3p and Pex12p-PrA. Upon induction, only a weak increase of Pex12p-PrA expression was detectable whereas expression of Fox3p increased from very low to clearly detectable levels (Fig. 5B).

Pex12p is an integral membrane protein

The subcellular localization of Pex12p was analyzed by conventional cell fractionation methods. To facilitate the detection of Pex12p by immunological techniques all localization experiments were performed with *pex12Δ* transformants carrying the low-copy plasmid pRS/*PEX12*. In sucrose density gradients of a homogenate derived from oleic acid-induced cells, Pex12p was found to co-migrate exclusively with the peroxisomal marker enzyme catalase and a marker protein for peroxisomal membranes, Pex3p, at a density of 1.22 g/cm^3, typical of peroxisome migration. Therefore we conclude that Pex12p is a peroxisomal protein (Fig. 6A).

The primary sequence of Pex12p strongly suggests that Pex12p is an integral membrane protein. To determine the subperoxisomal localization of Pex12p, a 25000*g* organellar sediment isolated from spheroplasts of oleic acid-induced transformants was subjected to successive extraction by 10 mM Tris-HCl, pH 8.0, and 100 mM · Na_2CO_3, pH 11.0. Pex12p was resistent to both low- and high-salt extraction (Fig. 6B). A comparable behavior was observed for the integral peroxisomal membrane protein Pex3p (Höhfeld et al., 1991) while the peroxisomal matrix protein thiolase (Fox3p) remained soluble. These data indicate that Pex12p is an integral component of the peroxisomal membrane. The intracellular localization of Pex12p was further confirmed by immuno-electron microscopy, using anti-Pex12p antibodies. *pex12*Δ cells complemented with either the *CEN* plasmid pRS/*PEX12* (Fig. 6C) or the *2μ* plasmid pYPGE/*PEX12* (Fig. 6D) were grown for 12 h on oleic acid medium and subsequently processed for immuno-electron microscopic analysis with a polyclonal antiserum against the Pex12p NH_2-terminus. Figure 6C shows an exclusive immunogold labeling of the peroxisomal periphery, consistent with Pex12p being a peroxisomal membrane protein. Note again that peroxisomes were found in clusters rather than as single organelles when Pex12p was expressed from the *CEN* plasmid pRS316. Furthermore, overexpression of Pex12p from the *2μ* plasmid pYGE15 resulted in significant membrane proliferation accompanied by a reduction in size of the peroxisomal lumen (Fig. 6D). Compared to wild-type cells, the peroxisomes shown in Figure 6D seemed to be surrounded by multiple-layers of membranous material that was specifically labeled with anti-Pex12p antibodies.

The Pex12p RING finger extends into the cytosol

To determine the orientation of Pex12p in the peroxisomal membrane, isolated organelles from *pex12*Δ cells expressing Pex12p from plasmid pRS/*PEX12* were incubated with varying amounts of trypsin in the presence or absence of detergent. Following protease incubation, samples were prepared for SDS-PAGE and Western blot analysis. Samples were tested for thiolase and Pex12p protection using specific anti-rabbit antibodies against the Pex12p amino- and carboxy-terminus and full-length thiolase (Fig. 7). Thiolase (Fox3p), an intraperoxisomal protein, was efficiently protected against protease digestion in the absence of detergent, but was degraded completely when detergent was present. Using a specific antibody against the NH_2-terminal amino acids 10–125 of Pex12p we could show that without detergent added (Fig. 7), a 31-kDa fragment of Pex12p is protected against exogenous protease. The protected 31-kDa polypeptide corresponds well to the calculated molecular weight of amino acids 1–286 of Pex12p (molecular mass: 33 kDa), a stretch which includes both of the predicted transmembrane segments (aa 191–202, 270–286). In contrast, using the anti-Pex12p peptide antibody, specific for the COOH-terminal amino acids 302 to 317, neither full-length Pex12p nor a Pex12p degradation product could be detected (Fig. 7). This result suggests that a significant portion of Pex12p extends into the peroxisomal matrix. Our data also indicate that Pex12p is characterized by at least one transmembrane segment and that the Pex12p NH_2-terminus is located in the peroxisomal lumen, while its COOH-terminus extends into the cytoplasm.

Pex12p interacts with other integral membrane proteins and the PTS1 receptor Pex5p

Since our data indicate a role of Pex12p in transport of proteins across the peroxisomal membrane, we investigated whether Pex12p interacts with components of the peroxisomal protein translocation machinery. To co-immunoprecipitate potential binding partners of Pex12p, solubilized peroxisomal mem-

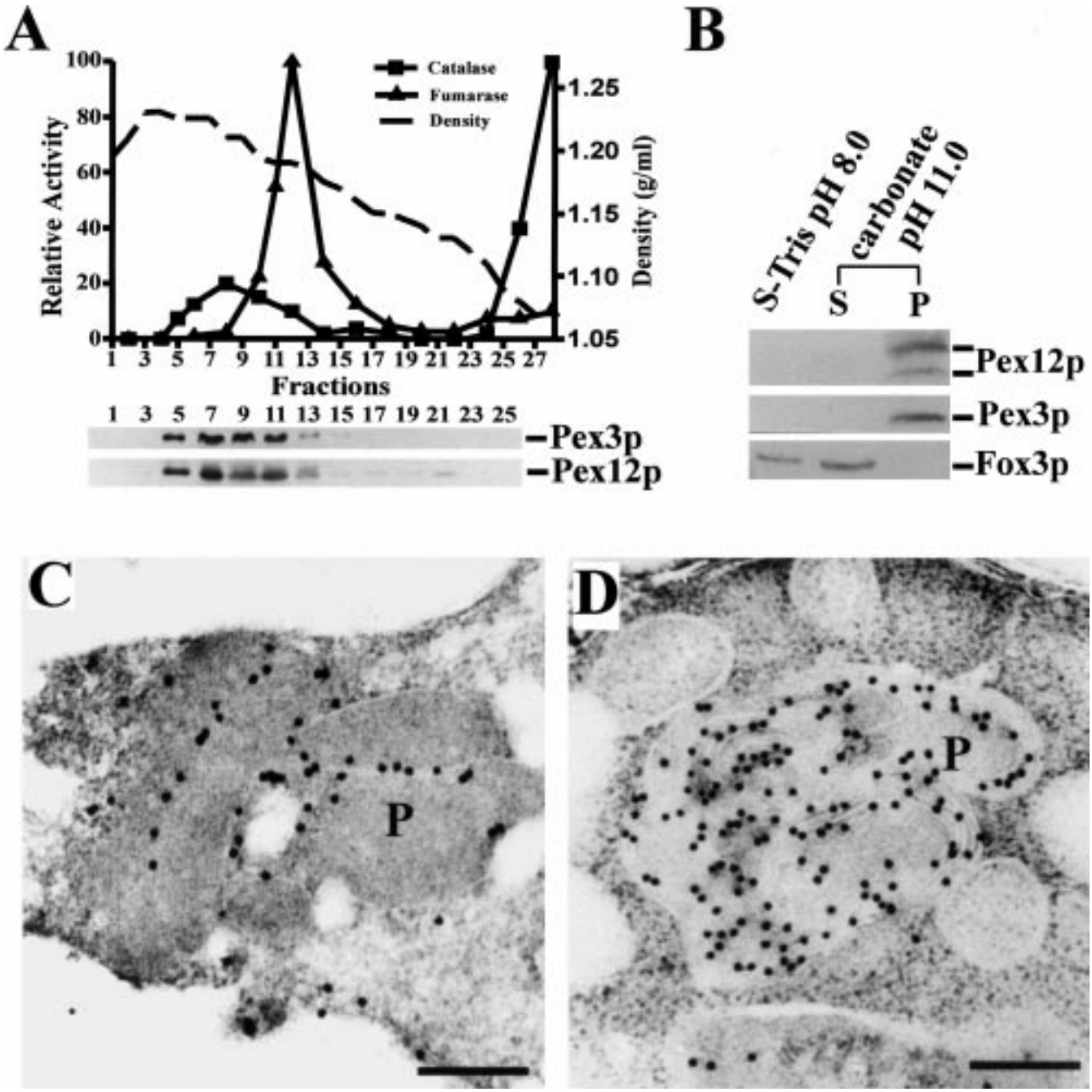

Fig. 6. Subcellular localization of Pex12p. (**A**) Pex12p is peroxisomal. Immunological detection of Pex12p and Pex3p in fractions obtained by isopycnic 20% – 54% sucrose density gradient centrifugation of cell-free homogenates from oleic acid-induced *pex12Δ* cells complemented with plasmid pRS/*PEX12*. The peroxisomal marker enzyme catalase as well as mitochondrial fumarase were monitored by activity measurements. Equal volumes of each fraction were immunologically analyzed for the presence of Pex12p and Pex3p using rabbit polyclonal antibodies against Pex12p NH2-terminus and Pex3p. The majority of Pex12p and Pex3p co-migrates with catalase at a density of 1.21 g/ml, which is characteristic of peroxisomes. (**B**) Pex12p is a peroxisomal integral membrane protein. An organelle-enriched fraction isolated from complemented *pex12*Δ mutant cells was successively extracted with 10 mM Tris-HCl, pH 8.0, and 100 mM · Na_2CO_3, pH 11. Equal amounts of the extracts (S) and membrane fraction (P) were separated by SDS-PAGE and analyzed by Western blot using antibodies against the Pex12p NH_2-terminus, thiolase (Fox3p) and Pex3p. Pex12p was resistant to low-salt and Na_2CO_3 treatment, characteristics which are typical of an integral membrane localization. (**C, D**) Immuno-electron microscopy localization of Pex12p in whole cells. Thin sections of Lowicryl-embedded complemented *pex12*Δ mutant cells (**C**) and *pex12*Δ cells overexpressing *PEX12* from plasmid pYPGE/*PEX12* (**D**). Cells were grown on oleic acid medium for 12 h and processed for immunocytochemical analysis with a polyclonal antiserum against the Pex12p NH_2-terminus. Immunogold labeling of the peroxisomal periphery is consistent with Pex12p being a peroxisomal membrane protein. Note that the overexpression of Pex12p from multi-copy plasmid pYPGE/*PEX12* effects membrane proliferation. P, peroxisome. Bar, 0.5 μm.

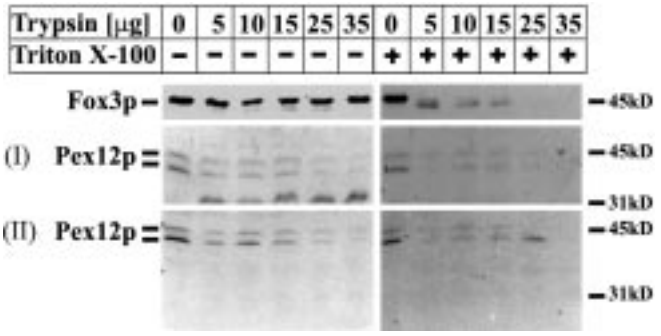

Fig. 7. The Pex12p NH_2-terminus is located in the peroxisomal matrix whereas the COOH-terminus faces the cytosol. Protease protection analysis of an organelle-enriched fraction isolated from spheroplasts of complemented *pex12*Δ mutant cells. Equal amounts of an organelle-enriched fraction were incubated for 30 min on ice with increasing amounts of trypsin in the presence or absence of detergent. Samples were analyzed by SDS-PAGE and Western blot using antibodies against the (I) NH_2- and (II) COOH-terminus of Pex12p and thiolase (Fox3p). The NH_2-terminus of Pex12p was protected against trypsin in the absence of detergent indicated by detection of a 31-kDa degradation product that was completely degraded upon addition of detergent. In contrast to the Pex12p NH_2-terminus the COOH-terminus was rapidly degraded even in the absence of detergent.

branes of the Pex12p-PrA strain were incubated with IgG coupled Dynabeads (Dynal, Hamburg, Germany), while membrane fractions of wild-type cells served as a control.

Judging from Western blot analysis, four peroxins, Pex5p (Brocard et al., 1994; van der Leij et al., 1993), Pex10p (Kunau et al., 1993), Pex13p (Elgersma et al., 1996; Erdmann and Blobel, 1996) and Pex14p (Albertini et al., 1997; Brocard et al., 1997) were co-immunoprecipitated together with Pex12p-PrA from the Pex12p-PrA strain but not from the wild-type control (Fig. 8A). Interestingly, with Pex10p a second RING finger protein was co-isolated in our preparation that also seems to be involved in matrix protein translocation (Chang et al., 1999; Okumoto et al., 2000).

To exclude the possibility that interaction of Pex12p and the membrane-bound peroxins Pex10p, Pex13p and Pex14p is indirect, due to e.g. Pex5p – mediated peroxisomal targeting of Pex12p, we repeated the co-immunoisolation experiment in a *PEX5* deletion strain. As shown in Fig. 8A, interaction of Pex12p with the peroxisomal membrane compounds Pex10p, Pex13p and Pex14p is independent of Pex5p. Whether Pex12p is directly associated with Pex13p and/or Pex14p at the peroxisomal membrane or if the interaction is Pex10p mediated (see below) remains unclear.

To exclude the possibility of nonspecific coprecipitation of proteins, we checked the precipitates for the presence of peroxisomal membrane protein Pex11p (Erdmann and Blobel, 1995; Marshall et al., 1995). This protein was not detected in any of the samples, indicating the specifity of the observed interactions (data not shown).

The Pex12p zinc RING finger motif: Pex10p interaction and functional relevance

Both the N-terminal region and the RING finger-containing COOH-terminal region of Pex10p are exposed to the cytosol, as assessed by an expression study of epitope-tagged human Pex10p (Okumoto et al., 2000). As the Pex12p RING finger also extends into the cytosol, we tested for in vivo protein interaction of the Pex10p and Pex12p COOH-termini using the two-hybrid system.

The COOH-terminal regions containing the RING finger of Pex12p and Pex10p were fused to the corresponding Gal4p domains on plasmids pPC86 and pPC97 and double transformants were analyzed for reporter gene expression by assaying β-galactosidase activity (Fig. 8B). Yeast cells co-expressing the Pex12p/GAL4-DB and the Pex10p/GAL4-AD RING finger produced significant amounts of β-galactosidase. The controls included in Figure 8B show that co-expression of either of the fusion proteins, together with respective Gal4p domains encoded by pPC86 and pPC97, did not support transcription activation of the reporter genes. This result demonstrates that the Pex12p carboxy-terminus is capable of binding the Pex10p carboxy-terminus in vivo (Fig. 8B). Therefore, it seems plausible that the two RING fingers are sufficient for physical Pex12p-Pex10p interaction. Recently, these data were independently confirmed by other laboratories for human and Chinese hamster Pex12p (Chang et al., 1999; Okumoto et al., 2000). It was also reported by (Chang et al., 1999) that the *hs*Pex12p RING finger motif is capable of binding the PTS1 receptor Pex5p in two-hybrid analysis (data not shown). However, when we performed an analogous experiment, we did not find a two-hybrid interaction between Pex5p and the COOH-terminal Pex12p RING finger fragment used in our experiments (data not shown).

To gain further insight into the function of Pex12p, we investigated the requirement of the RING finger motif for functional complementation of the *pex12Δ* onu$^-$ phenotype and peroxisomal targeting of Pex12p. A plasmid construct was generated to express a truncated version of Pex12p under the control of the alcohol dehydrogenase promotor (pYADE4/*PEX12*/DEL). The plasmid, deleted for amino acids 332 to 399 of Pex12p was introduced into wild-type and *pex12Δ* cells. Initial experiments showed $Pex12p_{aa1–332}$ to be insufficient for restoration of the wild-type phenotype in *pex12Δ*cells and therefore the RING finger to be essential for biological activity of Pex12p (data not shown).

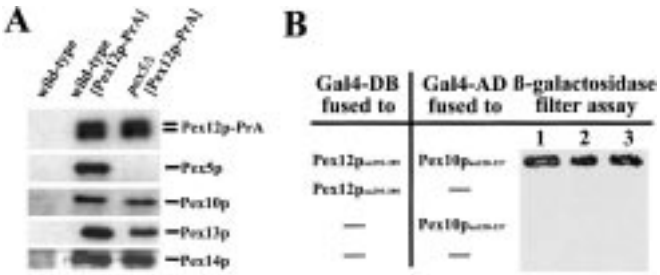

Fig. 8. Interaction of Pex12p with the PTS1-receptor Pex5p and the peroxisomal proteins Pex10p, Pex13p and Pex14p. (**A**) Co-immunoprecipitation of Pex12p-ProA with Pex5p, Pex10p, Pex13p and Pex14p. Pex12p-ProA was immunoprecipitated from sedimented membranes of wild-type (control), wild-type expressing tagged Pex12p and *pex5*Δ mutant cells expressing tagged Pex12p. Equal amounts of immunoprecipitates (corresponding to 10% of total) were separated by SDS-PAGE and subjected to immunoblot analysis with rabbit polyclonal antibodies against Pex5p, Pex10p, Pex13p and Pex14p. (**B**) The Pex12p zinc-binding domain interacts with the Pex10p RING finger. PCY2 double transformants expressing the indicated fusion protein combinations of Pex10p with Pex12p were analyzed for β-galactosidase activity by a filter assay using X-gal as a substrate. Three representative independent double-transformants are shown (1 – 3).

Although it has been demonstrated that membrane protein targeting and insertion is independent of the import pathway for matrix proteins in peroxisomes (Erdmann and Blobel, 1996; Gould et al., 1996), little is known about this process so far. Recently, it has been established that a subset of peroxisomal membrane proteins might be targeted to the peroxisome via the endoplasmic reticulum (ER) (Elgersma et al., 1997). One striking feature of these studies was the proliferation of ER membranes by overproduction of the peroxisomal membrane protein Pex15p, a phenotype we also see with Pex12p (data not shown). A consensus sequence (K/R-X-K/R-X-L-X_{9-10}-F/Y) involved in the targeting of this and other membrane proteins has been delimitated (Elgersma et al., 1997). We analyzed the Pex12p amino acid sequence but although we found two stretches of basic amino acids (aa 151–183, aa 222–250), no canonical sequence pattern could be identified. To investigate the function of the extreme COOH-terminus of Pex12p, the truncated Pex12$p_{aa1-332}$ lacking the RING finger was expressed in wild-type cells. Indicative of a peroxisomal localization we found Pex12$p_{aa1-332}$ co-migrating with the peroxisomal marker enzyme catalase in sucrose density gradients of cell-free homogenates from oleic acid-induced cells (Fig. 9). These data indicate that the RING region is not essential for targeting of Pex12p to peroxisomes.

Notably, we measured a severe dominant negative phenotype following expression of Pex12$p_{aa1-332}$ in wild-type cells. This observation is consistent with a predicted role of Pex12p in protein-protein interaction via its RING finger (Chang et al., 1999; Okumoto et al., 2000) and for Pex12p being a component of the peroxisomal import apparatus (this work). Therefore, integration of RING finger-deleted Pex12p into the import structure might disrupt the physical linkage between single components and/or functional subcomplexes.

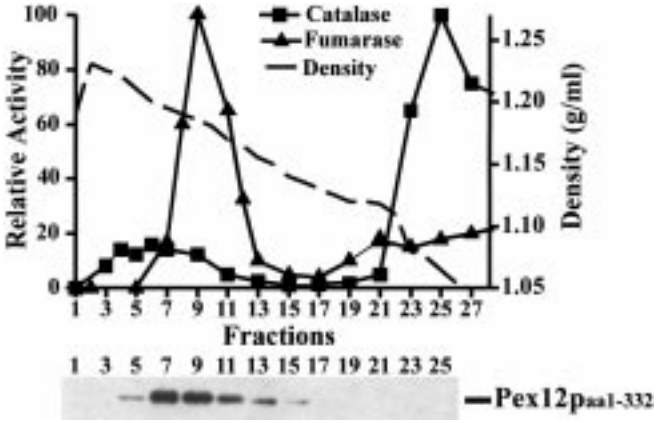

Fig. 9. The zinc-binding domain is not essential for the targeting of Pex12p to peroxisomes. A truncated version (aa1–332) of Pex12p, lacking the zinc-binding domain of Pex12p was expressed in wild-type cells from plasmid pYADE4/*PEX12/DEL* and immunologically detected in fractions obtained by isopycnic 20%–54% sucrose density gradient centrifugation of cell-free homogenates from oleic acid-induced transformants. The peroxisomal marker enzyme catalase as well as mitochondrial fumarase were monitored by activity measurements. Equal volumes of each fraction were immunologically analyzed for the presence of Pex12p_{1-332}. Pex12p_{1-332} co-localizes with the peroxisomal matrix protein catalase at a density of 1.21 g/ml, indicative of peroxisomes.

Discussion

Here we report the identification of the *PEX12* gene in the yeast *S. cerevisiae* and the molecular characterization of the *PEX12* gene product Pex12p as an essential component of the peroxisomal translocation machinery for matrix proteins. It has been reported before that Pex12p from other organisms interacts with the integral membrane protein Pex10p and the receptor for the peroxisomal targeting signal 1, Pex5p (Brocard et al., 1994; van der Leij et al., 1993). With respect to the Pex10p/Pex12p interaction our results confirm these findings and more importantly, reveal new aspects of Pex12p function by identification of two additional binding partners: the two docking proteins for the PTS1 and the PTS2 receptor, Pex13p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996) and Pex14p (Albertini et al., 1997; Fransen et al., 1998; Komori et al., 1997; Will et al., 1999).

We have isolated the *PEX12* gene by functional complementation of the original *pex12–1* mutant in *S. cerevisiae*. Typical of peroxisomal mutants, (Erdmann et al., 1989) the *pex12–1* mutant as well as the *pex12Δ* deletion mutant were unable to grow on oleic acid as a sole carbon source (Fig. 2A). Combined biochemical and immunofluorescence analyses further revealed that *pex12Δ* cells were affected in peroxisomal import of both the PTS1- and the PTS2-dependent peroxisomal matrix proteins (Figs. 2B and 2C). Consequently, lack of Pex12p results in the absence of normal, morphologically detectable peroxisomes (Fig. 3B).

Complementation of the *pex12Δ* mutant with the *CEN* plasmid pRS316 carrying the *PEX12* gene as well as its regulatory elements resulted in clustering of peroxisomes as judged by cell morphological studies and comparison to wild-type cells (Fig. 3C). As this phenotype cannot be explained by a difference in regulatory elements, we expect the plasmid-encoded version of the *PEX12* gene to be expressed at a slightly higher rate than its genomic copy. Comparable observations were reported previously (Elgersma et al., 1997). The finding that with specific antibodies against Pex12p, we were able to detect the protein in transformants but not in wild-type cells (data not shown) supports this assumption. Also, an even higher expression of *PEX12* from a *2μ* plasmid led to a more severe phenotype (Fig. 6D) characterized by strong membrane proliferation and clustering of peroxisomal membranes. Thus, we believe that Pex12p expression is low and that the protein is tightly regulated at the level of gene expression.

Observations from different laboratories indicate that peroxisomal matrix and membrane proteins are directed to their site of function through different pathways (Elgersma et al., 1996; Erdmann and Blobel, 1996). Evidently, mutant cells lacking components of the import machinery are blocked in matrix protein import yet still insert membrane proteins into residual peroxisomal membranes ("ghosts") (Albertini et al., 1997; Erdmann and Blobel, 1996; Gould et al., 1996; Huhse et al., 1998; Rehling et al., 2000). Only recently it was shown that the peroxins Pex3p and Pex19p are required for proper localization of peroxisomal membrane proteins in yeast (Hettema et al., 2000).

We have detected peroxisomal remnants at the level of resolution of immunofluorescence microscopy using the integral peroxisomal membrane protein Pex11p (Erdmann and Blobel, 1995) and the peripheral peroxisomal membrane protein Pex14p (Albertini et al., 1997) as markers (Fig. 4A).

Moreover, another indicator for peroxisomal membranes, the integral membrane protein Pex3p (Höhfeld et al., 1991), was detected in sucrose density fractions of *pex12Δ* cells characteristic of ghosts (Fig. 4B (Wiebel and Kunau, 1992)). Therefore, Pex12p is another example of a peroxin that plays a role in matrix protein import but not in peroxisomal membrane formation in *S. cerevisiae*.

Pex12p is orthologous to proteins from other species, including *Pichia pastoris* (Kalish et al., 1996), *Rattus norvegicus* (Okumoto and Fujiki, 1997), *Homo sapiens* (Chang et al., 1997) and as we learned while preparing this manuscript, Chinese hamster (Okumoto et al., 2000). A striking feature of all Pex12p orthologues is a carboxy-terminal motif referred to as the RING finger motif (Borden, 2000). This motif was originally defined by the consensus C-X_2-C-$X_{(9–39)}$-C-$X_{(1–3)}$-H-$X_{(2–3)}$-C-X_2-C-$X_{(3–47)}$-C-X_2-C (Freemont et al., 1991). Although detailed analysis of the *Sc*Pex12p amino acid sequence and other Pex12 proteins revealed that only five of the seven cysteines in Pex12p align with the consensus, the Pex12p RING finger is expected to be functional in zinc binding (Kalish et al., 1996). The classical RING finger domain was shown to bind two zinc ions (Barlow et al., 1994; Borden et al., 1995). Examination of the coordination sites for zinc binding in *Sc*Pex12p (data not shown) and other Pex12 proteins (Kalish et al., 1996; Okumoto and Fujiki, 1997) suggested binding of 1 zinc molecule for the Pex12p RING finger (data not shown).

We have demonstrated that Pex12p is an integral peroxisomal membrane protein with its COOH-terminus facing the cytosol and its NH_2-terminus extending into the peroxisomal lumen. Subfractionation studies revealed that Pex12p cosedimented with peroxisomes (Fig. 6A). Furthermore, computer algorithms of the Pex12p sequence (data not shown) predicted two hydrophobic regions that fulfill the criteria for transmembrane segments and led us to investigate the subperoxisomal localization of Pex12p. Membrane extraction experiments performed with purified peroxisomes demonstrated that Pex12p remains associated with the membrane during hypotonic lysis of organelles and incubation of organellar membranes with 100 mM Na_2CO_3, pH 11 (Fig. 6B). Moreover, protease protection experiments that were designed to determine the topology of Pex12p at the peroxisomal membrane showed that a 31-kDa amino-terminal fragment of Pex12p is protected against protease digestion by the membrane, while a COOH-terminal 15-kDa fragment is accessible to protease digestion. The cytosolic location for the Pex12p carboxy-terminus presented here is consistent with topology experiments performed for Pex12p in *Pichia pastoris*, Chinese hamster and human (Kalish et al., 1996; Okumoto et al., 2000; Chang et al., 1999). However, in contrast to our results, Okumoto et al. (1998) detected both the carboxy- and the amino-terminus of Pex12p to be exposed into the cytoplasm of rat cells. As yet, no information is available for the subcellular localization of the Pex12p NH_2-terminus in organisms other than *S. cerevisiae* and Chinese hamster. Future experiments will be needed to clarify if the membrane topology for Pex12 is divergent in different organisms.

Our data indicate that the Pex12p RING finger is essential for Pex12p function but not for its localization. While Pex12p deleted for the RING finger motif was still directed to peroxisomes (Fig. 9), it failed to complement the *pex12Δ* deletion strain (data not shown). In agreement with our results, Okumoto et al. (2000) and Chang et al. (1999) found that the RING finger was required for functional activity of Pex12p in mammals. In addition, neither mutagenesis nor deletion of the RING finger motif affected targeting of Pex12p to the peroxisome in CHO cells (Okumoto et al., 2000).

Surprisingly, no targeting signal for peroxisomal membrane proteins (mPTS) could be identified in the Pex12p amino acid sequence. Several mPTSs have been proposed for peroxisomal membrane proteins in mammals (Kammerer et al., 1998; Soukupova et al., 1999) and yeast (Baerends et al., 1996; Dyer et al., 1996; Elgersma et al., 1997; Höhfeld et al., 1991; Wiemer et al., 1996). Sequence comparison of these targeting sequences revealed a stretch of basic amino acids as their main feature. In our sequence analysis of Pex12p we detected two short regions (aa 151 – 183 and 222 – 250) that show an above average content of basic amino acids, however none of them exactly corresponds to a minimal consensus sequence predicted for the peroxisomal mPTS by Elgersma et al. (1997) or Dyer et al. (1996). The same sequence characteristics were reported for Pex12p from CHO cells (Okumoto et al., 2000). Beyond the data presented in this article, Okumoto et al. (2000) reported the amino-terminal amino acids 1 – 154 of Pex12p to be necessary for peroxisomal localization of Pex12p but not sufficient for its targeting to the peroxisomal membrane. Interestingly, most characterized yeast mPTSs were localized in the peroxisomal lumen (Baerends et al., 1996; Dyer et al., 1996; Elgersma et al., 1997; Höhfeld et al., 1991; Wiemer et al., 1996). Given the finding that the Pex12p carboxy-terminal amino acids 332 – 399 are not necessary for proper Pex12p localization and the fact that its NH_2-terminus extends into the peroxisomal matrix, we favor an amino-terminal mPTS in Pex12p from *S. cerevisiae*.

Pex12p is a strong candidate for an essential component of the import machinery for peroxisomal matrix proteins. Several lines of evidence support this idea: First, Pex12p is essential for import of proteins into the peroxisomal matrix (Fig. 2A – C). Secondly, Pex12p shares the same subperoxisomal localization at the membrane together with identified constituents of the translocation apparatus (Fig. 6A, B). Both aspects were addressed above. Thirdly, it has been reported independently from two different laboratories that the receptor for the PTS1, Pex5p, binds Pex12p at the peroxisomal membrane in human and CHO cells and also a Pex12p – interacting protein, Pex10p (Chang et al., 1999; Okumoto et al., 2000). Finally, we isolated Pex12p in a multi-protein complex from solubilized membranes, together with Pex5p (Brocard et al., 1994; Dodt et al., 1995; Fransen et al., 1995; van der Leij et al., 1993), Pex10p (Kunau et al., 1993; Okumoto and Fujiki, 1997; Warren et al., 1998) and the docking proteins at the peroxisomal membrane for both the PTS1 and the PTS2 receptor, Pex13p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996) and Pex14p (Albertini et al., 1997; Brocard et al., 1997; Komori et al., 1997), respectively (Fig. 8A). Taken together, these data strongly indicate a correlation between Pex12p and other characterized elements of the peroxisomal translocon.

To date, three peroxins have been described as members of the RING finger protein superfamily in yeast and mammals, Pex2p, Pex10p and Pex12p. All three proteins seem to have in common the cytosolic localization of their RING fingers (this work; Harano et al., 1999; Kalish et al., 1996; Okumoto et al., 2000; Shimozawa et al., 1992). An intriguing observation of this and other studies is that at least two of them, Pex12p and Pex10p, can be linked to the peroxisomal protein translocation machinery (this work; Chang et al., 1999; Kalish et al., 1996; Okumoto et al., 2000). It will be interesting to see if the third RING finger protein, Pex2p (Shimozawa et al., 1992; Tsuka-

moto et al., 1991, 1994) is also involved in protein translocation across the peroxisomal membrane.

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