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Albertini, M; Girzalsky, W; Veenhuis, M; Kunau, WH; Kunau, Wolf-H.

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

Markus Albertini1a, Wolfgang Girzalska2a, Marten Veenhuisb, Wolf-H. Kunau1a

a Abteilung für Zellbiochemie, Medizinische Fakultät der Ruhr-Universität Bochum, Bochum/Germany
b Laboratory for Electron Microscopy, University of Groningen, Biological Centre, Haren/The Netherlands

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Saccharomyces cerevisiae – peroxin – integral membrane protein – peroxisome biogenesis – RING finger

We have isolated the Saccharomyces cerevisiae pex12Δ1 mutant from a screen to identify mutants defective in peroxisome biogenesis. The pex12Δ gene 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 pex12Δ1 mutant strain and encodes a polypeptide of 399 amino acids. ScPex12p 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 NH2-terminus facing the peroxisomal lumen and with its COOH-terminus facing the cytosol. Pex12Δ – 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 immunoprecipitation 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.

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 NH2-terminal nonapeptide (RLXS(H/Q)I) termed PTS2 (Osumi et al., 1991; Swinkels et al., 1991) (for review see (De Hoop and Ab, 1992; Subramani, 1993)).
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 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/HindII fragment which comprised the full complementing activity.

Sequence analysis was carried out according to the dyeoxy 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/pex5Δ were generated as described by (Atchison et al., 1995). Primers KU571 (5′-ACATAAAC AACACACGATAAATGGGAAGTTGTGACAGGTATTAG-3′) and KU572 (5′-TGCTTGTAACACACAGGAAGGTTGGACCAAGAT GTACATGAAATCCTGCTGGATAACATTATCTATATACAG TTATTAG-3′) were used for PCR reactions.

To delete the PEX12 gene in wild-type strain UTL-7A, a complementing 3.5-kb EcoRI/HindII fragment of pRS/PEX12 was subcloned into pBlueScript vector (Stratagene, USA) resulting in pSK/PEX12/SE. The S. cerevisiae LEU2 gene of plasmid pJJS25 (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 gene, PRX12/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 leucinoprototrophic 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 diploids 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 promoter (Brunelli and Pall, 1993), was obtained as follows: The PEX12 open reading frame was amplified by PCR using oligonucleotides KU 11 (5′-GGAATTTCTCCCCGGAATGACGCTTTATTTCAA-3′) and 3′-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 leucinoprototrophic 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 diploids that were unable to grow on YNO agar. In addition, integration was confirmed by Southern blot analysis (Sambrook et al., 1989).

Materials and methods

Strains and growth conditions

Except for PCY2 (MATa, gal80Δ, gal10Δ2, URA3::GAL1-lacZ, lys2–801Δ162, his3–Δ200, trpl–Δ63, leu2–Δ128), all yeast strains used in this study were S. cerevisiae wild-type UTL-7A (MATa, ura3–52, trpl–2, leu2–3,112 (W. Duntze, Bochum)) and its derivatives pex5Δ (MATa, ura3–52, trpl, pex5::kanMX4 (Girzalsky et al., 1999)), and pex12Δ (MATa, ura3–52, trpl, 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).
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). Organelar 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-Pce60p, 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; Gritzalsky et al., 1999; Höhfeld et al., 1991). Rabbit polyclonal antibodies against the N-terminal amino acids 10–125 of Pex12p were raised against ananthranilate 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) corresponding to amino acids 302–337 of Pex12p. All antibodies were produced by Eurogentec (Seraing, Belgium). Electrophoresis and electrophoretic blotting 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-PaA 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’TCTATATGCGACCATGGGCAATTATTAGAAGAGGGG-3′) and KU673 (5’TCTATAGAGCTCGCGGCCGCTATTGCCGCAGGAC-3′) as well as genomic DNA as a template. The PCR fragment was cloned into SalI/NorI digested pPC97. This construct (pBA17) encoded a Gal4-Pex10 fusion protein consisting of 238–337 of Pex10p. A PEX12 fragment encoding aa 293–399 was amplified by PCR with primer set KU 13 (see above) and KU 228 (5’-CGAATTCGCCCGGGGATGACCCACACAGACT-3′) and cloned into Smal/BamHI digested pC86 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 organellar pellet enriched for peroxi-
somes and mitochondria has been described before (Cranze et al., 1994). For protease protection assays, an organellar pellet was resuspended in homogenization buffer (Erdmann et al., 1989) lacking protease inhibitors. Equal amount was incubated for 30 min on ice with increasing amounts of trypsin. After the protease was inhibited by the addition of trypsin inhibitor, samples were immediately precipitated with TCA and subsequently processed for SDS-PAGE.

Miscellaneous methods

Acetyl-CoA acetyltransferase (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 pex12Δ 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 pex12Δ mutant to be recessive. The PEX12 gene was isolated from a library of S. cerevisiae genomic DNA by functional complementation of the pex12Δ 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 (YMR206c). 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 (PpPex12p; (Kalish et al., 1996)) human (HsPex12p, (Chang et al., 1997)) and Rattus norvegicus (RnPex12p, (Ookoto and Fujiki, 1997)). Therefore, the newly identified gene was designated ScPex12p.

A PEX12 deletion strain (pex12Δ) 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* (ScPex12p), *Pichia pastoris* (PpPex12p; Kalish et al., 1995) *Homo sapiens* (HsPex12p, Chang et al., 1997) and *Rattus norvegicus* (RnPex12p, Okumoto and Fujiki, 1997). Conserved amino acids that are present in at least two of the four proteins are indicated. Identity of ScPex12p to PpPex12p, HsPex12p and RnPex12p is 32%, 23% and 22%, respectively.
**pex12Δ cells are deficient in peroxisomal matrix protein import**

Cells deficient in *PEXI2* 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 *pex12Δ* 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 *pex12Δ* cells (Fig. 2B). These data suggest that *pex12Δ* 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, *pex12Δ* and complemented *pex12Δ* cells. The different strains were grown on oleic acid and subjected to subcellular fractionation to give a 25 000g pellet enriched for peroxisomes and mitochondria and a 25 000g 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 25000g pellet of wild-type cells (Fig. 2C). In contrast, in *pex12Δ* cells all peroxisomal matrix proteins were preferentially localized to the 25000g 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.

**pex12Δ 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).
Conversely, pex12Δ 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 pex12Δ using sucrose density gradient centrifugation (Fig. 4B). Fumarase peaked at a sucrose density of 1.18 g/cm³, 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/cm³ (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 NH₂-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 pex12Δ transfectants 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 disulphide 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).

**Induction of Pex12p by oleic acid**

In S. cerevisiae, 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.
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 mutants expressing full-length Pex12p from plasmid pRS/PEX12. In sucrose density gradient centrifugation experiments, 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/cm³, 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 25 000 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 Na₂CO₃, pH 11.0. Pex12p was resistant 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 solubilized. These data indicate that Pex12p is an integral component of the peroxisomal membrane. The intracellular localization of Pex12p was further confirmed by immunoelectron microscopy, using anti-Pex12p antibodies. pex12A 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 immunoelectron microscopic analysis with a polyclonal antiserum against the Pex12p NH₂-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 pYPGE15 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 pex12A 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 NH₂-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 NH₂-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-
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 pex12Δ mutant cells was successively extracted with 10 mM Tris-HCl, pH 8.0, and 100 mM Na2CO3, 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 NH2-terminus, thiolase (Fox3p) and Pex3p. Pex12p was resistant to low-salt and Na2CO3 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 pex12Δ mutant cells (C) and pex12Δ 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 NH2-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.
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 (Egersma 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 specificity 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 hsPex12p 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 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 Pex12pΔmutant to be insufficient for restoration of the wild-type phenotype in pex12Δcells and therefore the RING finger to be essential for biological activity of Pex12p (data not shown).
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<sub>9–10</sub>-F/Y) involved in the targeting of this and other membrane proteins has been delimited (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 Pex12p<sub>aal–332</sub> lacking the RING finger was expressed in wild-type cells. Indicative of a peroxisomal localization we found Pex12p<sub>aal–332</sub> 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 Pex12p<sub>aal–332</sub> 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.

**Discussion**

Here we report the identification of the \textit{PEX12} gene in the yeast \textit{S. cerevisiae} and the molecular characterization of the \textit{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 \textit{PEX12} gene by functional complementation of the original \textit{pex12–1} mutant in \textit{S. cerevisiae}. Typical of peroxisomal mutants, (Erdmann et al., 1989) the \textit{pex12–1} mutant as well as the \textit{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 \textit{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 \textit{pex12Δ} mutant with the \textit{CEN} plasmid pRS316 carrying the \textit{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 \textit{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 \textit{PEX12} from a \textit{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-X₂-C-X₉₋₁₃-H-X₁₋₃-C-X₉₋₁₃-C-X₃₋₁₇-C-X₇₋₁₉-C (Freemont et al., 1991). Although detailed analysis of the ScPex12p 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 ScPex12p (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 NH₂-terminus extending into the peroxisomal lumen. Subfractionation studies revealed that Pex12p co-sedimented 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 organelar membranes with 100 mM Na₂CO₃, 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., 1997). 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-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 motif 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 NH₂-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. 6A – 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-
References


