Pex17p of *Saccharomyces cerevisiae* Is a Novel Peroxin and Component of the Peroxisomal Protein Translocation Machinery

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Abstract. The *Saccharomyces cerevisiae pex17-1* mutant was isolated from a screen to identify mutants defective in peroxisome biogenesis. *pex17-1* and *pex17* null mutants fail to import matrix proteins into peroxisomes via both PTS1- and PTS2-dependent pathways. The *PEX17* gene (formerly PAS9; Albertini, M., P. Rehling, R. Erdmann, W. Girzalsky, J.A.K.W. Kiel, M. Veenhuis, and W.-H Kunau. 1997. *Cell.* 89:83–92) encodes a polypeptide of 199 amino acids with one predicted membrane spanning region and two putative coiled-coil structures. However, localization studies demonstrate that Pex17p is a peripheral membrane protein located at the surface of peroxisomes. Particulate structures containing the peroxisomal integral membrane proteins Pex3p and Pex11p are evident in *pex17* mutant cells, indicating the existence of peroxisomal remnants ("ghosts"). This finding suggests that *pex17* null mutant cells are not impaired in peroxisomal membrane biogenesis. Two-hybrid studies showed that Pex17p directly binds to Pex14p, the recently proposed point of convergence for the two peroxisomal targeting signal (PTS)-dependent import pathways, and indirectly to Pex5p, the PTS1 receptor. The latter interaction requires Pex14p, indicating the potential of these three peroxins to form a trimeric complex. This conclusion is supported by immunoprecipitation experiments showing that Pex14p and Pex17p coprecipitate with both PTS receptors in the absence of Pex13p. From these and other studies we conclude that Pex17p, in addition to Pex13p and Pex14p, is the third identified component of the peroxisomal translocation machinery.

PROTEIN transport across the peroxisomal membrane from the cytoplasm into the peroxisomal matrix is thought to occur in a posttranslational manner (Lazarow and Fujiki, 1985). Two distinct peroxisomal targeting signals (PTSs)[1] that provide the molecular basis for the specificity of this process have been identified in peroxisomal matrix proteins. One of them, designated PTS1, is the carboxy-terminal tripeptide SKL, which was first discovered in firefly luciferase (Gould et al., 1989; for review see Subramani, 1993). This tripeptide or variants of it are found in the majority of the known intraperoxisomal proteins. In contrast, a smaller subset of peroxisomal matrix proteins is targeted to the peroxisomal lumen through an alternative signal termed PTS2. This sorting motif is located close to the amino terminus and has the more complex consensus sequence RLX_5H/QL (for review see DeHoop and Ab, 1992; Rehling et al., 1996*a*).

A major advance in understanding the mechanisms underlying peroxisomal protein import has been the identification and subsequent characterization of the two PTS receptors (signal sequence recognition factors) encoded by *PEX5* and *PEX7* (for review see Elgersma and Tabak, 1996; Erdmann et al., 1997). Cells deleted for either of the two genes display partial import deficiencies: *Δpex5* cells correctly import thiolase, a PTS2 protein, but are selectively compromised in the import of PTS1 proteins. *Δpex7* cells exhibit the reverse phenotype. These observations established the existence of at least two distinct import pathways for peroxisomal matrix proteins. The two PTS signals and their receptors are conserved from yeast to man (Subramani, 1997). Moreover, two out of eleven complementation groups (peroxisome biogenesis disorder [PBD] groups) among fibroblast cell lines derived from patients with peroxisomal disorders show the same partial import blocks as *Δpex5* and *Δpex7* yeast mutant cells (Motley et al., 1994; Slawecki et al., 1995).

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1. *Abbreviations used in this paper*: ORF, open reading frame; PTS, peroxisomal targeting signals.

The location of the two import receptors, Pex5p and Pex7p, and thus their site of function is still controversial: A predominantly cytoplasmic, membrane-bound, or even intraperoxisomal localization has been reported for either of them (for review see Rachubinski and Subramani, 1995). An attractive hypothesis to reconcile these different results is that Pex5p and Pex7p bind their cargo proteins in the cytoplasm, dock to specific proteins at the periphery of the peroxisomal membrane, and subsequently may even enter the peroxisome before they release their cargo and shuttle back to the cytoplasm. The notion that peroxisomes import both folded and oligomeric proteins seems to support this model (for review see McNew and Goodman, 1996). Recently, Dodt and Gould (1996) have provided data consistent with at least the first step of this cycle.

Two peroxisomal membrane proteins have been described that are essential for both PTS-dependent import pathways and have the necessary properties to serve as docking sites for the PTS receptors at the organelle. Pex13p, an integral peroxisomal membrane protein, specifically binds to the PTS1 receptor Pex5p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996). Pex13p contains an SH3 domain that appears to be required for the docking event since a single mutation in this domain severely disturbed the interaction. The second protein, Pex14p (Albertini et al., 1997; Komori et al., 1996), is a peripheral membrane protein located at the outer face of the peroxisome. Whereas the detected two-hybrid interactions of Pex13p were restricted to the PTS1 receptor, Pex14p was shown to physically interact with various Pex proteins: namely with Pex5p, Pex7p, and the SH3 domain of Pex13p. Thus, it has been proposed that this protein represents the point of convergence for both import pathways (Albertini et al., 1997). At present, only the two PTS receptors, Pex5p and Pex7p, and their binding partners at the peroxisomal membrane, Pex13p and Pex14p, have been characterized as integral parts of the peroxisomal sorting and translocation machinery. However, it seems reasonable to assume that among the membrane-bound members of the large collection of proteins encoded by *PEX* genes, other components of this machinery exist.

In this report, we describe the cloning of a novel peroxin that is encoded by the *PEX17* gene. Pex17p is a peroxisomal membrane protein located at the cytoplasmic side of peroxisomes and is required for import of both PTS1 and PTS2 proteins. However, it is not essential for the insertion of peroxisomal membrane proteins. Involvement of Pex17p in protein import into peroxisomes is indicated by the fact that it interacts with Pex14p. We further demonstrate that Pex17p binds to the PTS1 receptor, Pex5p, in a two-hybrid system but that this interaction is dependent on the presence of Pex14p. Taken together, these findings strongly suggest that Pex17p is a novel component of the peroxisomal import machinery.

Materials and Methods

Strains and Culture Conditions

Saccharomyces cerevisiae and *Escherichia coli* strains used in this study are listed in Table I.

Yeast complete (YPD) and minimal media (SD) 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. When necessary, auxotrophic requirements were added according to Ausubel et al. (1992).

Isolation of the pex17 Mutant Strain

The *pex17-1* 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 onto YNO agar plates, fractionation of yeast cells, and electron microscopy, all performed as described by Erdmann et al. (1989). Mutants were characterized by standard genetic techniques for *S. cerevisiae* (Ausubel et al., 1992).

Fractionation of Yeast Lysates and Purification of Peroxisomes

Preparation and fractionation of yeast lysates was performed as described by Erdmann et al. (1989). For further subfractionation by isopycnic sucrose density gradient centrifugation, cell lysates of wild-type and mutant strains were loaded onto continuous 20–53% sucrose density gradients. Centrifugation, fractionation of the gradient, and preparation of samples for SDS-PAGE were carried out as described by Höhfeld et al. (1991). Organelle pellets of oleate-induced wild-type and mutant strains were prepared according to Erdmann et al. (1989). Continuous nycodenz gradients (15–36%) were performed as described by Marzioch et al. (1994).

Cloning and Characterization of the PEX17 Gene

The *PEX17* gene was isolated from a *S. cerevisiae* genomic DNA library in the autonomously replicating *E. coli\yeast* shuttle vector YCp50 (Rose et al., 1987) by functional complementation of the *pex17*-1 mutation. Transformation was done by a modified lithium acetate method (Gietz and Sugino, 1988). Ura^+ colonies were screened on YNO agar plates for restoration of the ability to use oleic acid as the sole carbon source. Complementing plasmids were recovered by common isolation procedures. Standard recombinant DNA methodologies such as enzymatic modifica-

Table I. Yeast Strains Used in This Study

Name	Genotype	Source
UTL-7A	*MATa ura3-52 trp1 leu2-3/112*	W. Duntze (Medizinische Fakultät, der Ruhr-Universität Bochum)
pex17-1	*MATa pex17-1 ura3-52 trp1 leu2-3/112*	This study
Δpex17	*MATa pex17::LEU2 ura3-52 trp1 leu2-3/112*	This study
Δpex13	*MATa pex13::kanMX4 ura3-52 trp1 leu2-3/112*	This study
PCY2	*MATα, Δgal4, Δgal80, URA3::GAL1-lacZ, lys2-801^{amber}, his3-Δ200, trp1-Δ63, leu2 ade2-101^{ochre}*	P. Chevray (The Johns Hopkins University, Baltimore, MD)
PCY2Δ14	*MATα, pex14::kanMX4, Δgal4, Δgal80, URA3::GAL1-lacZ, lys2-801^{amber}, his3-Δ200, trp1-D63, leu2 ade2-101^{ochre}*	This study
PCY2Δ13	*MATα, pex13::kanMX4, Δgal4, gal80, URA3::GAL1-lacZ, lys2-801^{amber}, his3-Δ200, trp1-Δ63, leu2 ade2-101^{ochre}*	This study
PCY2Δ17	*MATα, pex17::kanMX4, Δgal4, Δgal80, URA3::GAL1-lacZ, lys2-801^{amber}, his3-Δ200, trp1-Δ63, leu2 ade2-101^{ochre}*	This study

tion of DNA, DNA fragment purification, and plasmid preparation were performed essentially as described in Ausubel et al. (1992).

DNA Sequencing

To sequence the 1.47-kb fragment of plasmid pRS9/1.4 (smallest complementing fragment), defined restriction fragments and deletion fragments generated by BAL31 exonuclease were subcloned into pBluescript vectors (Stratagene, La Jolla, CA). Sequencing was performed according to the dideoxy chain termination method (Sanger et al., 1977). The deduced Pex17p amino acid sequence was compared to other known protein sequences using the BLAST program of the Heidelberg Unix Sequence Analysis Resource (Husar 4.0; Deutsches Krebsforschungszentrum, Heidelberg, Germany).

Integrative Disruption of the PEX17 Gene

The *PEX17* gene deletion construct (pJJGD9) was constructed by subcloning a 236-bp EcoRI/ClaI fragment and a 316-bp DraI/HindIII fragment of the *PEX17* 5′ and 3′ noncoding region, respectively, into pBluescript vectors to generate appropriate restriction sites. The fragments were subsequently introduced into vector pJJ283 (Jones and Prakash, 1990), which carries the *LEU2* gene. Plasmid pJJGD9 was digested with PstI/SacI to liberate the *LEU2* gene flanked by the 5′ and 3′ noncoding region of the *PEX17* gene. The gene deletion fragment was introduced into the wild-type strain UTL-7A. One of the resultant leucine prototrophic transformants was mated with wild-type JKR101, the diploid was induced to sporulate, and the meiotic progeny were examined by standard tetrad analysis. In addition, integration was confirmed by Southern blot analysis (Sambrook et al., 1989).

Production of Anti-Pex17p Antibodies

Antibodies against the Pex17 protein were raised against a MS2-replicase-Pex17p fusion protein. Specifically, a 1.2-kb fragment of the *PEX17* gene containing both open reading frame and 3′ untranslated region and codes for amino acids 2–199 of Pex17p was inserted in the vector pLC2408 (Remaut et al., 1981). Furthermore, to generate suitable restriction sites for the correct in-frame fusion, a 1.2-kb *PEX17* gene fragment was excised with ClaI/HindIII and inserted into a ClaI/HindIII-digested pBluescript vector to generate pSK/AI-*PEX17*. Finally, a 1.2-kb SalI/HindIII (pSK/AI-*PEX17*) fragment was subcloned into the SalI/HindIII-digested vector pLC2408. Polyclonal antibodies against the purified hybrid protein were raised in rabbits. For affinity purification of anti-Pex17p antibodies according to Höhfeld et al. (1991), a glutathione-S-transferase (*GST)–PEX17* gene fusion was constructed. Therefore, a portion of the *PEX17* gene was amplified by PCR using primer HS9-1 (5′ATATAGAATTCACCCCAGTATCGTCGTTG3′) and HS9-2 (5′GGAATTCCTGCAGGTCGACTTACCTTGGCAATTGGC3′). The resulting fragment was subsequently cloned into vector pGEX4-T-1 (Pharmacia Biotech, Piscataway, NJ) using the primer-derived EcoRI and SalI sites. The construct encodes a GST–Pex17p fusion protein encompassing amino acids 90–199 of Pex17p. Expression and purification of the fusion protein was carried out according to the manufacturer's instructions.

Two-Hybrid Assay

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 construct the Gal4-(BD)-Pex17p fusion protein, plasmid pRS9/1.4 was digested with ClaI and EcoRV. To generate suitable restriction sites for cloning into plasmids pPC86 and pPC97, the resulting 1.2-kb *PEX17* fragment was ligated into the ClaI-SmaI site of pBluescript SK+ (pSK9/1.2). Finally, pSK9/1.2 was digested with SalI and SpeI, and the released *PEX17* insert was ligated into the SalI-SpeI sites of pPC86 and pPC97, respectively. The *GAL4PEX5* fusion construct was a kind gift of R. Erdmann (Medizinische Fakultät der Ruhr-Universität Bochum, Bochum, Germany) (Erdmann and Blobel, 1996). *GAL4PEX14* fusion constructs have been published previously (Albertini et al., 1997). Cotransformation of two-hybrid vectors into the strain *PCY2* was performed according to Gietz and Sugino (1988). 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*b*).

Construction of Knock Out Two-Hybrid Reporter Strains

To delete *PEX13*, *PEX14* and *PEX17* in the yeast two-hybrid reporter strain, and *PCY2* as well as *PEX13* in wild-type strain UTL-7A, the *kanMX4* gene was used as a selective marker for insertion into the genomic locus (Wach et al., 1994). Deletion cassettes containing the *kanMX4* gene and the 5′ and 3′ untranslated regions of the corresponding open reading frames (ORFs) were constructed by PCR using pFA6a-kanMX4 (Wach et al., 1994) as a template. To generate suitable constructs for a *PEX13* gene deletion, the primer set KU 274 (TATCTATAAATATCAAGGGGATTCTATACTATAACAATACCTGCGCGTACGCTGCAGGTCGAC) and KU 275 (TTTACTATATATATATGCGAATATATGTGTGCAAATATTGATGCAATCGATGAATTCGAGCTCG), for a *PEX14* gene deletion the primer set KU 289 (GAAAACTCAAG TAAAACAGAGAAGTTGTAAGGTGAATAAGGACGTACGCTGCAGGTCGAC) and KU 290 (AATTACAATTTCCGTTAAAAAACTAATTACTTACATAGAAATTGCGATCGATGAATTCGAGCTCG), and for a *PEX17* gene deletion the primer set KU 251 (TCCATCATTCTGATAAGCAGAACCACGTAAGG CAGACTAAAATCCGTACGCTGCAGGTCGAC) and KU 273 (ACGTGCACTAGAGCGTTTTAAATTCAATGCTATTATTTTTGATTGATCGATGAATTCGAGCTCG) were used. PCR and selection for geniticine resistance of transformants were performed according to Wach et al. (1994). Authenticity of each gene deletion by integration of the *kanMX4* gene was confirmed by PCR on genomic DNA of the putative knock out strains.

Immunofluorescence 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.

Membrane Preparation and Protease Protection Assay

Membrane preparation from an organelle pellet enriched for peroxisomes and mitochondria has been described by Crane et al. (1994). The subfractionation and extraction of purified peroxisomes was done according to Erdmann and Blobel (1995).

For protease protection assays, the peroxisomal peak fractions of a sucrose density gradient were pooled and subsequently diluted fivefold in gradient buffer (Erdmann and Blobel, 1995). Peroxisomes were concentrated by centrifugation at 25,000 *g* for 30 min. The resultant pellet was resuspended in homogenization buffer supplemented with 50 mM KCl (Erdmann et al., 1989) but without protease inhibitors. Equal amounts of isolated peroxisomes were incubated for 10 min on ice with increasing amounts of proteinase K. The proteinase was inhibited by the addition of 4 mM PMSF. The proteins were then precipitated with TCA and the samples were processed for SDS-PAGE.

Antibodies, Western Blotting, and Coimmunoprecipitation

Antithiolase (Fox3p) (Erdmann and Kunau, 1994), anti-Pcs60p (Blobel and Erdmann, 1996), anticatalase (Kragler et al., 1993), anti-Pex14p (Albertini et al., 1997), and anti-Pex3p antibodies (Höhfeld et al., 1991) have been described previously. Anti-Pex13p antibodies were kindly provided by R. Erdmann. Anti–rabbit or anti–mouse IgG-coupled HRP (Amersham Corp., Arlington Heights, IL) were used as secondary antibodies, and blots were developed using the ECL system (Amersham Corp.). Western blot analyses were performed according to standard protocols (Harlow and Lane, 1988).

Coimmunoprecipitation experiments were performed as described (Rehling et al., 1996*b*) with the exception that the 35,000-*g* sedimentation step was omitted.

Miscellaneous Methods

Protein concentrations were determined with a protein assay kit (BCA Protein Assay Reagent; Pierce Chemical Co., Rockford, IL) using BSA as standard. 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 according to established procedures (Moreno de la

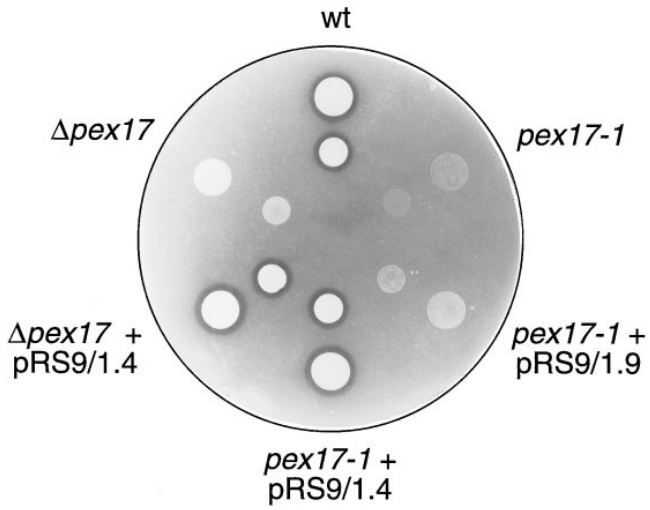

Figure 1. Growth of various *S. cerevisiae* strains on oleic acid medium (YNO). Equal amounts of cells were diluted in distilled H_2O, and aliquots were applied onto oleic acid plates (inner circle, 3 μl; outer circle, 5μl of dilution). Plates were subsequently incubated at 30°C for 7 d. In contrast to the wild-type strain UTL-7A (*wt*), the *pex17-1* mutant and the *Δpex17* null mutant are unable to grow on YNO. The plasmid pRS9/1.4, carrying a complete copy of *PEX17*, functionally complemented both *pex17-1* and *Δpex17*, whereas the plasmid pRS9/1.9 did not restore growth. Growth on YNO agar plates is indicated by a typical halo reflecting the consumption of oleic acid.

Garza et al., 1985; Veenhuis et al., 1987). Electron microscopy on intact yeast cells was performed as described by Erdmann et al. (1989).

Results

Isolation and Characterization of the pex17-1 Mutant

We have previously demonstrated that mutants of *S. cerevisiae* defective in various aspects of peroxisome function (e.g., *fox* mutants) and peroxisome biogenesis (*pex* mutants) can be isolated from a population of cells that were unable to grow on oleic acid (onu phenotype) as the sole carbon source (Erdmann et al., 1989). *pex17-1* was selected from such onu strains by two additional criteria: the mislocalization of peroxisomal marker enzymes to the cytosol and the lack of morphologically detectable peroxisomes as determined by electron microscopy.

pex17-1 carries the *pex17* defect as a single genetic lesion preventing growth on oleic acid as demonstrated by tetrad analysis (data not shown). Backcrosses of *pex17-1* to wild-type cells yielded diploids that regained the ability to use oleic acid, thereby indicating the recessive nature of the *pex17* mutation. Genetic complementation analysis revealed that *pex17-1* defines a complementation group distinct from *pex1-14* (Erdmann et al., 1997).

The onu phenotype of *pex17-1* is shown in Fig. 1. In contrast to the wild-type strain UTL-7A, the mutant is unable to grow on YNO agar and accumulates peroxisomal matrix enzymes in the cytosol as has been reported for other *pex* mutants (Kunau et al., 1993). Table II shows the distribution of a PTS2 (thiolase) as well as a PTS1 protein (catalase) between the organellar pellet fraction and the cytosolic

Table II. Distribution Pattern of Peroxisomal and Mitochondrial Marker Enzymes in the 25,000 g Supernatant and Pellet Fractions of Cell Lysates from Wild-Type and pex17 Mutants Grown for 15 h on YNO Medium

		Activity in 25,000 *g*		
Strain	Enzyme	Supernatant fraction (*A1*) (ncat/Fraction)	Pellet fraction (*A2*) (ncat/Fraction)	*A1/A2*
Wild-type	Thiolase	38.7	96.7	0.4
	Catalase	6.3×10^4	11.2×10^4	0.6
	Fumarase	50.0	78.3	0.6
pex17-1	Thiolase	62.5	7.7	8.1
	Catalase	27.7×10^4	0.5×10^4	55.4
	Fumarase	48.0	82.0	0.6
Δpex17	Thiolase	56.9	12.3	4.6
	Catalase	14.7×10^4	0.7×10^4	21.0
	Fumarase	45.0	192.0	0.2
pex17-1 +	Thiolase	60.0	75.0	0.9
YCp9/5.1	Catalase	12×10^4	16×10^4	0.8
	Fumarase	34.0	169.0	0.2
pex17-1 +	Thiolase	31.7	81.7	0.4
pRS9/1.4	Catalase	2.6×10^4	3.1×10^4	0.8
	Fumarase	50.0	76.7	0.6
Δpex17 +	Thiolase	50	163.3	0.3
pRS9/1.4	Catalase	1.0×10^4	2.5×10^4	0.4
	Fumarase	45.0	80.0	0.6

supernatant of cell lysates obtained from the mutant strain compared to wild-type cells. In *pex17-1*, these peroxisomal matrix proteins were predominantly found in the supernatant, whereas in wild-type cells the enzymes sedimented in the pellet fraction. These data indicate that in *pex17-1* cells both the PTS1 and PTS2 proteins are mislocalized to the cytosol and suggest that *pex17-1* cells may lack import-competent peroxisomes.

To address this question, mutant and isogenic wild-type cells were examined by electron microscopy. Wild-type cells grown in the presence of oleic acid show peroxisomes scattered throughout the cell (Fig. 2). In contrast, no peroxisomes or structures reminiscent of peroxisomes could be detected in thin sections of *pex17-1* cells grown under the same conditions.

Cloning and Sequence Analysis of the PEX17 Gene

The *PEX17* gene was cloned by functional complementation of *pex17-1* cells. Subcloning, sequencing, and complementation analysis (Fig. 3 *A*) revealed that an open reading frame of 597 bp corresponding to a putative protein of 199 amino acids with a predicted molecular mass of 23,187 D (Fig. 4) was responsible for complementing activity. More recently, this gene was identified in the genome sequencing project of *S. cerevisiae* during the sequencing of the left arm of chromosome XIV. (These data are available from GenBank/EMBL/DDBJ under accession number X78898, ORF N1319 [Coster et al., 1995].) The observation that the size of the encoded protein determined by Western blotting was ~25 kD (see below) strongly argues that the first ATG of the ORF is the translation initiation site.

A search of protein data bases (using the Husar 4.0 package based on the Wisconsin Genetics Computer Group program package version Unix-8.01 [1995]) revealed no striking similarity between Pex17p and any other known

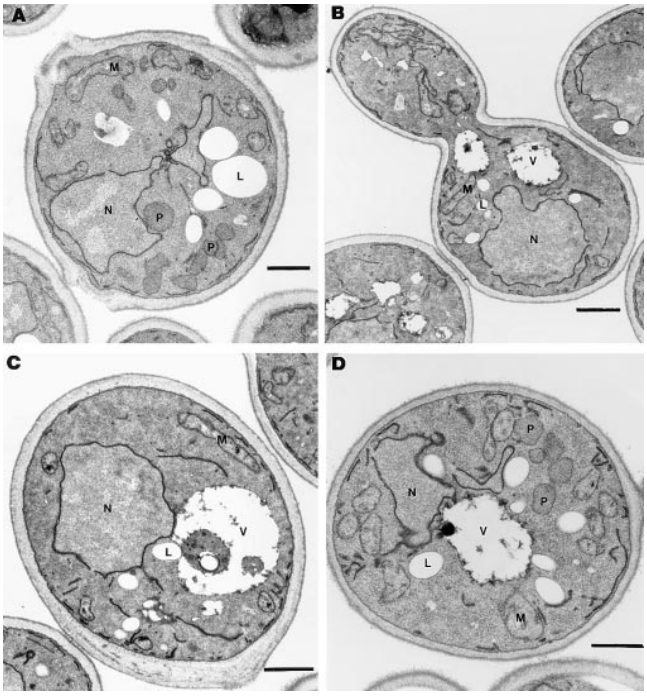

Figure 2. Sections of $KMnO_4$-fixed oleic acid–induced wild-type cells of *S. cerevisiae*, showing proliferation of peroxisomes (*A*). In contrast, peroxisomes could not be detected in oleic acid–induced *pex17-1* cells (*B*) and *Δpex17* null mutants cells (*C*) grown under the same conditions as the wild-type strain. In a thin section of a functionally complemented *pex17-1* mutant cell with plasmid pRS9/1.4, normal peroxisomes were evident (*D*). *P*, peroxisome; *M*, mitochondrion; *N*, nucleus; *V*, vacuole; *L*, lipid droplet. Bar, 0.5 μm.

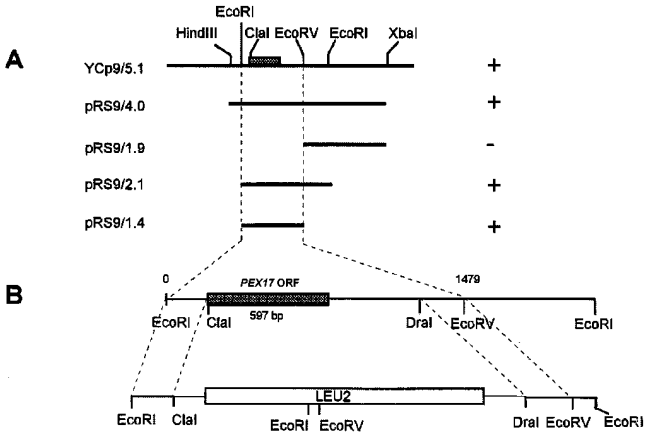

Figure 3. Complementation analysis and gene knock out strategy. (*A*) The solid black line indicates the 5.1-kb genomic fragment of plasmid YCp9/5.1, which was found to complement the *pex17-1* mutation. Subclones of this fragment are shown together with their complementing ability (+/−). The hatched bar indicates the position of the ORF of the *PEX17* gene. The strategy for the targeted disruption of the *PEX17* gene is depicted below (*B*). The ClaI/DraI fragment of the *PEX17*, which contains almost the entire *PEX17* ORF, was replaced by homologous recombination using the *LEU2* gene as a selective marker. The correct integration of the *LEU2* gene into the *PEX17* gene locus was confirmed by Southern blot analysis of genomic DNA (data not shown).

protein. Hydropathy analysis based on different algorithms (Kyte and Doolittle, 1982; Eisenberg et al., 1984; Klein et al., 1985; Roa and Argos, 1986) predicted Pex17p to contain a membrane-spanning region in the amino-terminal half of the protein (Fig. 4 *A*). Furthermore, neither of the two peroxisomal targeting signals, PTS1 and PTS2, is found in Pex17p (Subramani, 1993; Rehling et al., 1996*a*). This suggests that Pex17p is membrane associated rather than localized to the peroxisomal matrix. The use of computer programs that predict secondary structure suggested two segments in Pex17p with the potential to form α helices. According to the algorithm of Lupas (1996), these two regions, characterized by a heptad leucine motif, have a high probability to form a coiled-coil (amino acids 73–90 and 124–143) (Fig. 4 *B*).

Cells with a Deletion of the PEX17 Gene Have the Same Phenotype as pex17-1 Cells

A mutant strain of *S. cerevisiae* lacking the chromosomal copy of the *PEX17* gene was created by homologous recombination. For the replacement, a plasmid was constructed in which 590 out of 597 bp of the coding sequence were replaced by the *LEU2* gene (Fig. 3 *B*). The correct integration of the deletion construct was confirmed by Southern blot analysis (data not shown). Furthermore, the integration event was verified genetically by tetrad analysis. For this purpose, the *pex17*::*LEU2* (*Δpex17*) strain was mated with a wild-type strain, diploids were sporulated, and asci dissected into tetrads. Cosegregation of the onu^- and the $LEU2^+$ phenotype was observed in all cases (data not shown). In addition, diploids obtained after crossing of the *Δpex17* strain with the original mutant *pex17-1* displayed the onu phenotype, indicating that the *PEX17* gene product is essential for growth on oleic acid medium. The inability of *Δpex17* cells to metabolize oleic acid is shown in Fig. 1. The failure of *pex17*-1/*Δpex17* diploids or their progeny to grow on oleic acid demonstrated that the two genes are allelic, thereby confirming that the authentic *PEX17* gene and not a suppressor had been cloned. All spores from the tetrads were viable on YPD, demonstrating that the loss of *PEX17* is not lethal.

To analyze the *Δpex17* phenotype in more detail, a subcellular fractionation experiment was performed. A cell lysate obtained from spheroplasts of oleic acid–induced *Δpex17* cells was loaded onto a 20–53% continuous sucrose density gradient. After centrifugation, the gradient was drained into 30 1-ml fractions, and the subcellular distribution of peroxisomal marker proteins was determined both biochemically and immunologically. Consistent with our previous observation (Table II), thiolase and catalase were found to be present in the cytosolic fractions, indicating that both PTS-dependent import routes are compromised (Fig. 5). The sedimentation pattern of the mitochondrial marker enzyme fumarase with peak activity at a typical density of 1.18 g/cm^3 clearly demonstrates that mitochondria were intact. Thus, the observed mislocalization of peroxisomal matrix enzymes was not caused by disrupted peroxisomal organelles.

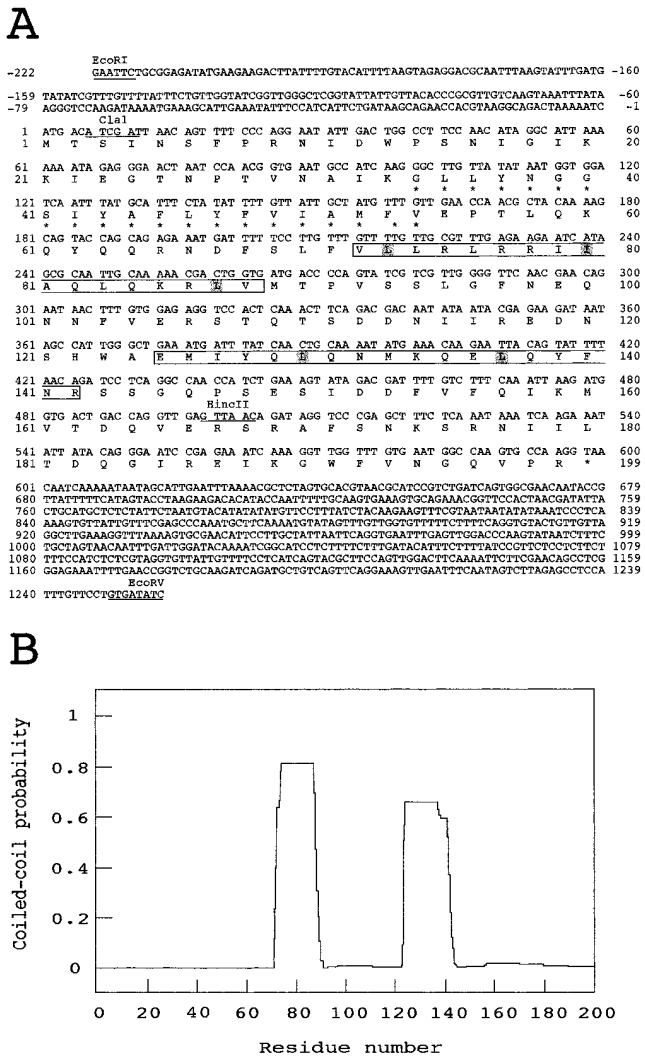

Figure 4. Nucleotide sequence of the *PEX17* gene and deduced amino acid sequence of Pex17p. (*A*) A few relevant restriction sites used for different constructs are indicated above the nucleotide sequence. A predicted membrane spanning region within the amino-terminal region of Pex17p is underlined with asterisks. Amino acid stretches marked by a box represent predicted coiled-coil structures within the protein. The hydrophobic amino acids of the heptad repeat are indicated by a hatched box. (*B*) Prediction of coiled-coil probability within Pex17p using the algorithm of Lupas (1996). These sequence data are available from EMBL/GenBank/DDBJ under accession number X78898 (N1319; Coster et al., 1995).

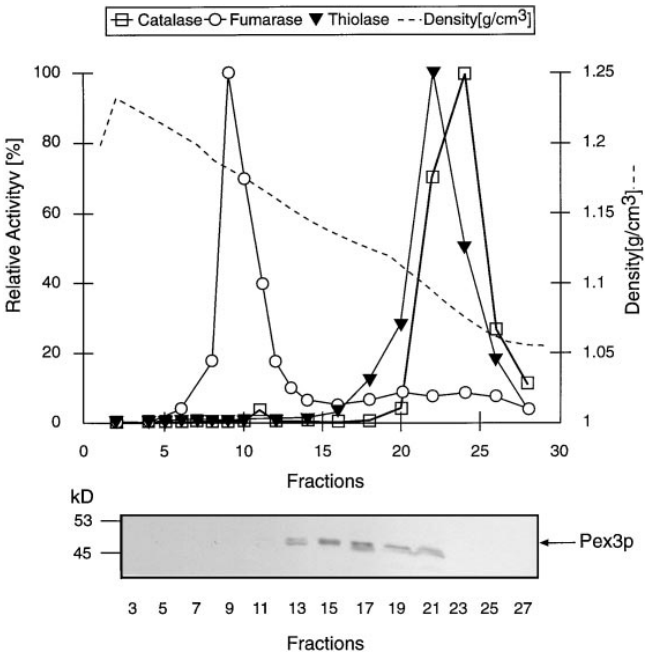

Figure 5. Activities of peroxisomal (catalase and thiolase) and mitochondrial (fumarase) marker enzymes in fractions of a continuous 20–53% sucrose density gradient of cell lysates obtained from *Δpex17* cells. The gradient was drained into 30 1-ml fractions. The lane numbers on the Western blot correspond to the fraction numbers, and 50 μl of the volume of each fraction was loaded per lane. The distribution of Pex3p was determined immunologically. Mitochondria peaked in fraction 9 at the expected density of 1.18 g/cm^3. As for most *pex* mutants, peroxisomal matrix proteins were found on top of the gradient, indicating their cytoplasmic localization. In contrast, the integral peroxisomal membrane protein Pex3p did not colocalize with peroxisomal matrix proteins but was instead predominantly localized to gradient fractions 15–17. This finding indicates an organelle associated localization of the Pex3p.

To address the question of where peroxisomal membranes are located within *Δpex17* cells, we determined the localization of the integral peroxisomal membrane protein Pex3p (Höhfeld et al., 1991) in the gradient fractions. We found Pex3p to be localized in considerable amounts not at the density of mature peroxisomes nor on top of the gradient but exclusively in a density region of 1.15–1.10 g/cm^3 (Fig. 5). This observation is in agreement with published data for the location of Pex3p in another peroxisome-deficient mutant of *S. cerevisiae* (*Δpex4*; Wiebel and Kunau, 1992). Moreover, we were able to show that Pex11p (Erdmann and Blobel, 1995; Marshall et al., 1995) comigrates together with Pex3p (data not shown). In addition, these membrane structures could be floated in a gradient and contain both Pex3p as well as Pex11p (Girzalsky, W., and R. Erdmann, unpublished results).

Micrographs obtained by transmission EM showed that the morphology of *Δpex17* was indistinguishable from that of *pex17-1*, in that no mature nor aberrant peroxisomes were detectable (Fig. 2). The same holds true for most other *pex* mutants of *S. cerevisiae* (Höhfeld et al., 1992). However, the expression of peroxisomal membrane proteins serving as organelle markers allowed the visualization of ghost-like structures either in immunofluorescence or immunoelectron microscopical studies (Purdue and Lazarow, 1995; Erdmann and Blobel, 1996; Albertini et al., 1997). Therefore, we expressed an HA-tagged Pex11p (Erdmann and Blobel, 1995; Marshall et al., 1995) in Δ*pex17*. In indirect double immunofluorescence analysis using anti-HA antibodies in combination with either antithiolase or anti-Pcs60p antibodies, a diffuse staining for thiolase (PTS2 protein) as well as for Pcs60p (PTS1 protein) was obtained (Fig. 6). This distribution reflects a cytoplasmic mislocalization of these enzymes in *Δpex17* (see above). When un-

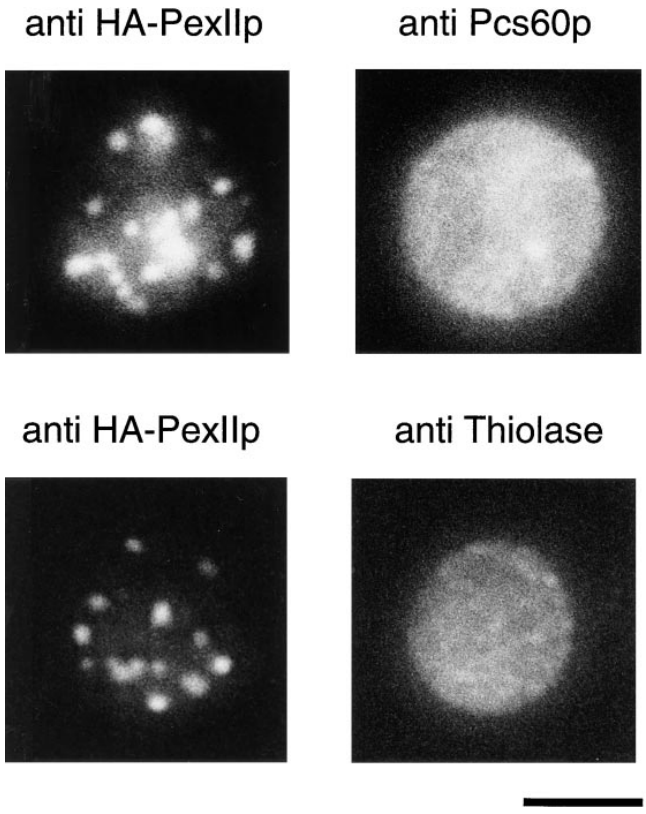

Figure 6. *Δpex17* cells contain peroxisomal membrane ghosts that are unable to import peroxisomal matrix proteins. Double immunofluorescence analysis of *Δpex17* cells expressing an HA-tagged version of Pex11p, a peroxisomal membrane protein (Erdmann and Blobel, 1995). Transformants were grown for 12 h in YNO medium and processed for indirect double immunofluorescence microscopy. Monoclonal antibodies against the HA-tag were used in combination with polyclonal antibodies for Pcs60p (PTS1 protein) or thiolase (PTS2 protein). One representative cell is shown in each row. While Pex11p gave rise to a punctate fluorescence pattern indicative of vesicle-like structures, Pcs60p and thiolase both gave a diffuse staining indicating a cytoplasmic mislocalization of these proteins. Bar, 0.5 μm.

transformed cells were probed with the anti-HA antibody, no staining was observed, indicating the specificity of the used anti-HA antibody. In contrast, a punctate fluorescence pattern was observed upon expression of HA-Pex11p in *Δpex17* cells (Fig. 6). These results correspond well to the previously described staining pattern of import-incompetent peroxisomal membrane structures discovered in other *pex* mutants (Erdmann and Blobel, 1996; Albertini et al., 1997). Taking all data into consideration, we conclude that Pex3p and Pex11p are localized to the membranes of "ghosts" in *Δpex17* cells.

Pex17p Localizes to Peroxisomes

To gain first insights into the function of Pex17p for peroxisome biogenesis, the intracellular location of Pex17p was investigated using antibodies specific for Pex17p. The crude Pex17p antiserum recognized a 25-kD polypeptide in a 25,000-*g* organelle pellet prepared from spheroplasts of

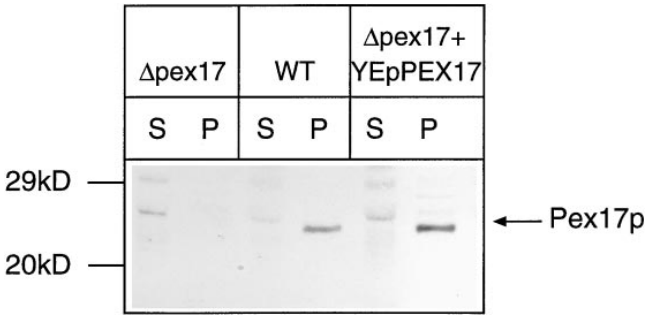

Figure 7. Pex17p is localized to the organelle pellet. In Western blot analysis using affinity-purified anti-Pex17p antibodies, a polypeptide of 23-kD was detected not only in oleate-induced wild-type (*WT*) but also in *Δpex17* cells overexpressing *PEX17* from a multicopy plasmid. In both cases, the protein was exclusively found in the 25,000-*g* pellet fraction. Fractions from *Δpex17* cells served as a negative control.

oleic acid–induced wild-type cells, whereas no such polypeptide was present in a 25,000-*g* organelle pellet of induced *Δpex17* cells (Fig. 7). Moreover, in a 25,000-*g* organelle pellet obtained from *Δpex17* cells expressing *PEX17* from a multicopy plasmid (YEp*PEX17*), the immunoreactive polypeptide was equally well detectable, verifying that the 25-kD protein is indeed Pex17p (Fig. 7). It should be noted that compared to the single copy (genomic) expression in wild-type cells, a substantial increase in the Pex17 protein level was not detectable in cells that express *PEX17* from a multicopy plasmid (Fig. 7). Even when *PEX17* was under the control of the strong, oleate-inducible promoter of the *FOX3* gene (thiolase gene; Einerhand et al., 1991), the amount of Pex17p, as assessed by Western blot analysis, could not be increased (data not shown).

Immunoblot analysis established that in wild-type cells, Pex17p was exclusively present in the organelle pellet enriched for peroxisomes and mitochondria (Fig. 7). From these findings we conclude an organelle associated localization of Pex17p.

To determine the subcellular localization of Pex17p more precisely, a nycodenz density gradient centrifugation was performed. A 25,000-*g* organelle pellet obtained from spheroplasts of induced wild-type cells was fractionated on a 15–35% continuous nycodenz gradient as described by Marzioch et al. (1994). Fractions were collected and assayed for the activities of peroxisomal (catalase) and mitochondrial (cytochrome-c) marker enzymes. In addition, the localization of Pex3p and Pex17p along the gradient was assessed. The result of the biochemical and immunological analysis is shown in Fig. 8. The enzyme profiles indicate a good separation of peroxisomes from mitochondria: peroxisomes sedimented at a density of 1.20 g/cm^3, whereas mitochondria peaked at a density of 1.15 g/cm^3. The second catalase activity peak in fractions with a lower density is most likely due to leakage of peroxisomes during preparation. Western blots with specific antibodies for Pex3p revealed a colocalization of this peroxisomal membrane protein with the catalase activity peak at a density of 1.20 g/cm^3. Pex17p and Pex3p were codistributed along the gradient, classifying Pex17p as a peroxisomal protein.

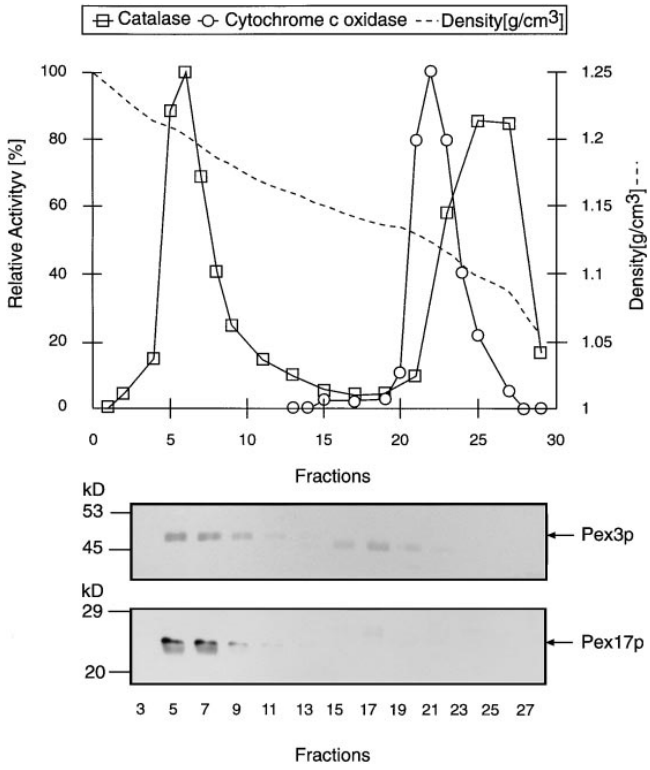

Figure 8. Determination of the subcellular location of Pex17p. Activities of peroxisomal (catalase) and mitochondrial (cytochrome c oxidase) marker enzymes were determined in fractions of a continuous 15–36% nycodenz density gradient of a crude organelle pellet obtained from oleic acid–induced wild-type cells. The gradient was drained into 30 1-ml fractions. The subcellular localization of Pex17p and the integral peroxisomal membrane protein Pex3p were determined immunologically using affinity-purified anti-Pex17p and anti-Pex3p antibodies. Pex17p was found to comigrate with the peroxisomal marker protein catalase and Pex3p, indicating a peroxisomal localization of the protein. 5% of the volume of each fraction was loaded per lane.

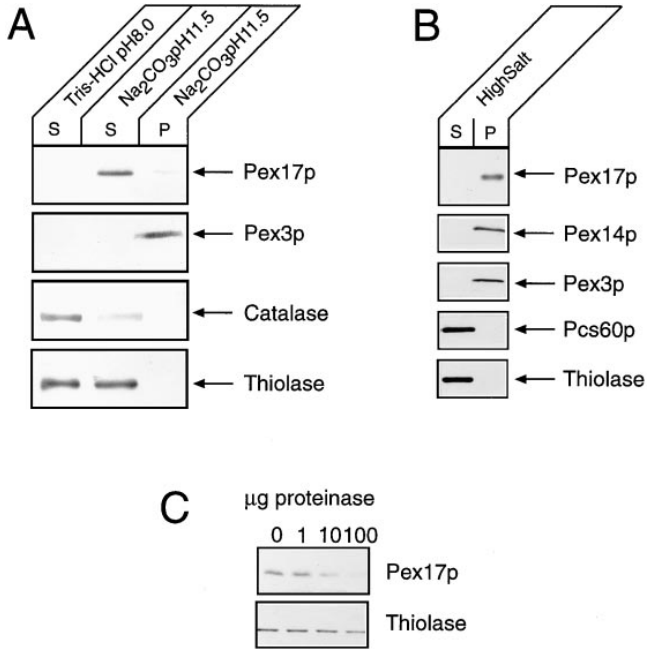

Figure 9. Pex17p is a peripheral peroxisomal membrane protein. (*A*) To separate membrane-bound (*P*) from soluble proteins (*S*), an organelle pellet prepared from oleate-induced wild-type cells was resuspended in 10 mM Tris-HCl, pH 8.0, and spun at 200,000 *g* for 30 min. The resultant pellet was extracted further with 100 mM Na_2CO_3, pH 11.5, to separate peripheral (Na_2CO_3 supernatant [*S*]) from integral membrane proteins (Na_2CO_3 pellet [*P*]). Equal amounts of the fractions were separated by SDS-PAGE and analyzed by Western blot using antibodies against Pex3p, catalase, and thiolase as well as affinity-purified antibodies against Pex17p. Pex17p is completely released from membranes by Na_2CO_3 treatment, suggesting a peripheral localization of the protein. (*B*) After purified peroxisomes were initially extracted with 10 mM Tris-HCl, the resultant membrane fraction was further extracted with a high-salt buffer (500 mM KCl). The supernatant (*S*) contains soluble or loosely membrane-associated proteins, whereas integral or tightly membrane-associated proteins are found in the pellet (*P*). In agreement with their subperoxisomal location, thiolase and Pcs60p were found in the supernatant. In contrast, Pex17p remained associated with membranes, and as such it shows identical extraction characteristics to the peripheral membrane protein Pex14p and the integral membrane protein Pex3p. (*C*) Pex17p is accessible to proteinase K treatment. Isolated peroxisomes were prepared from induced wild-type cells (see Materials and Methods). Equal amounts of isolated peroxisomes were incubated with increasing concentrations of proteinase K in the absence of detergent for 10 min on ice. The reaction was stopped by addition of PMSF (4 mM). Samples were precipitated with TCA and processed for SDS-PAGE. Equal amounts of samples were separated and analyzed by Western blot using specific antibodies for Pex17p and thiolase. While thiolase was resistant to protease, Pex17p was degraded.

Pex17p Is a Tightly Bound Peripheral Membrane Protein

As mentioned above, hydropathy analysis identified a region in the Pex17p amino acid sequence with the potential to span a membrane. To investigate this possibility, we extracted membrane proteins from crude organelle membranes and assayed for the solubility characteristics of Pex17p. The separation efficiency of these procedures was analyzed immunologically by monitoring the distribution of peroxisomal proteins known to reside in different subperoxisomal locations. A 25,000-*g* organelle pellet obtained from spheroplasts of oleic acid–induced wild-type cells was resuspended in 10 mM Tris-HCl, pH 8.0, and centrifuged at 200,000 *g* to separate membrane-bound from soluble proteins. The sediment (containing the membrane proteins) was subsequently subjected to a further extraction with sodium carbonate, pH 11 (Fujiki et al., 1982). As documented in Fig. 9 *A*, Pex17p was resistant to Tris-HCl, pH 8.0 treatment but was completely released from the membranes with sodium carbonate (Fig. 9 *A*). In contrast, the peroxisomal integral membrane protein Pex3p (formerly Pas3p; Höhfeld et al., 1991) was resistant to carbonate extraction. According to their localization in the peroxisomal matrix, thiolase (Erdmann and Kunau, 1994) and catalase were predominantly found in the Tris-HCl supernatant. This finding indicated that Pex17p is a peripheral membrane protein.

In a subsequent experiment, we determined how tightly Pex17p was associated with the peroxisomal membrane.

Therefore, purified peroxisomes were extracted with Tris-HCl to isolate membrane proteins, and the resultant membrane protein pellet was reextracted with a high-salt buffer (500 mM KCl). The samples were analyzed immunologically for the distribution of Pex17p (Fig. 9 *B*). Pcs60p (Blobel and Erdmann, 1996), which associates loosely with the peroxisomal membrane, and thiolase (Fox3p; Erdmann and Kunau, 1994) were efficiently released from the membranes into the supernatant. In contrast, Pex17p was detected in the membrane pellet, suggesting a tight association of Pex17p with its binding partner(s) at the peroxisomal membrane. In this respect it is interesting to note that Pex17p shares the same extraction properties as Pex14p, a recently identified component of the peroxisomal translocation machinery (Albertini et al., 1997).

Pex17p Localizes to the Cytosolic Face of the Peroxisomal Membrane

To determine whether Pex17p is attached to the outer or inner face of the peroxisomal membrane, a protease protection experiment with purified peroxisomes was performed. The proteinase K reaction was stopped after 10 min by the addition of 4 mM PMSF. The protease accessibility of Pex17p and the matrix protein thiolase was analyzed immunologically. Fig. 9 *C* shows the result of a representative experiment. In contrast to thiolase, which was protected against proteinase K degradation, Pex17p was consistently found to be degraded (Fig. 9 *C*). Similar results were obtained when trypsin was used instead of proteinase K (data not shown). From these data, we conclude that Pex17p is a peripheral membrane protein exposed to the cytosol.

Pex17p Interacts with Other Peroxins

Since the Pex17 protein sequence does not provide any functional information on what role Pex17p plays in peroxisome assembly, a yeast two-hybrid screen was performed. This approach has been used very successfully to elucidate the function of Pex14p, a central component of the peroxisomal import channel. To test whether any of the Pex proteins that are analyzed in our laboratory (Kunau et al., 1993) interact with Pex17p, a Gal4Pex17p was transformed into the reporter strain PCY2 in combination with a range of Gal4*pex* constructs. Double transformants were analyzed for reporter gene expression by assaying β-galactosidase activity (Fig. 10 *A*). In two double transformants, Gal4Pex17p\Gal4Pex5p and Gal4Pex17p\Gal4 Pex14p, β-galactosidase activity was detectable in considerable amounts (Fig. 10, *A* and *B*). This finding indicated a functional link between Pex17p and key components of the peroxisomal import machinery.

Albertini et al. (1997) have demonstrated recently that in vivo, Pex7p is part of a complex containing Pex5p, Pex14p, and Pex17p. This result supports the idea that the two-hybrid interaction between Pex17p and both Pex5p and Pex14p is physiologically relevant. To be certain that the Pex17p interactions represent a direct protein–protein interaction and are not mediated by one of the components identified in the in vivo complex, the two-hybrid tests were repeated in isogenic strains deleted for either *PEX14* (PCY2Δ*pex*14), *PEX13* (PCY2Δ*pex*13) or *PEX17*

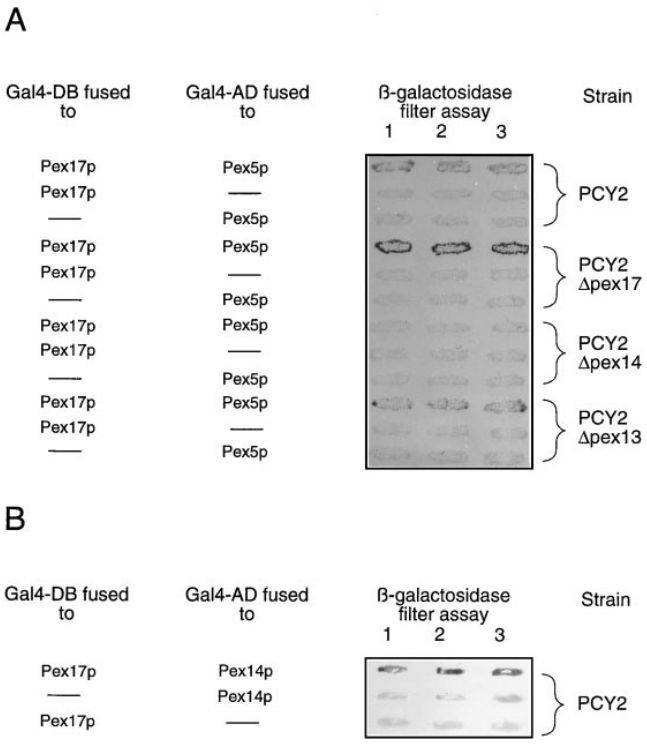

Figure 10. Identification of peroxins that interact with Pex17p in a yeast two-hybrid system. Pex17p fused to the Gal4 DNA-binding domain was tested for interactions with Pex5p (*A*) and Pex14p (*B*) both fused to the Gal4-activation domain. β-Galactosidase assays were carried out in the yeast reporter strain PCY2 or isogenic strains deleted for *PEX13*, *PEX14*, or *PEX17*, respectively. Double transformants expressing the indicated fusion proteins were selected, and β-galactosidase activity was determined by a filter assay using X-gal as a substrate. Three representative independent double transformants are shown (lanes *1–3*).

(PCY2Δ*pex17*). Double transformants were again analyzed for reporter gene expression by assaying β-galactosidase activity. As judged by Western blot analysis, the expression or stability of the Gal4 constructs in the individual gene deletion strains was not affected (data not shown).

In line with previous results, the Pex17p/Pex14p interaction appeared to be unaffected by either one of the three gene deletions (data not shown). However, the interaction between Pex5p and Pex17p was no longer detectable in the strain deleted for *PEX14* (Fig. 10 *A*). An involvement of Pex13p can be excluded since the *PEX*13 deletion did not influence the interaction.

Interestingly, the assayed β-galactosidase activity in the double transformant Gal4Pex17p\Gal4Pex5p was increased in a *Δpex17* background. A plausible explanation is that endogenously expressed Pex17p partially blocks or saturates the Pex17p binding site on Pex14p. As a consequence, only a limited amount of Pex14p is available to mediate the Pex17p/Pex5p interaction.

To confirm by an independent method the conclusion that Pex17p associates with Pex14p and that this interaction does not require Pex13p, we used a functional mycPex7p fusion protein (Marzioch et al., 1997) to isolate a PTS receptor–associated protein complex from different strains. In immunoprecipitates from wild-type and Δpex7

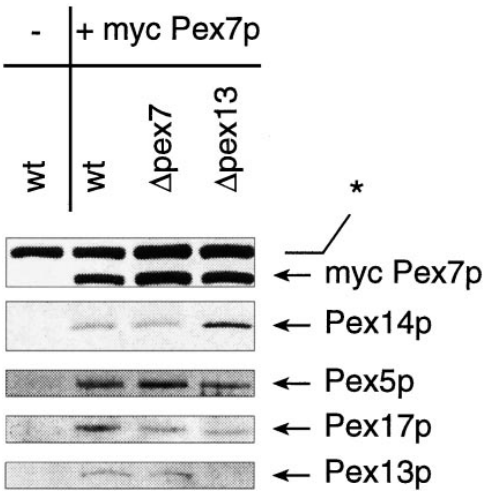

Figure 11. Coimmunoprecipitation of mycPex7p, Pex5p, Pex13p, Pex14p, and Pex17p. Whole-cell extracts of wild-type *(UTL-7A)* cells and of wild-type, *Δpex7*, and *Δpex13* cells expressing myc Pex7p were immunoprecipitated using antibodies against the c-Myc epitope. Equal amounts of immunoprecipitates were separated by SDS-PAGE and subjected to immunoblot analysis using antibodies against c-Myc, Pex14p, Pex13p, Pex5p, and Pex17p. The asterisk indicates the heavy chain of the anti-myc antibodies that were used for precipitation.

cells expressing the mycPex7p fusion protein Pex14p, Pex5p, Pex13p, as well as Pex17p were detected by Western blotting (Fig. 11). When the same precipitation was performed in a *Δpex13* background, we found the same peroxins (with the expected exception of Pex13p) in comparable amounts to wild-type or complemented Δpex7 cells. This result indicates that by using mycPex7p as a bait a receptor associated group of peroxins can be precipitated which includes Pex17p. Thus, suggesting that the association of both receptors with Pex14p as well as the interaction between Pex14p and Pex17p does not require Pex13p. When the precipitates were analyzed for the presence of another peroxisomal membrane protein (Pex3p) no signal was observed. This result indicates the specificity of the interactions indicated by the immunoprecipitation experiment (Fig. 11).

Discussion

There are several key questions concerning how matrix proteins are properly imported into peroxisomes. It is of great importance to define the nature of the peroxins involved and how they physically and functionally interact with each other. Furthermore, how these peroxins interact with the PTS receptors and with the proteins to be imported needs to be established. As a first step to tackle these questions, a yeast two-hybrid system was used successfully to identify interacting peroxins. The physiological relevance of these interactions was subsequently confirmed by coimmunoprecipitation. These and other studies led to the discovery of Pex13p (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996) and Pex14p (Albertini et al., 1997) as the first two components of the peroxisomal translocation machinery.

Here we report the isolation of the *PEX17* gene by functional complementation. Pex17p is required for the biogenesis of peroxisomes and therefore can be classified as a peroxin (Distel et al., 1996). Lack of Pex17p compromises both PTS-dependent import pathways for matrix proteins and as a consequence leads to the absence of morphologically detectable peroxisomes in *pex17* null mutant cells.

Recent evidence from different laboratories suggests that the insertion of peroxisomal membrane proteins occurs independently of the matrix protein import pathways (for review see Subramani, 1996). This finding also appears to be true for *Δpex17* cells. Localization experiments using the integral peroxisomal membrane proteins Pex3p (Höhfeld et al., 1991) and Pex11p (Erdmann and Blobel, 1995; Marshall et al., 1995) as markers indicated the presence of peroxisomal membrane "ghosts" in these cells (Figs. 5 and 6). We conclude that Pex17p plays a role in matrix protein import and not in peroxisomal membrane formation.

We demonstrate that Pex17p is a peripheral membrane protein that is tightly bound to the outer face of the peroxisomal membrane. Subfractionation studies revealed that Pex17p cosedimented with peroxisomes (Figs. 7 and 8). Computer analyses of the Pex17p sequence by independent algorithms predicted a transmembrane domain in the amino terminus of the protein (Fig. 4). However, membrane extraction experiments performed with purified peroxisomes demonstrated that Pex17p is a peripheral membrane protein since it was not extractable from the membranes with Tris-HCl but completely released upon alkaline treatment (Fig. 9). Moreover, washes with high salt established that Pex17p is tightly associated with the peroxisomal membrane. Further support for this conclusion comes from protease protection experiments that were designed to determine the topology of Pex17p at the peroxisomal membrane. In this experiment, Pex17p was not only accessible to proteinase K but was completely degraded, implying the lack of a membrane anchor (Fig. 9).

A strong candidate to serve as a binding site for Pex17p at the peroxisomal membrane is Pex14p, a peroxin that has recently been identified as a peripheral peroxisomal membrane protein located at the outer face of the organelle (Albertini et al., 1997). Several findings support the hypothesis that Pex14p anchors Pex17p. First of all, both proteins share the same subperoxisomal localization at the membrane. Secondly, a physical interaction of these proteins in vivo was detected in a yeast two-hybrid system (Fig. 10). Finally, immunoprecipitation studies in which Pex17p and Pex14p were found in the same precipitate (Fig. 11) corroborate the physiological relevance of the two-hybrid data. Taken together, these findings strongly suggest that Pex14p and Pex17p bind to each other at the outer face of the peroxisomal membrane. In this respect, it is interesting to note that despite the use of different expression systems to overexpress *PEX17*, e.g., under the control of the *FOX3* promoter (data not shown) or from a multicopy plasmid (Fig. 7), a significant increase in Pex17 protein levels was not achieved. This observation may indicate that association of Pex17p with the peroxisomal membrane is stoichiometrically dependent on another protein. Interestingly, in support of this the amount of Pex17p in *pex14* null mutant cells is drastically decreased (Rehling, P., and W.-H. Kunau, manuscript in preparation).

A very intriguing observation of this study is that in the yeast two-hybrid system Pex17p interacted with the PTS1 receptor, Pex5p, in a Pex14p-dependent manner (Fig. 10). An interpretation of these findings is that Pex5p and Pex17p have adjacent binding sites on Pex14p and that Pex14p bridges an interaction between these proteins. Alternatively, it is also possible to envisage that Pex14p induces a conformational state in Pex17p that allows it to associate with Pex5p. Both models, however, implicate direct binding of Pex14p to Pex17p.

This data strongly argues that Pex17p is directly involved in peroxisomal matrix protein import and is the third component of the recently identified protein translocation complex at the peroxisomal membrane. The other constituents of this complex, Pex13p and Pex14p (Albertini et al., 1997), both bind the PTS1 receptor, and Pex14p also binds the PTS2 receptor. On the basis of these properties, Pex14p was proposed to be the point of convergence for the two PTS-dependent import pathways, indicating that both use a common translocation machinery. The notion that Pex17p is an essential component of this translocation machinery is supported by the fact that its deficiency impairs both import pathways.

As we show in this paper, Pex17p clearly plays a role in the protein import through the PTS1 and PTS2 pathway, presumably via its interaction with Pex14p. However, at present it is not known whether the components of the proposed translocation machinery form a stable or transient complex (Albertini et al., 1997). The fact that the deletion of Pex13p did not prevent the interaction between Pex7p, Pex5p, and Pex17p with Pex14p serving as a bridging molecule (Figs. 10 and 11) suggests that just these four peroxins have the potential to form a heterooligomeric complex even in the absence of Pex13p. Moreover, our data suggests that Pex13p is not a prerequisite for binding of the two PTS receptors to Pex14p. Whether Pex13p is responsible for complex dissociation is another aspect that needs to be addressed. It is tempting to speculate that Pex17p regulates the affinity of Pex14p for its binding partners, and thereby could play a key role in the dynamics of any complex formed during peroxisomal protein import. Our laboratory is currently investigating this model. Further studies will have to clarify the importance of Pex17p for the formation of the import complex and hence increase our understanding of peroxisome biogenesis.

We are indebted to M. Bürger and S. Wüthrich for technical assistance. We are grateful to Ralf Erdmann and all members of our lab for stimulating discussions and critical comments on the manuscript. We thank F. Mitchell (Institute of Cancer Research, London, UK) for her helpful comments on the manuscript. We would like to thank P. Chevray (The Johns Hopkins University School of Medicine, Baltimore, MD) for two-hybrid plasmids.

This work was supported by the Deutsche Forschungsgemeinschaft (KU-329/17-1 and 329/17-2) and by the Fond der Chemischen Industrie.

Received for publication 1 August 1997 and in revised form 10 November 1997.

References

Albertini, M., P. Rehling, R. Erdmann, W. Girzalsky, J.A.K.W Kiel, M. Veenhuis, and W.-H Kunau. 1997. Pex14p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. *Cell.* 89: 83–92.

Ausubel, F.J., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1992. Current Protocols in Molecular Biology. Greene Publishing Associates, New York.

Blobel, F., and R. Erdmann. 1996. Identification of a peroxisomal member of the AMP-binding protein family. *Eur. J. Biochem.* 240:468–476.

Chevray, P.M., and D. Nathans. 1992. Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. *Proc. Natl. Acad. Sci. USA.* 89:5789–5793.

Coster, F., L. Van Dyck, J.L. Jonniaux, B. Prunelle, and A. Goffeau. 1995. The sequence of a 13.5 kb DNA segment from the left arm of chromosome XIV reveals MER1; RAP1; a new putative member of the DNA replication complex and a new putative serine/threonine phosphatase gene. *Yeast.* 11:95–91.

Crane, D.I., J.E. Kalish, and S.J. Gould. 1994. The Pichia pastoris *PAS4* gene encodes a ubiquitin-conjugation enzyme required for peroxisome assembly. *J. Biol. Chem.* 269:21835–21844.

De Hoop, M.J., and G. Ab. 1992. Import of proteins into peroxisomes and other microbodies. *Biochem. J.* 286:657–669.

Distel, B., R. Erdmann, S.J. Gould, G. Blobel, D.I. Crane, J.M. Cregg, G. Dodt, Y. Fujiki, J.M. Goodman, W.W. Just, et al. 1996. A unified nomenclature for peroxisome biogenesis. *J. Cell Biol.* 135:1–3.

Dodt, G., and S.J. Gould. 1996. Multiple *PEX* genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. *J. Cell Biol.* 135: 1763–1774.

Einerhand, A.W.C., T.M. Voorn-Brouwer, R. Erdmann, W.-H. Kunau, and H.F. Tabak. 1991. Regulation of transcription of the gene coding for peroxisomal 3-oxoacyl CoA thiolase of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 200:113–122.

Eisenberg, E., E. Schwarz, M. Komaromy, and R. Wall. 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* 179:125–142.

Elgersma, Y., and H.F. Tabak. 1996. Proteins involved in peroxisome biogenesis and functioning. *Biochim. Biophys. Acta.* 1286(3):269–283.

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

Erdmann, R. 1994. The peroxisomal targeting signal of 3-oxoacyl-CoA thiolase from *Saccharomyces cerevisiae*. *Yeast.* 10:935–944.

Erdmann, R., and G. Blobel. 1995. Giant peroxisomes in oleic acid-induced *Saccharomyces cerevisiae* lacking the peroxisomal membrane protein Pmp27p. *J. Cell Biol.* 128:509–523.

Erdmann, R., and G. Blobel. 1996. Identification of a peroxisomal membrane receptor for the COOH-terminal tripeptide signal recognition factor. *J. Cell Biol.* 135:111–121.

Erdmann, R., and W.-H. Kunau. 1994. Purification and immunolocalization of the peroxisomal 3-oxoacyl-CoA thiolase from *Saccharomyces cerevisiae*. *Yeast.* 10:1173–1182.

Erdmann, R., M. Veenhuis, D. Mertens, and W.-H. Kunau. 1989. Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 86:5419–5423.

Erdmann, R., M. Veenhuis, and W.-H. Kunau. 1997. Peroxisomes: organelles at the cross-road. *Trends Cell Biol.* 7:400–407.

Fields, S., and O.K. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature.* 340:245–246.

Fujiki, Y., A.L. Hubbard, S. Fowler, and P.B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: applications to the endoplasmic reticulum. *J. Cell Biol.* 93:97–102

Gietz, R.D., and A. Sugino. 1988. New yeast-*E. coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene.* 74:527–534.

Gould, S.J., G.A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108:1657–1664.

Gould, S.J., J.E. Kalish, J.C. Morrell, J.B. Jorkman, A.J. Urquhart, and D.I. Crane. 1996. An SH3 protein in the peroxisome membrane is a docking factor for the PTS1 receptor. *J. Cell Biol.* 135:85–95.

Harlow, E., and D. Lane. 1988. Antibodies—A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Höhfeld, J., M. Veenhuis, and W.H. Kunau. 1991. *PAS3*, a *Saccharomyces cerevisiae* gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. *J. Cell Biol.* 114:1167–1178.

Höhfeld, J., D. Mertens, F.F. Wiebel, and W.-H. Kunau. 1992. Defining components required for peroxisome assembly in *Saccharomyces cerevisiae*. *In* New Comprehensive Biochemistry Series: Membrane Biogenesis and Protein Targeting. W. Neupert and R. Lill, editors. Elsevier Science Publishing Co., New York. 185–207.

Jones, J.S., and L. Prakash. 1990. Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast.* 6:363–366.

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

Komori, M., S.W. Rasmussen, J.A.K.W. Kiel, R.J.S. Baerends, J.M. Cregg, I.J. van der Klei, and M. Veenhuis. 1996. The Hansenula polymorpha *PEX*14 gene encodes a novel peroxisomal membrane protein involved in matrix protein import. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:44–53.

Kragler, F., A. Langeder, J. Raupachova, M. Binder, and A. Hartig. 1993. Two

independent peroxisomal targeting signals in catalase A of *Saccharomyces cerevisiae*. *J. Cell Biol.* 120:665–673.
Kunau, W.-H., A. Beyer, T. Franken, K. Götte, M. Marzioch, J. Saidowsky, A. Skaletz-Rorowski, and F.F. Wiebel. 1993. Two complementary approaches to study peroxisome biogenesis in *Saccharomyces cerevisiae*: forward and reversed genetics. *Biochimie (Paris)*. 75:209–224.
Kyte, J., and R.F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.
Lazarow, P.B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489–530.
Lupas, A. 1996. Prediction and analysis of coiled-coil structures. *Methods Enzymol.* 266:513–525.
Marshall, P.A., Y.I. Krimkevich, R.H. Lark, J.M. Deyer, M. Veenhuis, and J.M. Goodman. 1995. PMP27 promotes peroxisome proliferation. *J. Cell Biol.* 129:345–355.
Marzioch, M., R. Erdmann, M. Veenhuis, and W.-H. Kunau. 1994. *PAS7* encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4908–4918.
McNew, J.A., and J.M. Goodman. 1996. The targeting and assembly of peroxisomal proteins: some old rules do not apply. *Trends Biochem. Sci.* 21:54–58.
Moreno de la Garza, M., U. Schulz-Borchard, J.W. Crabb, and W.H. Kunau. 1985. Peroxisomal β-oxidation system of *Candida tropicalis*. *Eur. J. Biochem.* 148:285–291.
Motley, A., E. Hettema, B. Distel, and H. Tabak. 1994. Differential protein import deficiencies in human peroxisome assembly disorders. *J. Cell Biol.* 125: 755–767.
Purdue, P.E., and P.B. Lazarow. 1995. Identification of peroxisomal membrane ghosts with an epitope-tagged integral membrane protein in yeast mutants lacking peroxisomes. *Yeast*. 11:1045–1060.
Rachubinski, R.A., and S. Subramani. 1995. How to penetrate peroxisomes. *Cell.* 83:525–528.
Rehling, P., M. Albertini, and W.-H. Kunau. 1996*a*. Protein import into peroxisomes: new developments. *In* Peroxisomes: Biology and Role in Toxicology and Disease. *Ann. NY Acad. Sci.* 804:34–46.
Rehling, P., M. Marzioch, F. Niesen, E. Wittke, M. Veenhuis, and W.-H. Kunau. 1996*b*. The import receptor for the peroxisomal targeting signal 2 PTS2 in *Saccharomyces cerevisiae* is encoded by the *PAS7* gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2901–2913.
Remaut, E., P. Stanssens, and W. Fiers. 1981. Plasmid vectors for high-efficiency expression controlled by the pL promotor of coliphage Lambda. *Gene.* 15:81–93.
Roa, M.J.K., and P. Argos. 1986. A conformational preference parameter to predict helices in integral membrane proteins. *Biochim. Biophys. Acta.* 869: 197–214.
Rose, M.D., P. Novick, J.H. Thomas, D. Botstein, and G.R. Fink. 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene.* 60:237–243.
Rout, M.P., and J.V. Kilmartin. 1990. Components of the yeast spindle pole body. *J. Cell. Biol.* 111:1913–1927.
Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 74:5463–5467.
Sherman, F., G.R. Fink, and C.W. Lawrence. 1979. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Slawecki, M.L., G. Dodt, S. Steinberg, A.B. Moser, H. Moser, and S.J. Gould. 1995. Identification of three distinct peroxisomal protein import defects in patients with peroxisome biogenesis disorders. *J. Cell Sci.* 108:1817–1829.
Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol.* 9:445–478.
Subramani, S. 1996. Protein translocation into peroxisomes. *J. Biol. Chem.* 271: 32483–32486.
Subramani, S. 1997. *PEX* genes on the rise. *Nat. Genet*. 15:331–332.
Veenhuis, M., M. Mateblowski, W.-H. Kunau, and W. Harder. 1987. Proliferation of microbodies in *Saccharomyces cerevisiae*. *Yeast.* 3:77–84.
Wach, A., A. Brachat, R. Poehlmann, and P. Philippsen. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*. 10:1793–1808.
Wiebel, F.F., and W.-H. Kunau. 1992. The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. *Nature.* 359:73–76.