A Mobile PTS2 Receptor for Peroxisomal Protein Import in *Pichia pastoris*

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Abstract. Using a new screening procedure for the isolation of peroxisomal import mutants in *Pichia pastoris*, we have isolated a mutant (*pex7*) that is specifically disturbed in the peroxisomal import of proteins containing a peroxisomal targeting signal type II (PTS2). Like its *Saccharomyces cerevisiae* homologue, *PpPex7p* interacted with the PTS2 in the two-hybrid system, suggesting that *Pex7p* functions as a receptor. The *pex7Δ* mutant was not impaired for growth on methanol, indicating that there are no PTS2-containing enzymes involved in peroxisomal methanol metabolism. In contrast, *pex7Δ* cells failed to grow on oleate, but growth on oleate could be partially restored by expressing thiolase (a PTS2-containing enzyme) fused to the PTS1.

Because the subcellular location and mechanism of action of this protein are controversial, we used various methods to demonstrate that *Pex7p* is both cytosolic and intraperoxisomal. This suggests that *Pex7p* functions as a mobile receptor, shuttling PTS2-containing proteins from the cytosol to the peroxisomes. In addition, we used *PpPex7p* as a model protein to understand the effect of the *Pex7p* mutations found in human patients with rhizomelic chondrodysplasia punctata. The corresponding *PpPex7p* mutant proteins were stably expressed in *P. pastoris*, but they failed to complement the *pex7Δ* mutant and were impaired in binding to the PTS2 sequence.

The sorting of proteins to distinct subcellular compartments is achieved by the coordinated action of organelle-specific targeting signals and receptors. Studies on protein targeting across biological membranes have uncovered two general paradigms for the action of targeting signal receptors. In certain cases, such as the SecA/B-dependent secretion of proteins across the bacterial inner membrane and the signal recognition particle (SRP)-dependent insertion of proteins across ER membranes or transport across the nuclear pore, the targeting signal is recognized by a mobile receptor that typically recognizes the signal in the cytosol and then shuttles the cargo to the target organelle (for review see Schatz and Dobberstein, 1996; Görlich and Mattaj, 1996). These mobile receptors cycle either between the cytosol and the organelle membrane, as seen for SecA/B and SRP, or they cycle into and out of the organelle, as observed for importin and transportin. In other instances, such as mitochondrial (Schatz and Dobberstein, 1996) and chloroplast import (Schnell, 1995), the receptors are located on the target membrane (membrane-associated receptors), where they bind the targeting signal but their mobility is believed to be limited to the plane of the membrane.

Peroxisomal matrix proteins are synthesized on free polyribosomes and are posttranslationally imported into the peroxisome (for review see Lazarow and Fujiki, 1985). Sorting to peroxisomes requires the presence of a peroxisomal targeting signal (PTS). Most peroxisomal matrix proteins contain a PTS1 sequence that consists of the COOH-terminal tripeptide SKL or other variants (Gould et al., 1987, 1989). A second peroxisomal targeting signal (PTS2) was first identified in the NH2 terminus of rat peroxisomal thiolase (Osumi et al., 1991; Swinkels et al., 1991) and has been identified in several other proteins since (for review see Elgersma and Tabak, 1996). Alignments and site-directed mutagenesis of these proteins suggest a consensus PTS2
sequence of R/K-L/V/I-X,R-H/Q-L/A (Gietl et al., 1994; Glover et al., 1994a; Tsukamoto et al., 1994).

Additional evidence for at least two pathways for peroxisomal protein import was provided by cloning of the PTS1 and the PTS2 receptors. The PTS1 receptor, Pex5p, has seven tetra-tricopeptide repeat (TPRs) in the COOH-terminal half of the protein, but surprisingly, it lacks a hydrophobic domain that could anchor the protein in the peroxisomal membrane (McCollum et al., 1993; Van der Leij et al., 1993; Dodt et al., 1995; Fransen et al., 1995; Nuttley et al., 1995; Szilard et al., 1995; Van der Klei et al., 1995; Wiemer et al., 1995). There has been much debate about the localization of Pex5p, but current evidence suggests that in most organisms, at least a fraction of this protein is cytosolic (Dodt et al., 1995; Van der Klei et al., 1995; Wiemer et al., 1995; Elgersma et al., 1996; Gould et al., 1996), while the rest is peroxisome associated. This led to the proposal that Pex5p functions as a mobile receptor, shuttling between the cytosol and the peroxisome. This model is further supported by the characterization of the SH3 domain–containing peroxisomal membrane protein, Pex13p, and another protein, Pex14p, which serve as docking proteins for Pex5p (Elgersma et al., 1996a; Erdmann and Blobel, 1996; Gould et al., 1996; Albertini et al., 1997). Furthermore, kinetic evidence for Pex5p as a mobile receptor has been uncovered recently (Dodt and Gould, 1996).

The Saccharomyces cerevisiae (Sc) pex7 mutant is characterized by the mislocalization of thiolase, whereas import of other proteins analyzed is unaffected. A phenotype analogous to that of the pex7 mutant has also been described for a human patient cell line (Motley et al., 1994; Slawecck et al., 1995). Cloning of the yeast, and more recently the human, PEX7 genes led to the identification of the PTS2 receptor (Pex7p; Marzioch et al., 1994; Zhang and Lazarow, 1995; Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997). Pex7p belongs to the WD-40 repeat (β-transducin) family, which is characterized by the presence of a 43–amino acid repeat domain. Like TPRs, the WD repeats are probably involved in multiple protein–protein interactions (Komachi et al., 1994). Interestingly, a functional relationship between proteins belonging to the TPR and the β-transducin (WD-40) families has been described (for review see Goebi and Yanagida, 1991; Van der Voorn and Ploegh, 1992). Although it is not yet clear whether Pex5p and Pex7p interact directly, evidence exists that they share a common import machinery. In humans, it has been shown that a certain cell line that is defective in the PEX5 gene is not only deficient in the import of PTS1-containing proteins, but is also deficient in the import of PTS2-containing proteins (Dodt et al., 1995; Albertini et al., 1997). Moreover, a peroxin (Pex) has been identified (ScPex14p) that is required for both import pathways and which interacts with both Pex5p and Pex7p (Albertini et al., 1997).

The location of Pex7p is not clear. An HA-tagged Pex7p (Pex7p-HA3) was found to be entirely intraperoxisomal in S. cerevisiae, whereas a Myc-tagged (overexpressed) ScPex7p (Myc-Pex7p) was predominantly cytosolic (Marzioch et al., 1994; Zhang and Lazarow, 1995). Human Myc-Pex7p expressed from the strong cytomegalovirus (CMV) promoter was found predominantly in the cytosol, and no peroxisome-associated Pex7p was detected (Braverman et al., 1997). Because antibodies suitable for studying endogenous Pex7p are lacking, it is not clear which of these tagged or overexpressed proteins reflects its real location.

The model for the action of Pex7p as a receptor has remained elusive because of uncertainties regarding the precise subcellular location of this protein. The cytosolic localization of epitope-tagged yeast or human Pex7p has been cited as evidence for a mobile receptor, shuttling between the cytosol and the peroxisome (Marzioch et al., 1994; Rehling et al., 1996; Braverman et al., 1997). In contrast, the presence of an intraperoxisomal receptor that acts from within the organelle to pull proteins in would be novel in comparison with the existing paradigms for targeting signal receptors described earlier (Zhang and Lazarow, 1995, 1996).

The finding that the PTS2 import pathway may not be induced in methanol-grown Hansenula polymorpha (Farber et al., 1994) suggests that this pathway may not be required for growth on methanol. This may explain why despite elaborate screening procedures, mutants deficient in the PTS2 import pathway have not yet been described in methylotrophic yeasts, since the primary screening was performed by selecting mutants that were unable to grow on methanol (Cregg et al., 1990; Gould et al., 1992; Liu et al., 1992). To obtain more insight into the mechanism of import of PTS2-containing proteins in Pichia pastoris, we have used a new screening procedure that is more selective for the PTS2 import pathway. We have isolated the P. pastoris (Pp) PEX7 gene, studied the function and localization of endogenous PpPex7p, and examined the use of the PTS2-dependent import pathway upon growth of P. pastoris on different carbon sources. Furthermore, because recent studies have identified several mutations in human Pex7p that are responsible for the disease rhizomelic chondrodysplasia punctata (RCDP; Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997), we used the corresponding mutations in PpPex7p to provide insights regarding why the mutant proteins were nonfunctional.

Materials and Methods

Strains and Media

P. pastoris strains used in this study were as follows: PPY12 (arg4, his4; parental wild-type strain); pex7.1 (his4, pex7, ARG4-pTW84 (PTS2-GFP (S65T))/GAPDH); PEX7 (his4, pex7, PpARG4); and fox3∆ (his4, fox3::PpARG4). S. cerevisiae strains used in this study were as follows: BJ1991 (MATα, leu2, trp1, ura3-251, prb1-1122, pep4-5) (wild-type strain); pex7 (pex7, ura3-251, leu2, his3); and L40 (MATa, his3Δ200, trp1-901, leu2-3,112, aude2, lys2::(lexAop)- HIS3, URA3::(lexAop)-lacZ (for two-hybrid assays). Excherichia coli strains used in this study were as follows: DH5a (recA, hisdR, supE, endA, gyrA96, thi-1, relA, lacZ; for cloning procedures), and S131000 (sul2’, strr’, rifr’, lac’a, gal’, mtl’, F-, recA-; for protein expression).

Minimal methanol contained the following: 0.25% (NH4)2SO4, 0.02% MgSO4, 0.39% NaH2PO4, 0.05% yeast extract, 0.1% (vol/vol) Vinnich minerals mix, 0.1% vitamins, 20 mg/ml amino acids as needed, and 0.5% methanol as carbon source. Minimal oleate medium contained 0.2% oleate and 0.02% Tween 40 as carbon source. Minimal oleate plates were made according to Elgersma et al. (1993). Other media used in this study were described by Wiemer et al. (1996).

Isolation of pex Mutants

A culture of P. pastoris PPY12+pTW84 (PTS2-GFP(S65T))/GAPDH,
PTS2-BLE/GAPDHJ) cells were grown for 15 h on rich glucose medium (YPD), diluted 12-fold in 600 ml YPD, and grown for an additional 8 h. Cells were pelleted and washed twice with water and once with 100 mM sodium citrate, pH 5.5. The cells were then resuspended in 150 ml 100 mM Na-citrate, pH 5.5, and 25-ml batches were treated with concentrations (0-2%) N-methyl-N-nitro-nitrosoguanidine (NTG) for 30 min. Mutagenized cells were washed three times with 100 mM sodium citrate, pH 5.5, resuspended in 20 ml YPD, and recovered for 2 h. Small aliquots were used to determine the survival rate, and the remainder was frozen in glycerol at -80°C. Cells with an NTG survival of 4% were used to inoculate 50 ml YPD and were grown for 24 h. These cells were pelleted by centrifugation at 2,500 × g, washed with water, and transferred to 300 ml rich oleate medium (YPO) and induced for 15 h. These cells were collected, resuspended in 250 ml YPO, aliquoted in five portions of 50 ml, and induced in oleate medium for an additional 2 h. Additional amounts of phleomycin were added to these cultures (to concentrations of 0–100 μg/ml), and the cells were incubated for 3 h at 30°C. The phleomycin-treated cells were washed twice with water and resuspended in YPD to recover for 2 h at 30°C. Cells were diluted and plated on YPD to determine the survival rate. Colonies obtained from the batch showing a 5% survival rate were tested for growth on minimal oleate and methanol medium, and they were analyzed for PTS2-green fluorescent protein (GFP) expression by fluorescence microscopy.

Cloning of the PpPex7 and PpPAT1 Genes

A genomic library was transformed into pex7.1 cells. Three different plasmids (pM1, pM2, and pM3), which restored growth on oleate, were isolated. Subclone analysis revealed that pM3 contained ~1 kb of the 5' end of a gene (PpPAT1) that is able to suppress the pex7.1 phenotype. pM2 and pM3 had an overlapping fragment of 2.8 kb. This 2.8-kb insert contained the PpPex7 gene, which was sequenced on both strands. The PpPex7 sequence data have been submitted to the GenBank database under accession number AF021797.

Construction of Plasmids Expressing PpPex7p

A 2.6-kb genomic fragment containing the PEX7 gene was cloned in a three-fragment ligation, as a Sall-EcoRI and EcoRI-BamHI fragment in a Sall/BamHI-digested pUC18, resulting in pM19. The PEX7 gene knockout was obtained by cloning the PyAR1 gene as a blunt fragment in the blunt NsiI sites (86 bp upstream of the ATG and 28 bp downstream of the stop codon of PEX7) of pM19. The poly linker of pUC18 was modified by ligating adaptors P43 and P44 (see Table I) into the EcoRI and BamHI sites so that these sites were destroyed and the BglII, KpnI, EcoRV, NsiI, and XbaI sites were introduced (pM24). The PEX7 gene was amplified by PCR using primers P33 and P34, thereby introducing a KpnI site immediately upstream of the ATG, and was cloned as a KpnI-NsiI fragment in pM24. To eliminate PCR errors, the BamHI-Nsi fragment was replaced by the BamHI-Nsi fragment of the genomic clone, and the remainder of the PEX7 gene was sequenced. The PEX7 gene on this plasmid (pM27) was used for further manipulations.

For NH tagging, a linker encoding the NH tag (primers P26/P28) was ligated in the EcoRI site of pUC18, resulting in pEL210. PEX7 was cloned as a KpnI-NLS fragment from pM27 in the Km/PstI sites of pEL210, resulting in pM22. The NH-tagged PEX7 was cloned downstream of the ACOI promoter in the BglII/EcoRI-digested pTW72 (a pHILD2-based plasmid in which the alcohol oxidase promoter was replaced by the ACOI promoter), using the BglIII/HindIII fragment from pM22 and EcoRI/ HindIII adaptor (P49/P50), resulting in plasmid pM39. The non-tagged gene was cloned downstream of the ACOI promoter by replacing the BglII fragment from pM27 (encoding NH-PpPex7) with another fragment from pM27 (encoding PEX7), resulting in pM70. The ACOI promoter was replaced by the endogenous PEX7 promoter by replacing the NdeI/BamHI fragment from pM39, containing the ACOI promoter, with the genomic Xhol/BamHI fragment and the NdeI/XhoI adapter (P122/P123), resulting in pM78.

To introduce the C347R mutation, we ligated linker P124/P125 in an EcoRI/Sall-digested pM27, resulting in pM75, and sequenced the inserted linker. To introduce the G249V and A248R point mutations in PEX7, we designed primers that introduced the desired mutation and a unique restriction site at the location of the mutation. The 5' part of the gene was amplified by PCR with primers P32 (internal primer just upstream of the BamHI site) and P128 (G249V) or P126 (A248R). The 3' part of the gene was amplified by PCR with primers P35 (a primer just downstream of PEX7) and P129 (G249V) or P127 (A248R). The PCR fragments corresponding to the 5' segments of PEX7 were digested with BamHI and PstI (G249V) or BamHI and XbaI (A248R), and the PCR fragments corresponding to the 3' segments of PEX7 were digested with PstI and EcoRI (G249V) or XbaI and EcoRI (A248R). These fragments were ligated in a three-fragment ligation into the BamHI/EcoRI-digested pM27, resulting in pM76 (G249V) and pM77 (A248R). The BamHI/EcoRI inserts were sequenced. The mutated PEX7 genes were cloned as BamHI/Sall fragments, downstream of the ACOI promoter in a BamHI/XhoI-digested pM70, resulting in pM58 (C347R), pM86 (G249V), and pM87 (A248R). The mutated PEX7 genes were cloned as BamHI/Sall fragments downstream of the PEX7 promoter in a BamHI/XhoI-digested pM78, resulting in pM82 (C347R), pM83 (A248R), and pM84 (G249V).

PpFox3-SKL was amplified by PCR using primers based on the sequence of the PpFOX3 gene so that a BamHI site was introduced directly upstream of the ATG, and an SKL sequence and an XbaI site were added at the 3' end of the gene. The PCR product was digested with BamHI and XbaI and was cloned downstream of the ACOI promoter between the BglII/Sall sites of pM39.

For the yeast two-hybrid analysis, plasmids were generated that expressed appropriate protein fusions to the DNA binding (DB) domain of LexA, and the activation domain (AD) of VP16. The LexA(DB)-ScFox3p (DB-ScFox3p) fusion protein was made by cloning ScFOX3 as a BamHI/XhoI fragment in pBTM116B, resulting in pM35. pBTM116 (Bartlett et al., 1995) was modified by introducing a poly linker in all three open reading frames, resulting in pBTM116A, B, and C. The DB-ScPTS2 fusion protein was made by digesting pM35 with NciI/Sall, followed by a filling-in reaction using Klenow polymerase and religation, thereby deleting the PTS2 signal of ScFOX3 (pM51). The AD-PpPex7p fusion protein was made by cloning PpPEX7 as a BglII/Sall fragment from pM27 in the BamHI/Sall-digested pVP16C, resulting in pM34. pVP16 (Hollenberg et al., 1995) was modified by introducing a polylinker in all three open reading frames, resulting in pVP16A, B, and C. The RCDP mutations were introduced in the two-hybrid vector as BamHI/Sall fragments into a BamHI/Sall-digested pM34, resulting in pM79 (PpPex7p(G249V)), pM80 (PpPex7p(A248R)), and pM81 (PpPex7p(C347R)).

Raising Antibodies against PpPex7p

To raise antibodies against PpPex7p, we cloned the DNA encoding amino acids 1–143 of PpPex7p as a KpnI-HpaI fragment from pM27 into pQE30 (Qiagen Inc., Chatsworth, CA), which was first digested with HindIII, blunted with Klenow polymerase, and subsequently digested with KpnI. The (His)_6-tagged protein was expressed in E. coli SG13009 and purified

Table I. Oligonucleotides Used in This Study

| Primer | Sequence |
|--------|----------|
| P26    | 5'-AATTCAGGCTCATGCAAACCTTGCAAGAAATGCA-3' |
| P28    | 5'-AATTCGGGACCTACCTGTGCGTGGTCTTTTCG-3' |
| P32    | 5'-CTTGGTGTGACTAGCTGTCG-3' |
| P33    | 5'-AACCTGGTGACACTATCGACTACGG-3' |
| P34    | 5'-GGTACCATGTTTGAATGTCCAAAC-3' |
| P35    | 5'-GGCAGGAGATGTATCTAACCCCC-3' |
| P43    | 5'-AATGATTGCTGAGCATGATCATCGACTTACAC-3' |
| P49    | 5'-AGCTTACTAGTCTAGGCCCCCTTCCGAG-3' |
| P50    | 5'-AATTTCCGAGGCCTTTAGGACTGAATA-3' |
| P122   | 5'-ATGTTGCGCTTGGCTGAGG-3' |
| P123   | 5'-TCGACCTTCAAGAAGCCAGAAAC-3' |
| P124   | 5'-AATTTGGTTGACCTGAG-3' |
| P125   | 5'-TCGACCTCAGACCATTA-3' |
| P126   | 5'-GATCCTCTAGAGGTTCTGAGAACCTGCAGAAGTCC-3' |
| P127   | 5'-GATCTCTAAGAAGCACTACAAAGGTCCATGAGTAC-3' |
| P128   | 5'-GCAGCTGCTAGTTGAGCATGCTGAAAGGTCAT-3' |
| P129   | 5'-GATCCTACTGCGAAGAAGCATAAGAAAAAGTCATGATC-3' |

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under denaturing conditions on Ni2+-NTA beads according to the manufac-
turer’s manual (QIAGEN Inc.). The protein was further purified by SDS-
PAGE, excised with 0.25 M MOPS, pH 7.4, and subsequently excised and eluted from the gel in elution buffer (50 mM Tris, pH 8.0, 0.1% SDS, 0.1 mM EDTA, 5 mM DTT, 0.15 M NaCl). This purified pro-
tein was used to immunize rabbits.

The antibody was purified using a pex7Δ depletion column and, subse-
duant, a Pex7p affinity column. The depletion column was made by cou-
pling overnight ~20 mg of proteins from a cell-free pex7Δ lysate to 0.6 g of
cyanogen bromide–activated Sepharose 4B (Sigma Immunochemicals, St.
Louis, MO) in coupling buffer (0.1 M NaHCO3, 0.5 M NaCl). After block-
ing the column for 2 h with blocking buffer (1 M methanol, 0.1 M NaHCO3, 0.5 M NaCl, pH 8.0), the column was washed extensively, alter-
ning between coupling buffer and low pH wash buffer (20 mM sodium acetate 0.35% [vol/vol] acetic acid, 0.5 M NaCl, pH 3.5) and subsequently with PBS, followed by 0.2 M glycine, 1 mM EGTA, pH 2.4, followed by PBS. After overnight incubation with 10 ml of serum, the serum was drained off and collected together with the first 3 ml of PBS wash. This purified serum was used for further purification on an affinity column. This was done essentially in the same manner as the depletion column, ex-
cept that 2 mg of purified Pex7p was bound to the column, and after bind-
ing the serum to the column, it was washed extensively with PBS, low pH wash buffer (0.3% [vol/vol] acetic acid, 50 mM sodium acetate, 1 M NaCl, pH 4.5), high pH wash (0.1 M NaHCO3, 1 M NaCl, pH 9.0), and again with PBS. The antibodies were eluted with 15 ml of 0.2 M glycine, 1 mM EGTA, pH 2.4, and immediately neutralized to pH 7.0 with 1 M Tris base. Finally, the antibodies were concentrated to ~1 ml using a Centriprep 30 (Amicon, Beverly, MA). The antibody was used at a 1:10,000 dilution for Western blotting.

Subcellular Fractionation and Nycodenz Gradients

Subcellular fractions of oleate-grown cells were essentially per-
formed as described by Van der Leij et al. (1992), in the presence of pro-
tease inhibitors (1 mM PMSF, 0.2 mg/ml NaF, and 2 mM of chymostatin, leupeptin, antipain, and pepstatin). 6 ml of a 1,000 g postnuclear superna-
tant was layered on a continuous 16–35% Nycodenz gradient (30 ml), with a 4-mM cufl of 42% Nycodenz dissolved in 5 mM MES, pH 6.0, 1 mM EDTA, 1 mM KCl, and 8.5% sucrose. The sealed tubes were centrifuged for 2.5 h in a vertical rotor at 29,000 g at 4°C. Afterwards, fractions were collected and analyzed by SDS-PAGE and Western blotting.

Microscopy Analysis

Fluorescence microscopy for GFP analysis was done as described by Monosov et al. (1996). Fluorescence microscopy of semithin sections and immuno-EM analysis of ultrathin sections was done as follows: P. pastoris cells were grown to log phase and fixed in either periodate-lysine-parafomaldehyde (1:1:5 M MOPS, 0.2 mg/ml NaF, and 2 mg/ml of chymostatin, leupeptin, antipain, and pepstatin) or 4% paraformaldehyde in 10 mM sodium periodate, pH 6.2) for 6 h, or in paraformaldehyde (75 mM phosphate buffer containing 2% formalde-
hyde, 70 mM lysine, and 10 mM sodium periodate, pH 6.2) for 6 h, or in 4% paraformaldehyde contained in 100 mM phosphate buffer, pH 7.4, for 18-24 h at 4°C. The cells were washed, pelleted, and embedded in 1% ul-
tralow temperature gelling agarose, and were trimmed into 1-mm3 blocks. The cells were cryoprotected in 2.3 M sucrose containing 20% polyvinyl pyrollidone for 1 h, mounted on aluminum cryopins, and frozen in liquid nitrogen. Ultrathin cryosections were then cut on a Reichert Uälcuttec E

Miscellaneous

Digitonin titrations were essentially performed as described by Zhang and Lazarow (1995), with modifications as described in Eilersma et al. (1996a).

For TCA lysates, cells (30 ml of culture OD600 = 0.7) were pelleted by

Results

Isolation of PTS2 Import–deficient Mutants

The negative screening procedure for isolating P. pastoris pex mutants that has been used thus far (Gould et al., 1992; Liu et al., 1992) has not resulted in the isolation of mutants that are specifically disturbed in the PTS2 import pathway. Therefore, we developed a positive selection procedure that would enrich for such mutants. In S. cerevi-
siae, such a scheme has been developed, which is based on the ability of peroxisomes to import the bleomycin resistance protein (BLE) fused to the PTS1 (Elgersma et al., 1993). In wild-type cells, BLE-PTS1 is imported into peroxi-
somes, thereby questrering the BLE protein from its toxic ligand, phleomycin. Peroxisomal import mutants (pex) are unable to import BLE-PTS1 into their peroxi-
somes, which allows the protein to bind phleomycin, thereby preventing its toxicity. We modified this procedure by fusing the BLE protein to the PTS2. Since a PTS2 sequence of an endogenous P. pastoris protein had not been identified, we tested the PTS2 sequence of S. cerevi-
siae thiolase (Fox3p) in P. pastoris. We therefore made a fusion of the first 17 amino acids of thiolase (containing the PTS2 signal) to GFP (PTS2-GFP). When expressed in oleate-grown P. pastoris, we observed a punctate GFP ex-
pression pattern, suggesting that PTS2-GFP was imported into the peroxisomes and that the ScPTS2 is functional in P. pastoris (Fig. 1 E). We also observed some cytosolic label-
ing, however, suggesting that PTS2-GFP import was not as efficient as GFP-SKL import (Fig. 1, E versus C).

A yeast strain (PPY12 + pTW84) that expressed PTS2-GFP was made, and the BLE fused to the PTS2 sequence (PTS2-BLE) under the control of the glyceraldehyde 3-phosphate dehydrogenase promoter (GAPDH) from a plasmid integrated in the genome. These cells were mu-
tagenized, induced on oleate, and treated with phleomycin. We expected that PTS2 import mutants would be able to grow on methanol because none of the known enzymes involved in methanol metabolism contain a PTS2 import signal. Conversely, because thiolase is required for growth on oleate, we expected that a PTS2 import–deficient mut-
ent would be unable to grow on oleate. Therefore, colo-
nies that had survived the phleomycin selection were screened for their ability to grow on methanol but not on oleate. Two mutants (fox3.1 and pex7.1) clearly fulfilled this criterion (shown for pex7Δa in a growth curve in Fig. 2, A and B). Inspection of PTS2-GFP import revealed, how-
ever, that one of these mutants (fox3.1) was unaffected in PTS2 protein import. Cloning of the complementing gene revealed that this mutant was deficient in the thiolase gene itself (Koller, A., and S. Subramani, unpublished results).

pex7.1 Cells Are Deficient in PTS2 Import Only

The second mutant (pex7.1) was not only specifically dis-
turbed in its growth on oleate (Fig. 2, A and B), but was also unable to import PTS2-GFP (Fig. 1 F), whereas import of GFP-SKL was normal (Fig. 1 D). This suggested a general deficiency of pex7.1 cells in the import of PTS2-containing proteins. The pex7.1 mutant was therefore further characterized by biochemical subcellular fractionation. In wild-type cells, we observed an equal distribution of thiolase between the organellar pellet fraction and the cytosolic supernatant fraction. As expected, thiolase was exclusively present (27,000 g) in the cytosolic supernatant fraction of pex7.1, providing additional evidence that the import of PTS2-containing proteins was disturbed (Fig. 2 C). Targeting of the membrane protein Pex3p was normal, as was the import of acyl CoA oxidase (Aco1p), because these proteins were predominantly present (27,000 g) in the organellar pellet fraction. Furthermore, the import of presumed PTS1-containing proteins such as catalase (Cta1p) and trifunctional enzyme (Tfe1p) was unaffected, suggesting that the import deficiency of pex7.1 is specific for PTS2-containing proteins only.

**Cloning of pex7.1 Complementing Genes**

The pex7.1 mutant was complemented for growth on oleate medium using a genomic library. Two plasmids (pM1 and pM2) with nonoverlapping inserts restored growth on oleate, although growth was better when using plasmid pM2 (Fig. 2 D). Further analysis of plasmid pM1 revealed that complementation of the oleate-minus phenotype was caused by a truncated protein (amino acids 1–342) with highest homology to ScPat1p, a peroxisomal ABC trans-
porter (Hettema et al., 1996). Interestingly, it has been reported that the peroxisomal ABC transporter Pmp70 can suppress the peroxisomal protein import deficiency of a mammalian pex2-deficient cell line (Gartner et al., 1994). Therefore, we tested whether plasmid pM1 was able to restore the PTS2-GFP import in the pex7.1 mutant. Microscopy analysis did not reveal any restoration of import of PTS2-GFP in these cells, suggesting that this protein did not act as a suppressor of the PTS2 import deficiency (data not shown). It has been recently demonstrated that ScPat1p and ScPat2p are involved in the transport of (activated) long-chain fatty acids across the peroxisomal membrane (Hettema et al., 1996). We therefore speculate that the truncated PpPat1p somehow disturbed the normal functioning of the Pat1p/Pat2p complex, thereby allowing leakage of the partially completed \( \beta \) oxidation product (3-oxoacyl-CoA) into the cytosol, allowing cytosolic thiolase to complete the \( \beta \) oxidation in this pex7.1 mutant.

The second clone (pM2) was not only able to restore growth on oleate of the pex7.1 mutant, but it also corrected the import deficiency of PTS2-GFP (data not shown). Further analysis revealed that the complementing gene encoded a protein of 376 amino acids with a calculated molecular mass of 42.4 kD. The protein contains six WD-40 repeats and has 43% sequence identity with ScPex7p (Fig. 3). Evidence that we cloned a true orthologue of ScPex7p was obtained by expressing the gene in \( S. \) cerevisiae. It was able to complement the growth defect of \( S. \) cerevisiae pex7 (Fig. 4 A); hence, the gene is designated \( PpPEX7 \).

**Pex7p Interacts with the PTS2**

Pex7p from \( S. \) cerevisiae has been shown to interact with the PTS2 sequence in multiple ways (Rehling et al., 1996; Zhang and Lazarow, 1996). Because PpPex7p complements the Scpex7p mutant, we expected PpPex7p to interact with the PTS2 sequence. This was tested using the yeast two-hybrid system (Fields and Song, 1989). Therefore, we fused Pex7p to the LexA activation domain (AD-PpPex7p) and Sc-thiolase to the LexA DNA–binding domain (DB-ScFox3p). These plasmids were coexpressed in \( S. \) cerevisiae L40 cells with either an empty control plasmid or with each other. High induction of the \( HIS3 \) gene and \( \beta \)-galactosidase was obtained when AD-PpPex7p and DB-ScFox3p were coexpressed, whereas no induction was observed when AD-PpPex7p or DB-ScFox3p was coexpressed with a control plasmid (shown for \( HIS3 \) expression in Fig. 4 B). This indicates that indeed Pex7p interacts with Fox3p in vivo. To test whether this interaction was dependent upon the presence of the PTS2, we made a construct from which the 17 NH\(_2\)-terminal amino acids of thiolase were removed (DB-ScFox3p\( \Delta \)PTS2). No induction of the \( HIS3 \) gene and \( \beta \)-galactosidase was observed when DB-ScFox3p\( \Delta \)PTS2 was coexpressed with AD-PpPex7p, indicating that the interaction of PpPex7p with thiolase was PTS2 dependent. To test whether the PTS2 alone would be sufficient to bind to PpPex7p, we fused the first 17 amino acids of ScFox3p to the LexA DNA–binding domain (DB-ScPTS2). When DB-ScPTS2 was coexpressed with AD-PpPex7p, we found again a strong induction of the \( HIS3 \) gene and \( \beta \)-galactosidase (shown for \( HIS3 \) expression in Fig. 4 B). These results indicate that the ScPTS2 sequence alone is sufficient for interaction with PpPex7p in vivo, supporting a receptor-like function for this protein.

**Fox3p-SKL Is Efficiently Imported into Peroxisomes of pex7\( \Delta \) Cells**

To study the function of Pex7p, we made a \( PEX7 \) deletion mutant (\( pex7\Delta \)) in which the entire open reading frame was replaced by the \( PpARG4 \) gene. Subcellular fractionation of this mutant showed the same phenotype as was observed for the pex7.1 mutant: a specific import deficiency of the PTS2-containing enzyme thiolase (shown later in Figs. 5 B and 6, B and D).

Like the original \( pex7.1 \) mutant, \( pex7\Delta \) did grow on methanol medium, suggesting that peroxisomal import of PTS2-containing enzymes is not necessary for growth on methanol (Fig. 2 A). Conversely, \( pex7\Delta \) did not grow on oleate, indicating that import of at least one PTS2-containing protein is required for growth on this medium (Fig. 2 B). We investigated whether the growth defect on oleate was caused solely by the mistargeting of thiolase. There-
thiolase was exclusively cytosolic in untransformed cells. Alternatively, the results suggest that thiolase easily leaks out of peroxisomes during subcellular fractionation. This is supported by the observation that PTS2-GFP is also largely cytosolic and only partially peroxisomal (Fig. 5 A).

The import efficiency of Fox3p-SKL was studied by subcellular fractionation (Fig. 5 B). We observed a considerable amount of endogenous thiolase in the cytosolic fraction in the control (untransformed wild-type) cells, whereas thiolase was exclusively cytosolic in import-deficient fox3Δ cells. The control enzyme, Aco1p, was predominantly present in the organellar pellet fractions (Fig. 5 B). These results suggest that thiolase easily leaks out of peroxisomes during subcellular fractionation. Alternatively, the PTS2 import pathway in P. pastoris is not very efficient. This is supported by the observation that PTS2-GFP is also largely cytosolic and only partially peroxisomal (Fig. 1, E and G), although we cannot rule out that this localization is caused by the chimeric protein itself. Interestingly, Fox3p-SKL was fully imported into peroxisomes of fox3Δ cells, with no protein detectable in the cytosolic fraction. In fox3Δ cells expressing Fox3p-SKL, thiolase was equally distributed between the pellet and supernatant fractions (Fig. 5 B). From the small difference in molecular weight between Fox3p-SKL and endogenous Fox3p, we conclude that Fox3p-SKL is fully imported into these cells via its PTS1 sequence, whereas endogenous thiolase is almost exclusively cytosolic, as in the untransformed fox3Δ control cells (Fig. 5 B). These results suggest that the PTS2 import pathway in P. pastoris may be less efficient than the PTS1 import pathway (see Discussion).

**Antibodies against Pex7p**

The lack of useful antibodies against endogenous Pex7p has greatly hampered the localization analysis of this protein in S. cerevisiae and in human cells. We therefore wanted to raise antibodies against PpPex7p to analyze its localization. We expressed the full-length Pex7p in E. coli fused to a (His)₆ tag, but the expression of this protein was very poor. We were unable to increase its expression by fusing it to dihydrofolate reductase, nor could we increase the expression by fusing three different truncated versions of Pex7p to dihydrofolate reductase. Good expression in E. coli was obtained for a truncated protein corresponding to amino acids 1–143 fused to the (His)₆ epitope. This puri-
fied protein, \((\text{His})_6\text{Pex7p}(143)\), was used to immunize rabbits. The antiserum that was obtained recognized several proteins in the wild-type strain and in the \textit{pex7Δ} strains (Fig. 6 A, lanes 1 and 2). Because this could indicate that a (nonfunctional) pseudogene of \textit{PEX7} might be present, we checked this possibility with PCR and low stringency Southern blotting, but did not find any evidence for the existence of such a gene (data not shown). We purified the antiserum with an immunodepletion column, using a lysate obtained from \textit{pex7Δ} cells. The flow-through of this column was used to purify the antiserum on an affinity column using the coupled\((\text{His})_6\text{Pex7p}(143)\) protein. The eluted antibody recognized two prominent bands in lysates of wild-type cells (Fig. 6, lane 4). The signal obtained from the lower molecular weight (cytosolic) protein was present at the same intensity in \textit{pex7Δ} cells (Fig. 6, lane 3) and could be eliminated by using more stringent washing conditions (see figures below). The higher molecular weight band was largely reduced in \textit{pex7Δ} cells, indicating that this signal is from Pex7p (Fig. 6 A, lane 3 versus lane 4). This could be confirmed by overexpressing Pex7p from the acyl-CoA oxidase promoter (Fig. 6, lane 5). A 25-fold dilution of this lysate (compared to a wild-type and \textit{pex7Δ} lysate) gave only one signal at the expected molecular weight (Fig. 6 A, lane 6). Because there is still a weak signal present at this molecular weight in a \textit{pex7Δ} lysate (from a mitochondrial protein, see below), in our subsequent analyses, we included the \textit{pex7Δ} control and separated the peroxisomes from mitochondria where necessary.

### PpPex7p Is Distributed between the Peroxisomes and the Cytosol

The intracellular location of Pex7p was determined by subcellular fractionation. Wild-type and \textit{pex7Δ} cells were fractionated, and the organellar pellet and the cytosolic supernatant were analyzed. Aco1p, Pex3p, and thiolase (Fox3p) were present in the organellar \((27,000\, g)\) pellet fraction of wild-type cells, although varying portions of some of these proteins were also present in the cytosolic \((27,000\, g)\) supernatant fraction, possibly because of leakage (Fig. 6 B). A similar distribution was observed for \textit{pex7Δ} cells, with the exception of thiolase, which was exclusively present in the cytosolic fraction, reflecting the PTS2 import deficiency of \textit{pex7Δ} (Fig. 6 B). Pex7p was present in both the organellar pellet (varying from 10 to 30%) and the cytosol fraction of wild-type cells, suggesting that about a quarter of the protein is associated with peroxisomes. In Fig. 6 B, the mitochondrial protein that sometimes cross-reacts with the anti-Pex7p antibodies (see Fig. 6, A and D) would not affect our conclusion that Pex7p is partially found in the cytosol because (a) we did not observe the cross-reacting band in this particular experiment (see H and P fractions of control \textit{pex7Δ} cells), and (b) the cross-reacting mitochondrial band was never found in the supernatant fraction during subcellular fractionation. The protein band found in the cytosolic fraction can therefore be only Pex7p.

To determine whether the pelletable Pex7p was indeed associated with peroxisomes, we used a postnuclear supernatant for further analysis on a Nycodenz gradient. A portion of Pex7p comigrated with the peroxisomal marker enzymes Aco1p and Fox3p, suggesting that at least some Pex7p is associated with peroxisomes (Fig. 6 C). We also found significant amounts of \(\alpha\text{Pex7p}–\text{cross-reacting mate-}
rial at the top of the gradient (fractions 14–20), representing mitochondrial and cytosolic proteins. In these gradients, the mitochondrial protein (Fig. 6 D, fractions 14–18) that cross-reacted with the antibody against Pex7p was never found at the top of the gradient in fractions 19 and 20, where cytosolic proteins would be found. The presence of Pex7p in fractions 19 and 20 of the gradient from wild-type cells (Fig. 6 D) shows that some Pex7p is indeed cytosolic. Because of the cross-reacting protein present in the mitochondrial fractions (see fractions 14–18 in the pex7Δ control gradient, Fig. 6 D), however, it is impossible to estimate the exact amount of Pex7p present in the cytosol in this experiment.

To study the localization of Pex7p by an independent biochemical method, we selectively permeabilized spheroplasts of wild-type cells, pex7Δ and pex3Δ (a mutant disturbed in the import of PTS1- and PTS2-containing proteins; Wiemer et al., 1996) by digitonin, and we followed the subsequent leakage of Pex7p and control proteins from the cells. In this procedure, cytosolic enzymes are released at low concentrations of digitonin, whereas peroxisomal matrix enzymes are released at much higher concentrations of digitonin (Zhang and Lazarow, 1995). As can be seen in Fig. 7, the cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PDH) was completely released at low concentrations (50 μg/ml) of digitonin. This was also observed for catalase in pex3Δ, as well as thiolase in the pex7Δ and pex3Δ strain, which reflects the peroxisomal import deficiency of these mutants. In contrast, thiolase and catalase were released at significantly higher concentrations of digitonin in wild-type cells, starting at 150 μg/ml digitonin, and release was not complete until 300–400 μg/ml digitonin was added. The membrane protein Pex3p could be completely released at only very high concentrations of digitonin (>600 μg/ml; Fig. 7). Using the αPex7p antibody, we observed release of the mitochondrial αPex7p-cross-reacting protein in pex7Δ cells at digitonin concentrations of 300–400 μg/ml. In wild-type cells, a substantial amount of Pex7p was already released before release of peroxisomal matrix proteins took place (before 150 μg/ml digitonin). In contrast to the cytosolic markers, however, release was not complete until 300–400 μg/ml digitonin, which is similar to what was observed for the release of Cta1p and Fox3p. In pex3Δ cells, Pex7p was completely released at 50 μg/ml. These results confirm that Pex7p is distributed at the peroxisome and the cytosol. Furthermore, since complete release of Pex7p is observed at digitonin concentrations <300 μg/ml, these results also suggest that some Pex7p is present in the lumen of peroxisomes.

Overexpressed PpPex7p Is Primarily Located in the Cytosol

If Pex7p functions solely as an intraperoxisomal receptor, it probably has a PTS that directs it efficiently to the peroxisomal matrix. Some experiments in S. cerevisiae suggest that this is indeed the case (Zhang and Lazarow, 1996). To test whether PpPex7p can direct a reporter protein to the peroxisome, we fused PpPex7p to the peroxisomal malate dehydrogenase of S. cerevisiae, which lacks its PTS1 (NH-Mdh3pPTS1). It has previously been shown that NH-Mdh3pPTS1 is cytosolic and that it can not complement the growth defect on oleate of S. cerevisiae mdh3Δ cells (Elgersma et al., 1996b). When PpPex7p was fused to NH-Mdh3pPTS1 (NH-Mdh3p-PpPex7p) and expressed in S. cerevisiae mdh3Δ cells under the control of the ScCTA1 promoter, we observed some complementation of the mdh3Δ mutant (data not shown). This suggests that PpPex7p does indeed have a signal that can direct it to the peroxisomal matrix.

To study the effect of overexpressing and/or epitope-tagging Pex7p, the expression level of Pex7p was increased by expressing it under the control of the ACO1 promoter. Western blot analysis revealed that this resulted in an ~25-fold higher expression level (see Fig. 6 A). The overexpressed protein fully complemented the growth deficiency of pex7Δ cells (Fig. 8 A). Full complementation was also observed when we epitope tagged Pex7p by fusing it to the NH tag (Elgersma et al., 1996b) and expressed it under the control of the ACO1 promoter (NH-Pex7p/ACO1) (Fig. 8 A). Neither tagging nor overexpression affected its ability to restore the peroxisomal import of thiolase in pex7Δ cells, as shown by subcellular fractionation (Fig. 8 B). Interestingly, we found on average >90% of overexpressed Pex7p in the cytosol (Fig. 8 B). Comparison of the absolute amount present in the organelar pellet revealed that the amount of pelletable Pex7p was only increased 5–10-fold (Fig. 8 C) compared to untransformed cells. These experiments suggest that targeting of PpPex7p to the peroxisomal matrix is not as efficient as the PTS1 or PTS2 import pathway, and that an unknown component is limiting the import of Pex7p.

The localization of overexpressed NH-Pex7p was also addressed by fluorescence microscopy of semithin sections of yeast cells labeled with the NH antibody. This antibody

![Figure 7. Location of Pex7p in digitonin-permeabilized, wild-type, pex3Δ and pex7Δ cells. Oleate-induced cells were spheroplasted, aliquoted in identical portions, and incubated for 10 min at 30°C with increasing amounts of digitonin dissolved in isotonic buffer. After quickly cooling the samples on ice, the spheroplasts were pelleted, and the supernatants were used for Western blotting with the antibodies as indicated. Triton, the samples have been incubated with 0.2% Triton X-100 in hypotonic buffer to get complete release of all proteins. Digitonin concentrations are given in micrograms per milliliter.](image-url)
These results confirm the predominantly cytosolic and indeed present in the peroxisomal matrix (Fig. 10, EM analysis. This revealed that a portion of NH-Pex7p is cytosolic. To verify the nature of the punctate staining, we used data that a large amount of overexpressed Pex7p is cytosolic (Fig. 9, indicating efficient import of NH-Mdh3p into peroxisomes.

To test whether the mutations were nonfunctional because of instability, mistargeting, or failure to bind the PTS2 sequence, we made the PpPex7p equivalents of the RCDP G217R, A218V, and L292ter mutations (A248R, G249V, and C347ter, respectively, as indicated in Fig. 3). The mutated PpPex7p proteins were expressed under the control of the PEX7 and ACO1 promoters, and they were tested for the ability to correct the growth deficiency of pex7Δ on oleate. As shown in Fig. 11 A, all mutated proteins were unable to restore the growth defect on oleate. When (over)expressed under the control of the ACO1 promoter, we observed some oleate usage by Pex7p(G249V), as judged by the formation of a halo on an oleate plate (data not shown). This correlates with the finding that patients with this corresponding allele have a phenotype milder than in classical RCDP (Braverman et al., 1997).

We made lysates of pex7Δ cells expressing the mutated Pex7p. As shown in Fig. 11 B, all proteins were expressed stably, suggesting that the deficiency is not caused by protein instability. We then tested whether the mutated proteins were able to bind the PTS2 sequence, using the two-hybrid system as described above. Interestingly, all mutated proteins had lost the ability to bind the PTS2 sequence (Fig. 11 C). This suggests that the mutations lead to impaired PTS2 binding.

To test whether the mutations affected the peroxisomal targeting of Pex7p, we expressed the mutated proteins in pex7Δ cells and compared their targeting to that observed for wild-type Pex7p by Nycodenz gradient analysis. We...
did not observe significant differences between the distribution of wild-type or mutated proteins (data not shown). This indicates that peroxisomal targeting of the mutated Pex7p proteins is not affected. Moreover, this suggests that binding of the PTS2 sequence is not a prerequisite for targeting Pex7p to the peroxisome.

**Discussion**

**PpPex7p Is a Receptor for PTS2 Protein Import**

Using a new screening procedure devised to yield mutants specifically compromised in the PTS2 import pathway, we have obtained a *P. pastoris pex7* mutant, cloned the *PpPEX7* gene, and shown in several ways that PpPex7p is the homologue of ScPex7p, the PTS2 receptor for protein import (Rehling et al., 1996, Zhang and Lazarow, 1996): (a) PpPex7p has high sequence identity with ScPex7 (43%), (b) *PpPEX7* complements the ScPex7 mutant, (c) *PpPex7Δ* is specifically disturbed in PTS2 protein import, and (d) PpPex7p interacts with the PTS2 sequence in the two-hybrid system. The observation that *PpPex7Δ* is unaffected in its growth on methanol medium suggests that there are no PTS2-containing enzymes or peroxins required for growth on this medium. Furthermore, it explains why screening procedures for *pex* mutants, based on their inability to grow on methanol, did not result in the isolation of this mutant.

**PpPex7p Functions Probably as a Mobile Receptor**

The role of Pex7p in PTS2 import has been a matter of debate. Zhang and Lazarow (1995, 1996) concluded that ScPex7p is an intraperoxisomal receptor and functions by pulling PTS2 proteins inside, analogous to the role of mitochondrial HSP70. Although they found significant amounts of a triple-tagged Pex7p (Pex7p-HA3) in the cytosol upon fractionation, they observed in digitonin experiments a strikingly similar pattern of latency of Pex7p-HA3 compared to peroxisomal matrix proteins. Therefore, they concluded that the cytosolic pool found in fractionation studies was probably caused by leakage of Pex7p-HA3. In contrast, others proposed that Pex7p functions as a mobile cytosolic receptor because most of the (overexpressed and tagged) Pex7p protein was found in the cytosol by biochemical fractionation (Marzioch et al., 1994; Rehling et al., 1996) or by immunofluorescence (Braverman et al., 1997).

We raised antibodies against the endogenous Pex7p and studied its localization. Subcellular fractionation and digitonin experiments suggest that Pex7p is largely cytosolic.
The mitochondrial F1β subunit of F1 ATPase is shown as a construct that is similar to the amounts of protein that were layered from each lysate. (C) Mutated PpPex7p fails to bind to ScFox3p in a two-hybrid analysis. S. cerevisiae L40 cells were transformed with the plasmids as indicated, grown on glucose plates lacking leucine and tryptophan, spotted on plates lacking histidine, and incubated for 2 d. ScFox3p was cloned in frame with the LexA DNA-binding domain. Pex7p and mutated Pex7p were cloned in frame with the LexA transcription-activation domain.

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but that some of the protein is peroxisomal. Evidence that the peroxisome-associated protein is present in the matrix was deduced from digitonin experiments and immuno-EM using NH-tagged Pex7p, which showed clear peroxisomal matrix labeling and no obvious membrane labeling. Interestingly, a 25-fold overexpression of Pex7p results in a modest 5–10-fold increase in peroxisome-associated Pex7p and in a large increase of cytosolic Pex7p. This explains why others found an almost exclusively cytosolic localization for Pex7p when they studied the localization of overexpressed Pex7p (Marzioch et al., 1994; Rehling et al., 1996; Braverman et al., 1997).

Our analyses indicate that most of Pex7p is cytosolic (varying from 70 to 90%), suggesting a role in the cytosol where it binds to PTS2-containing proteins and delivers them at the peroxisomal membrane. The identification of Pex14p as a putative peroxisomal docking protein for Pex7p supports this model (Albertini et al., 1997). A similar model has also been proposed for the PTS1 receptor Pex5p, which binds to the peroxisomal membrane protein Pex13p (Elgersma et al., 1996a; Erdmann and Blobel, 1996; Gould et al., 1996).

In addition to the cytosolic pool of Pex7p, we found also a significant fraction of Pex7p in the peroxisomal matrix (varying from 10 to 30%). Therefore, it is possible that Pex7p is also involved in the subsequent translocation step. In that case, we expect that Pex7p has to get recycled to the cytosol because thiolase expression is at least 25-fold higher than Pex7p expression. The observation that the amount of intraperoxisomal Pex7p did not increase proportionally upon overexpression suggests that the Pex7p import signal is not very efficient. Therefore, it is conceivable that intraperoxisomal Pex7p does not have a role at all, but that some Pex7p enters the peroxisome coincidentally and remains in the peroxisomal matrix without any function. Further kinetic experiments are required to address this point.

**Targeting of Pex7p to the Peroxisomal Matrix**

The ability of Pex7p to enter the peroxisomes, albeit at low efficiency, appears to be real. Some amounts of the wild-type and overexpressed Pex7p were intraperoxisomal, as judged by digitonin permeabilization and immuno-EM (Figs. 7 and 10). Furthermore, NH-Mdh3p-PpPex7p was able to complement an mdh3Δ strain of *S. cerevisiae* (data not shown). The mechanism involved in this targeting is unknown. Because protein unfolding is not essential for the import of proteins into the peroxisomal matrix, Pex7p might enter the matrix solely by association with PTS2-containing proteins. This is unlikely, however, because the A248R, G249V, and C347ter mutants cannot interact with the PTS2 sequence and show the same subcellular location as wild-type Pex7p. Moreover, within experimental error, we did not observe a different distribution of Pex7p when we deleted the FOX3 gene or when we raised its expression twofold by overexpressing it from the ACO1 promoter. An alternative possibility is that Pex7p has a new targeting signal or that it enters the peroxisome in association with some other protein. Such a targeting or protein association signal might reside in the first 56 amino acids of ScPex7p, as suggested by the experiments of Zhang and Lazarow (1996).

**Yeast Model System for Human Peroxisome Biogenesis Defects**

The cloning of yeast *PEX* genes has tremendously facilitated the characterization of their human orthologues (for review see Subramani, 1997). For instance, the human *PEX7* gene was cloned by screening databases using the yeast *PEX7* gene (Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997). Our studies show a new role for the yeast model system: the rapid analysis of the effect of the mutations found in the *PEX* genes of patients. We introduced the mutations found in the RCDP patients at the corresponding positions in the *PpPex7* gene and found that this resulted in impaired growth on oleate. Furthermore, we found that the mutated Pex7p proteins in *P. pastoris* are stably expressed and not disturbed in peroxisomal targeting, but that they are unable to bind to the PTS2 sequence. The application of a similar strategy to other human *PEX* genes involved in disease is likely to provide useful insights regarding the effect of mutations.

**Fox3p-SKL Only Partially Restores Growth on Oleate When Expressed in pex7Δ Cells**

*Pp*Δ did not grow on oleate plates, but this deficiency could be partially suppressed by expressing Fox3p-SKL in these cells. This indicates that the lack of thiolase import is possibly the main, but certainly not the only, reason that *pex7Δ* cells do not grow on oleate. Hence, we expect that other PTS2-containing proteins are also required for ole-
ate metabolism. Surprisingly, it appeared that Fox3p-SKL was either imported or retained in peroxisomes much more efficiently than endogenous thiolase. Moreover, it should be noted that we did not observe significant import of endogenous thiolase in per7a cells expressing Fox3p-SKL. This indicates that the formation and import of heterodimers of Fox3p-SKL with endogenous thiolase did not take place, in contrast to what has been observed for S. cerevisiae cells expressing thiolase with and without a PTS2 (Glover et al., 1994b). This may suggest that the import of Fox3p-SKL via the PTS1 pathway is much faster than the import of endogenous thiolase via the PTS2 pathway, and that import of Fox3p-SKL takes place before the folding and oligomerization of Fox3p-SKL is completed.

Further kinetic experiments, as conducted by Ruigrok et al. (1996), are necessary to validate this hypothesis.

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