Receptor-mediated import of proteins into peroxisomes
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The peroxisomal localization of the PTS1 receptor Pex5p in *Saccharomyces cerevisiae* is regulated by growth conditions and is dependent on the peroxisomal membrane proteins Pex14p and Pex17p

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**SUMMARY**

The mobile receptor for import of proteins containing a peroxisomal targeting signal type 1 (PTS1), Pex5p, is primarily localized in the cytosol of *Saccharomyces cerevisiae* cells grown in oleate-containing media. Here we report that in wild-type cells grown in glucose media, Pex5p is predominantly present in the 25,000 x g organellar pellet. The subcellular distribution of Pex5p was not changed by variations in the expression of peroxisomal matrix proteins in either glucose-grown cells or in oleate-grown cells. In addition, dramatic variations in expression levels of Pex5p did also not affect the overall subcellular distribution of the protein in either of the two growth conditions. To identify proteins that are involved in the peroxisomal association of Pex5p, the localization of Green Fluorescent Protein–tagged Pex5p (GFP-Pex5p) was monitored by fluorescence microscopy in the currently known *S. cerevisiae* PEX deletion strains. Of the *pexΔ* strains containing peroxisomal membrane remnants, only cells lacking the *PEX14* or the *PEX17* gene completely mislocalized GFP-Pex5p to the cytosol. Upon induction of the Pex14 protein in *pexl4Δ* cells, Pex5p redistributed to peroxisomal membranes, indicating a direct correlation between Pex14p expression and peroxisomal localization of Pex5p. However, overexpression of Pex14p did not change the subcellular distribution of Pex5p. Together these results show that Pex14p, and likely Pex17p, are required for stable association of Pex5p with the peroxisome, whereas the distribution of Pex5p over peroxisome and cytoplasm is regulated by factors that are influenced by growth conditions of the yeast cell.
INTRODUCTION

Peroxisomes are ubiquitous organelles that are present in almost all eukaryotic cells. Depending on the demands of the cell, the number and size of peroxisomes may vary, underscoring the dynamic behaviour of this organelle. The single membrane that bounds the peroxisomes forms a barrier across which proteins and metabolites must pass. By means of genetic screens in yeasts and in Chinese hamster ovary (CHO) cell-lines a large number of genes encoding for proteins that are involved in peroxisome biogenesis (PEX genes) have been identified (Hettema et al., 1999; Tabak et al., 1999). One of the PEX genes has been implicated to play a role in peroxisome proliferation (Erdmann and Blobel, 1995; Schrader et al., 1998; Van Roermund et al., 2000), while others have a chaperone-like function (Purdue et al., 1998; Titorenko et al., 1998), play a role in insertion and stability of membrane proteins (Hettema et al., 2000; South and Gould, 1999; Subramani et al., 2000), or are involved in transport of proteins across the membrane (reviewed by Subramani et al., 2000). The import of peroxisomal proteins is facilitated by proteins that act in the cytosol and by a set of proteins that are present at the peroxisomal membrane. The exact mechanism of protein import into peroxisomes is however largely unknown. Import of most matrix proteins is depending on a peroxisomal targeting sequence (PTS) at the extreme C-terminus, which is composed of the conserved amino acid tripeptide Ser-Lys-Leu or a derivative thereof (PTS1). Alternatively, a few proteins contain a PTS at their N-terminus (PTS2). PTS1 and PTS2 proteins are recognized and bound by their receptors Pex5p and Pex7p, respectively. In yeast the two PTS targeting pathways can function independently of each other (reviewed by Hettema et al, 1999). In mammalian cells the PTS1 receptor exists in two different isoforms; a short form and an alternatively spliced longer form that contains an extra exon encoding 37 amino acids (Braverman et al., 1998; Otera et al., 1998). The longer form of Pex5p is essential for Pex7p-mediated import of PTS2 proteins (Braverman et al., 1998; Matsumura et al., 2000; Otera et al., 2000). With the exception of Yarrowia lipolytica Pex5p (Szilard et al., 1995), the subcellular distribution of Pex5p and Pex7p is predominantly cytoplasmic, with a minor amount of receptor associated to peroxisomes (de Walque et al., 1999; Dodt and Gould, 1996; Elgersma et al., 1996a; Marzioch et al., 1994; Otera et al., 2000; Wiemer et al., 1995; Wimmer et al., 1998), or in sometimes even inside peroxisomes (Elgersma et al., 1998; Gouveia et al., 2000; Van der Klei et al., 1995). In human fibroblasts, Pex5p is able to move from the cytoplasm to the peroxisome and vice versa, suggesting continuous cycling of the receptor (Dodt and Gould, 1996). The dual localization of Pex5p and its capacity to cycle between the peroxisome and the cytoplasm suggests that the PTS receptor binds its cargo in the cytoplasm and brings it to the peroxisomal membrane, where the receptor-cargo complex docks and the cargo protein is handed over to the import system. In the yeast S. cerevisiae Pex5p and Pex7p are able to bind the membrane proteins Pex13p and Pex14p at the cytoplasmic face of the peroxisomal membrane (Albertini et al., 1997; Brocard et al., 1997; Elgersma et al., 1996a; Erdmann and
The Src Homology 3 (SH3) domain of the integral membrane protein Pex13p interacts with both Pex5p and the peripheral membrane protein Pex14p (Barnette et al., 2000; Bottger et al., 2000; Girzalsky et al., 1999). The binding site for Pex7p has been suggested to be localized in the N-terminus of Pex13p (Girzalsky et al., 1999). Although the interaction of Pex5p with Pex13p has only been shown in S. cerevisiae and in P. pastoris (Gould et al., 1996; Urquhart et al., 2000), the specific interaction of Pex5p with Pex14p is conserved between various species including human and rat (Albertini et al., 1997; Fransen et al., 1998; Gouveia et al., 2000; Otera et al., 2000; Schliebs et al., 1999; Urquhart et al., 2000; Will et al., 1999). The SH3 domain-mediated interaction of Pex13p with Pex14p and the presence of stoichiometric amounts of Pex13p and Pex14p are essential for functional peroxisomosomal protein import, underscoring close cooperation between the two proteins (Bottger et al., 2000; Girzalsky et al., 1999). Recently it has been reported that in human and CHO cells, another membrane protein, Pex12p, interacts with Pex5p, but that the interaction occurs downstream of the docking event of Pex5p at the peroxisomal membrane (Chang et al., 1999; Okumoto et al., 2000).

Here we have investigated the localization of Pex5p in the yeast S. cerevisiae under different growth conditions. We show that in glucose-grown cells Pex5p is predominantly present in the 25,000 x g organellar pellet. Changing growth conditions to oleate media results in subcellular redistribution of Pex5p from the organellar pellet to the supernatant. To investigate whether one or more PEX genes are responsible for peroxisomal localization of Pex5p, we determined the subcellular localization of Pex5p fused to the Green Fluorescent Protein (GFP) in all available pex deletion strains. Of the fifteen yeast strains with morphologically recognizable peroxisomal remnants only the pex14Δ and pex17Δ strains were devoid of peroxisomal labeling of Pex5p. Induction of Pex14p in a pex14 deletion strain resulted in redistribution of Pex5p to the peroxisome, establishing a correlation between expression of Pex14p and peroxisomal localization of Pex5p.

**EXPERIMENTAL PROCEDURES**

**Yeast strains and growth conditions**

Yeast strains used in this study were S.cerevisiae BJ1991 (MATα, leu2, trpl, ura3-251, prb1-1122, pep4-3, gal2). Previously described BJ1991 pexΔ strains are: pex3Δ and pex19Δ (Hettema et al., 2000), pex5Δ (Van der Leij et al., 1993), pex6Δ (Voorn-Brouwer et al., 1993), pex11Δ (Van Roermund et al., 2000), pex13Δ (Elgersma et al., 1996a), pex15Δ (Elgersma et al., 1997), dip1Δ (Hettema et al., 1998) and pip2Δ (Rottensteiner et al., 1996). The remaining pexΔ strains were generated by one-step PCR-mediated gene disruption as described by Wach et al. (Wach et al., 1994).

Cell culture conditions: cells were pregrown overnight on minimal 0.3% glucose medium (0.3% glucose, 0.67% yeast nitrogen base (YNB; Difco) and amino acids (20-30 µg/ml) as required). These cultures were inoculated 1:3 in fresh 0.3% glucose medium and grown to log
phase for 3 h (glucose-grown cultures) or further grown in oleate-containing growth medium (0.5% potassium phosphate buffer pH 6.0, 0.3% yeast extract, 0.5% peptone, 0.1% (v/v) oleate and 0.2% (v/v) Tween-40) for 16 hours (oleate-grown cultures). Induction of Gal11/10 promoter controled Pex14p: pex14Δ strain was transformed with pGAL-PEX14 (pGB60). Transformants were grown overnight on selective liquid medium containing 2% glucose, 0.67% YNB (Difco) and amino acids as required, cells were harvested and resuspended in selective 2% galactose medium supplemented with 0.67% YNB (Difco) and amino acids as required.

**Plasmids and cloning procedures**

Plasmids used were: pNV8 (PPGK-NHMdh3p) (Verleuer et al., 1997); p20.19: NH-tagged PEX13 cloned downstream of the CTA1 promoter (Elgersma et al., 1996a); p21.27: NH-tagged PEX15 cloned downstream of the CTA1 promoter (Elgersma et al., 1997); pEW90: pEW88 encoding GFP-SKL (Hetteema et al., 1998) was digested with SacI and XbaI and the GFP-SKL fragment was ligated between the SacI and XbaI sites of a YEplac181 (47) derivative containing the CTA1 promoter (45). pGB52: The BamHI-HindIII fragment encoding the open reading frame of wild-type PEX5 from pTi98 (Bottger et al., 2000) was ligated between the BamHI-HindIII sites of pA67 (a plasmid derived from YCplac33 in which the PCR product of the PEX6 promoter region was ligated between the EcoRI-SacI sites), generating a plasmid for PEX6 promoter-controlled Pex5p expression; PCTA1-PEX5: The BamHI-HindIII fragment encoding the open reading frame of wild-type PEX5 from pTi98 (Bottger et al., 2000) was cloned behind the CTA1 promoter by ligation between the BamHI-HindIII sites of pEL43 (Elgersma et al., 1996b); pGB39: pGB4 (Bottger et al., 2000) was digested with BamHI and PsI, the obtained fragment containing the PEX14 open reading frame was ligated between the BamHI-PsI sites of pA20 (Stroobants et al., 1999), generating a plasmid for GAL11/10-promoter-controlled Pex14p expression; pGB60: pGB39 was digested with EcoRI and HindIII, generating a HindIII-HindIII fragment containing the C-terminus of PEX14 and a EcoRI-HindIII fragment containing the GAL11/10 promoter and the N-terminus of PEX14. The two fragments were inserted between the EcoRI-HindIII sites (3-point ligation) of YCplac33 (Gietz and Sugino, 1988); pGB61: GFP was fused to Pex5p by insertion of a BglII-digested PCR product of GFP between the BamHI restriction site of the PEX5 expression plasmid pTi98 (Bottger et al., 2000) generating pAN13. This construct was digested with NraI and PsI and the insert was ligated between the NraI and PsI sites of YCplac111 (Gietz and Sugino, 1988).

**GFP-Pex5p localization in pexΔ strains**

Wild-type and pexΔ strains were transformed with pGB61 encoding PEX5 promoter-controlled GFP-Pex5p. Transformants were grown overnight on minimal 2% glucose medium. Transformants were transferred to minimal 0.3% glucose and grown overnight. Cultures were inoculated 1:3 in fresh 0.3% glucose media and grown for another 3-4 h. Cells were harvested, washed in water and directly analyzed by fluorescence microscopy.

**Subcellular fractionation and gradient analysis**

Subcellular fractionation experiments were performed as previously described (Bottger et al., 2000). For flotation gradient analysis, a 25,000 x g organellar pellet was resuspended in 1 ml 60% wt/wt sucrose solution in hypotonic lysis buffer (0.65 M Sorbitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 2(N-morpholino)ethane sulfonic acid (MES) pH 5.5, 1 mM KCl, 1 mM EDTA) and loaded on the bottom of the tube. The 60% sucrose solution was overlayed with 2 ml of subsequently 48%, 42%, 38%, 30%, 25% and 20% wt/wt sucrose in hypotonic lysis buffer. Gradients were spun in a TST 41.14 swing-out rotor for 16 h at 36,000 rpm. Fractions of 1 ml were collected from the top to the bottom of the gradient and protein was precipitated with 10% Trichloric acid (TCA) on ice for 1 h. Precipitated proteins were pelleted by spinning in an eppendorf centrifuge for 30 min at maximum speed and were resuspended in Laemml sample
buffer (Sambrook et al., 1989). SDS-PAGE and Western blotting were performed as previously described (Bottger et al., 2000).

**Antibodies**

Generation of antibodies against catalase A, 3-ketoacyl-CoA thiolase, Pex5p and Pex13p are described in Elgersma et al. (1996a); antibodies against Pex14p are described in (Bottger et al., 2000).

**Immunofluorescence analysis**

Immunofluorescence analysis was carried out essentially as described by Erdmann, (1994) with the following modifications: spheroplasts were resuspended into 100 μl isotonic buffer (1.2 M Sorbitol; 1 mM PMSF; 5 mM MES pH 5.5; 1 mM EDTA; 1 mM KCl) and spread on coverslips coated with 0.1% Poly-L-lysine (Sigma). The coverslips were soaked in methanol for 1 min and incubated in blockbuffer (0.5% BSA in PBS-0.1% Tween-20) for 15 min. Coverslips were subsequently incubated for 1 hour in primary antibody diluted in blockbuffer (α acyl-CoA oxidase 1:100, α catalase A 1:200, α NH 1:100), washed 4 times in blockbuffer and then incubated for 1 hour in secondary antibody (Goat-anti-rabbit conjugated with Texas Red, 1:200 diluted in blockbuffer). Coverslips were dipped into water, mounted in Mowioil (Hoechst) and instantly viewed under the fluorescence microscope (Zeiss axioplan).

**RESULTS**

**Pex5p is localized in the organellar pellet fraction of glucose-grown wild-type cells and redistributes to the supernatant fraction upon oleate induction**

The number and size of peroxisomes in *Saccharomyces cerevisiae* is subject to change depending on growth conditions. Upon changing growth conditions from glucose to oleate media, the cell responds with induction of transcription of several peroxisomal matrix proteins (Karpichev and Small, 1998; Rottensteiner et al., 1996) resulting in extensive proliferation and enlargement of peroxisomes (Karpichev and Small, 1998; Veenhuis et al., 1987). Expression of most *PEX* proteins, however, remains constant under these growth conditions (Kal et al., 1999). Here we have investigated the subcellular distribution of Pex5p, the PTS1 receptor, in cells grown on either glucose or oleate media. Cleared homogenates of glucose and oleate-grown cells (see experimental procedures for detailed growth conditions) were centrifugated at 25,000 x g and pellet and supernatant fractions were analyzed for Pex5p by immunoblotting. In oleate-grown wild-type cells most of Pex5p could be found in the cytoplasm and a minor fraction of Pex5p was associated with the organellar pellet. In contrast, in glucose-grown cells Pex5p was primarily located in the 25,000 x g pellet (Figure 1A). A similar fractionation experiment was performed with a *pex3Δ* strain, which lacks detectable peroxisomal membranes (Baerends et al., 1996; Hettema et al., 2000; Wiemer et al., 1996). The majority of Pex5p in *pex3Δ* cells grown in glucose media appeared in the supernatant fraction, suggesting that the subcellular distribution of Pex5p in glucose-grown wild-type cells is specifically related to the
Peroxisomal localization of Pex5p requires Pex14p and Pex17p

Figure 1. Pex5p is mainly associated with the 25,000 x g pellet in homogenates of glucose-grown wild-type cells and redistributes to the supernatant upon growth on oleate. A) Cleared homogenates (H) of wild-type and pex3Δ cells grown in glucose media or oleate media were subjected to centrifugation at 25,000 x g and the obtained organelar pellet fractions (P) and cytosolic supernatant fractions (S) were analyzed for Pex5p by immunoblotting. B) Wild-type cells were precultured in minimal glucose media (t = 0) and subsequently shifted to oleate containing media. At indicated time points the cells were spheroplasted and homogenates were separated into 25,000 x g pellet (P) and 25,000 x g supernatant (S) fractions.

presence of peroxisomes. The marked difference in Pex5p localization in glucose versus oleate-grown cells was further investigated in a time-course experiment. Wild-type yeast cells were grown to log-phase in 0.3% glucose liquid culture media. These cells were harvested, inoculated in liquid oleate medium and continued to grow. At indicated time points cells were spheroplasted and cleared homogenates were centrifuged at 25,000 x g. The obtained pellet and supernatant fractions were analyzed by immunoblotting with antibodies specific for Pex5p. Figure 1B shows that during the course of the experiment more Pex5p appeared in the supernatant fraction. After approximately 8 hours of growth on oleate, the subcellular distribution of Pex5p was comparable to that of cells grown for 16 hours on oleate (fractions of oleate-grown wild-type cells in Figure 1A).
**Pex5p distribution is not depending on PTS1 protein expression**

To investigate whether Pex5p localization is correlated with the expression of PTS1 proteins, we carried out two types of experiments. In the first experiment a PTS1 protein, NH-tagged Mdh3p, was overexpressed under the control of the strong PGK promoter in both wild-type and pex5Δ cells grown on glucose. It has been shown that this Mdh3p is imported into peroxisomes of glucose-grown cells (Verleur et al., 1997). In line with this NH-Mdh3p could be recovered from the 25,000 x g pellet fraction in wild-type cells, whereas this protein primarily localized in the supernatant fraction in pex5Δ cells (Figure 2A), implying that peroxisomal import was Pex5p-mediated. The overexpression of Mdh3p had no effect on the subcellular distribution of Pex5p (Figure 2A). We conclude therefore that alterations in PTS1 protein expression in glucose-grown cells do not affect the localization of Pex5p.

In the second experiment we reduced the expression of PTS1 proteins in oleate-grown cells. For this purpose we made use of the pip2Δ strain, which lacks one of the transcription factors involved in oleate-induced transcription of peroxisomal matrix proteins (Karpichev and Small, 1998; Rottensteiner et al., 1996). Consequently, oleate-grown pip2Δ cells contain a relatively low amount of peroxisomal matrix proteins. Subcellular fractionation of oleate-grown wild-type and pip2Δ cells revealed no clear differences in the distribution between the two strains. Taken together, these results show that neither increased, nor decreased peroxisomal matrix protein synthesis affect the subcellular localization of Pex5p.

**Figure 2.** Subcellular distribution of Pex5p is not dependent on PTS1 protein expression in either glucose-grown or oleate-grown cells.

A) Glucose-grown wild-type cells and pex5Δ cells expressing an NH-tagged version of the PTS1 protein Mdh3p under the control of the PGK promoter (PPGK-NHMdh3p) were converted to spheroplasts and cleared homogenates (H) were separated into 25,000 x g pellet fractions (P) and 25,000 x g supernatant fractions (S). Fractions were subjected to immunoblot analysis with antibodies specific for the NH epitope and for Pex5p. B) Cleared homogenates (H) of oleate-grown wild-type and pip2Δ cells were separated into 25,000 x g pellet (P) and supernatant (S) fractions. Fractions were analyzed by immunoblotting using anti-bodies specific for Pex5p.
Overexpression of Pex5p does not affect the subcellular distribution of Pex5p

To investigate whether the large cytoplasmic pool of Pex5p in oleate-grown cells is caused by saturation of Pex5p binding sites on the peroxisomal membrane, we expressed Pex5p under the control of different promoters in the pex5Δ strain. The promoters used were: 1) the PEX5 promoter (PPEX5), or 2) the weak promoter of the PEX6 gene (PPEX6), or 3) the strong CTA1 promoter (PCTA1). The messenger RNA (mRNA) levels of the PEX6 gene in glucose-grown cells are 1.5 times lower, and in oleate-grown cells 4 times lower than the mRNA levels of PEX5, whereas mRNA levels of CTA1 in glucose-grown cells are 80 times higher, and in oleate-grown cells 200 times higher than mRNA levels of PEX5 (M. Groot-Koerkamp, A.N. Mul, G. Hardy and H.F. Tabak, unpublished observations). The PEX5 expression constructs were able to complement the growth defect of the pex5Δ strain on oleate, although cells transformed with PPEX6-Pex5p showed a slight delay in growth (data not shown). Transformants were grown on glucose media or on oleate media and cleared homogenates were separated into 25,000 x g pellet and 25,000 x g supernatant fractions. The obtained fractions were analyzed for Pex5p by immunoblotting (Figure 3). Interestingly, the majority of Pex5p in glucose-grown cells overexpressing Pex5p from the CTA1 promoter could be recovered from the pellet fraction, while under oleate growth conditions, Pex5p expressed from the weak PEX6 promoter still appeared in the supernatant fraction (Figure 3). The expression level of Pex5p in glucose-grown cells transformed with PPEX6-Pex5p was too low to be detected by our antibodies. Thus, overexpressed and underexpressed forms of Pex5p showed distribution patterns in glucose-grown and oleate-grown cells comparable to that of PPEX5-Pex5p under these growth conditions. We conclude therefore that the subcellular distribution of Pex5p between the 25,000 x g pellet and supernatant fractions is not affected by the absolute amount of Pex5p in the cell.

**Figure 3.** The subcellular distribution of Pex5p is not affected by its expression level. Pex5Δ cells expressing Pex5p under the control of the PEX5 promoter (PPEX5-Pex5p), the CTA1 promoter (PCTA1-Pex5p), or the PEX6 promoter (PPEX6-Pex5p) were grown in glucose or oleate media, converted to spheroplasts and subjected to differential centrifugation. The homogenates (H), 25,000 x g pellet fractions (P) and 25,000 x g supernatant fractions (S) were analyzed by immunoblotting with antibodies specific for Pex5p.
GFP-Pex5p is targeted to peroxisomes in wild-type cells

For rapid analysis of Pex5p localization we generated an N-terminal fusion of full-length Pex5p with the green fluorescent protein (GFP-Pex5p). DNA encoding the fusion protein was cloned downstream of the PEX5 promoter. Wild-type cells expressing GFP-Pex5p were grown on glucose and prepared for indirect immunofluorescence with antibodies specific for catalase and Texas Red-labeled secondary antibodies. Fluorescence analysis revealed a congruent fluorescence pattern of GFP-Pex5p and catalase A, indicating that the GFP-tagged version of Pex5p was targeted to peroxisomes (Figure 4A). To determine whether GFP-Pex5p was associated with peroxisomal membranes, the 25,000 x g pellet fractions of wild-type and pex3Δ cells were subjected to sucrose-density flotation gradient analysis. Gradient fractions were analyzed by immunoblotting with antibodies specific for Pex5p, the peroxisomal membrane protein Pex13p and the mitochondrial marker.
Peroxisomal localization of Pex5p requires Pex14p and Pex17p

protein Hsp60. Figure 5B shows that GFP-Pex5p in wild-type cells migrated into the gradient, where it co-localized with Pex13p, whereas GFP-Pex5p from pex3Δ cells remained in the loading zone of the gradient. Pex13p was not detectable in the pex3Δ gradient due to instability of the membrane protein (Hettema et al., 2000). Immunodetection of Hsp60 showed that mitochondria migrate at somewhat lower density (peak at fraction 7) than wild-type peroxisomes (peak at fraction 6). It should be noted that protein levels of endogenous Pex5p in the gradient fractions were too low to be detected with our antibody specific for Pex5p. Together these results indicate that GFP-Pex5p is localized to peroxisomes in glucose-grown wild-type cells and that this fusion protein can be used as a reporter for the subcellular localization of Pex5p. It should be noted, however, that the GFP-Pex5p fusion does not complement the growth defect on oleate of pex5Δ cells (A. Klein, unpublished results) and must therefore be considered as a non-functional protein. Localization of GFP-Pex5p in pex deletion strains. screen for PEX proteins that are responsible for the association of Pex5p with peroxisomes, all available Saccharomyces cerevisiae pexΔ strains were analyzed for GFP-Pex5p localization. The pexΔ strains were transformed with the construct encoding GFP-Pex5p, grown on liquid 0.3% glucose medium and analyzed for GFP labeling using direct fluorescence microscopy. Table 1 summarizes the results of this screen. Four different groups of GFP-Pex5p distribution patterns can be distinguished: 1) exclusively cytosolic labeling (pex14Δ and pex17Δ strains); 2) exclusively peroxisome associated labeling (wild-type and dip1Δ strains); 3) peroxisome associated and cytosolic labeling (pex1Δ, pex2Δ, pex4Δ, pex5Δ, pex6Δ, pex7Δ, pex8Δ, pex10Δ, pex11Δ, pex12Δ, pex13Δ and pex15Δ strains) and 4) cytosolic labeling combined with labeling of aberrant structures (pex3Δ and pex19Δ strains). Of the pex mutants that contain peroxisomes or peroxisomal membrane structures (Hettema et al., 2000) only Pex14p and Pex17p (group 1) displayed an exclusively cytosolic staining of GFP-Pex5p. The pex mutants in group 3 showed a clear cytosolic labeling of GFP-Pex5p combined with weakly labeled punctated structures and occasionally aberrant fluorescence consisting of 1-3 fluorescent dots. To test whether GFP-Pex5p is targeted to peroxisomal remnants in these cells, several pexΔ strains were co-transformed with GFP-Pex5p and NH-tagged versions of the peroxisomal membrane proteins (PMPs) Pex13p or Pex15p. Indirect immunofluorescence with antibodies for the NH-tag identified the GFP-Pex5p labeled structures as peroxisomes (pex7Δ strain and wild-type cells) or peroxisomal remnants (Figure 4B and data not shown). These remnants could also be detected with the NH-antibody in the pex14Δ and pex17Δ strains and were morphologically distinct from the diffuse fluorescence pattern of GFP-Pex5p (Figure 4B and data not shown), indicating that GFP-Pex5p is not associated with recognizable peroxisomal ghosts in pex14Δ and pex17Δ cells. The pex3Δ and pex19Δ strains that lack morphologically recognizable peroxisomal remnants showed aberrant labeling. GFP-Pex5p in pex3Δ cells is present in 1-3 punctated structures per cell in combination with a clear cytoplasmic staining. In pex19Δ cells GFP-Pex5p also appears to be
primarily cytoplasmic with occasionally a cell exhibiting one fluorescent dot. GFP-Pex5p in *pex*3Δ cells, however behaves as a soluble protein in a flotation gradients (Figure 5A) Together these results suggest that of the peroxins identified so far Pex14p and Pex17p are the only two proteins responsible for peroxisomal localization of Pex5p. To investigate whether Pex5p is soluble in the *pex*14Δ strain, cells transformed with GFP-Pex5p were grown in 0.3% glucose media, spheroplasted and cleared homogenates were centrifuged at 25,000 x g. Pellet and supernatant fractions were analyzed using SDS-PAGE. Western blotting and immunodetection with antibodies specific for Pex5p. In contrast to the distribution of GFP-Pex5p in wild-type cells, the majority of GFP-Pex5p and of endogenous Pex5p in *pex*14Δ cells was recovered in the supernatant fraction (Figure 5A). However, a significant amount of both proteins was present in the pellet fraction. This fraction was subjected to sucrose flotation gradient analysis and gradient fractions were analyzed by immunoblotting. Peroxisomal membrane ghosts in *pex*14Δ cells were visualized with an antibody specific for Pex13p and appeared in the top of the gradient (Figure 5B). GFP-Pex5p and endogenous Pex5p did not co-localize with peroxisomal membrane.

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Strains were transformed with GFP-tagged Pex5p and analyzed by direct fluorescence microscopy. a): punctated structures correspond to either peroxisomes or peroxisomal membrane remnants as shown by colocalization with peroxisomal membrane proteins (see figure 4B) Diffuse staining corresponds to cytosolic localization of GFP-Pex5p. Labeling intensities are indicated by “++” (strong), “+” (weak), or “-” (absent). The *pex*3Δ and *pex*19Δ strains show aberrant staining of GFP-Pex5p, indicated by “*” , consisting of 1-3 clear fluorescent spots.
Figure 5. GFP-Pex5p mislocalizes to the cytosol in pex14Δ cells. A) Wild-type and pex14Δ cells expressing GFP-Pex5p were spheroplasted and cleared homogenates (H) were separated into 25,000 x g pellet (P) and 25,000 x g supernatant (S) fractions and analyzed for Pex5p and GFP-Pex5p using immunoblotting with the Pex5p antibody. B) Glucose-grown wild-type and pexΔ cells expressing GFP-Pex5p were converted to spheroplasts and cleared homogenates were centrifuged at 25,000 x g. The obtained pellets were subjected to flotation gradient analysis. Fractions were analyzed by immunoblotting using antibodies specific for Pex5p, Pex13p and mitochondrial Hsp60. Fraction 1 contains the load (60% wt/wt sucrose); fraction 11 contains 20-25% wt/wt sucrose. Endogenous Pex5p is only visible in the pex14Δ gradient. The asterisk indicates an aspecific cross-reacting band.
remnants and remained in the loading zone of the gradient, indicating that neither of the Pex5 proteins were associated with ghosts or other membrane structures. Together with the fluorescence data it can be concluded that in \textit{pex14}\textDelta cells Pex5p is located in the cytoplasm.

**GFP-Pex5p redistributes to peroxisomes in pex14\textDelta cells upon Pex14p expression**

To further investigate the role of Pex14p in Pex5p localization, the \textit{PEX14} gene was placed under the control of the galactose-inducible \textit{Gal1/10} promoter. The resulting construct (pGAL-\textit{PEX14}) was transformed to \textit{pex14}\textDelta cells. To investigate the expression of Pex14p in these transformants, cells were precultured on 2% glucose media (repressed condition), harvested and inoculated in 2% galactose containing medium (induced condition). At various time-points after galactose-induction, cells were lysed and equal amounts of protein were analyzed by SDS-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
Time on galactose (h): & 0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 20 \\
\hline
A & Pex14p & & & & & & & & & \\
\hline
 & Kar2p & & & & & & & & & \\
\hline
B & GFP-Pex5p & & & & & & & & & \\
\hline
 & GFP-SKL & & & & & & & & & \\
\hline
C & Wild-type & \textit{pex14}\textDelta & \textit{pex14}\textDelta & & & & & & & \\
& H & P & S & H & P & S & H & P & S & \\
\hline
\end{tabular}
\caption{induction of Pex14p expression in the \textit{pex14}\textDelta strain partially restores PTS1 and PTS2 protein import and localizes Pex5p to peroxisomes. A) \textit{Pex14} cells transformed with pGAL-\textit{PEX14} were precultured on glucose media and shifted to 2% galactose media. At indicated timepoints cells were lysed and samples were analyzed for Pex14p and the ER protein Kar2p by immunoblotting. B) \textit{Pex14} cells transformed with pGAL-\textit{PEX14} and a plasmid encoding GFP-Pex5p or GFP-SKL were followed with time after galactose induction. Photographs were taken at indicated time points. Bar = 5 \textmu m. C) Pex14\textDelta cells expressing pGAL-\textit{PEX14} and untransformed \textit{pex14}\textDelta and wild-type cells were converted to spheroplasts and cleared homogenates (H) were separated into 25,000 x g pellet (P) and a 25,000 x g supernatant (S) fractions. Fractions were analyzed for 3-ketoacyl-CoA thiolase (thiolase) by immunoblotting.}
\end{table}
Peroxisomal localization of Pex5p requires Pex14p and Pex17p

PAGE and immunoblotting with antibodies specific for Pex14p. As a control for the amount of protein loaded, samples were analyzed for the ER protein Kar2p. Figure 6A shows that in glucose-grown cells (t = 0) Pex14p is not detectable. Expression of Pex14p becomes visible after 2 h of induction on galactose and reaches a steady-state level after 6 h. Pex14Δ cells were transformed with pGAL-PEX14 and with the construct for expression of GFP-Pex5p. The transformants were grown on 2% glucose media, harvested and resuspended in liquid media containing 2% galactose. At different time points after galactose-induction the subcellular localization of GFP-Pex5p was monitored by fluorescence microscopy. Glucose-grown transformants showed exclusively cytosolic GFP-Pex5p labeling (Figure 6B, t = 0). After 2 h induction on galactose some cells exhibited a few fluorescent spots, that were absent in control cells (untransformed pex14Δ cells). After 4 h growth on 2% galactose medium approximately 30-40% of the cells showed punctated GFP-Pex5p labeling (Figure 6B, t = 4), that appeared in a few distinct dots at the periphery of the cell. After 8 h of growth in galactose-medium, most of the cells exhibited punctated GFP-Pex5p-containing structures that in some individual yeast cells had distributed throughout the cell. In parallel cultures of untransformed pex14Δ cells GFP-Pex5p localized to the cytoplasm, whereas wild-type cells exhibited a punctated fluorescence pattern, indicating that growth on galactose alone did not affect the subcellular distribution GFP-Pex5p (data not shown). These fluorescence data suggest a correlation between Pex14p expression and redistribution of GFP-Pex5p from a diffuse cytosolic labeling to a punctated peroxisomal staining.

To investigate whether import of PTS1 proteins is restored in pex14Δ cells, a galactose- induction experiment was performed with cells co-expressing pGAL-PEX14 and the PTS1 reporter protein GFP-SKL. After 4 h on galactose GFP-SKL started to concentrate in punctated structures, suggesting peroxisomal import of the reporter protein (Figure 6B). The import of GFP-SKL did not appear to be efficient, since after 8 h of growth on galactose media, only 50% of the population exhibited punctated fluorescence combined with clear cytoplasmic labeling. The subcellular distribution of the peroxisomal matrix protein 3-ketoacyl-CoA thiolase, which is localized to the 25,000 x g supernatant of fractionated pex14Δ cells, could partially be recovered from the pellet fraction of pex14Δ cells expressing pGAL-PEX14, indicating partial rescue of PTS2 protein import (Figure 6C). The observed partial import defect of PTS1 and PTS2 proteins in pex14Δ cells expressing Gal1/10 promoter controled Pex14p is probably due to dramatic overexpression of Pex14p (estimated mRNA levels are at least 65 times higher than normal). Overproduction of Pex14p has previously been shown to inhibit normal peroxisome biogenesis (Bottger et al., 2000; Komori et al., 1997; Otera et al., 2000; Will et al., 1999).
DISCUSSION

The subcellular distribution of Pex5p depends on the growth conditions

The subcellular distribution of the PTS receptors, in particular that of the PTS1 receptor Pex5p, has been analyzed in a wide variety of organisms using different experimental setups. These studies have resulted in the general view that the receptors are predominantly cytoplasmic with a small, but significant fraction present at the peroxisomal membrane (de Walque et al., 1999; Dodt and Gould, 1996; Gould et al., 1996; Gould and Valle, 2000; Gouveia et al., 2000; Okumoto et al., 2000; Otera et al., 2000) or even in the peroxisomal matrix (Dodt and Gould, 1996; Van der Klei et al., 1995; Wimmer et al., 1998). A predominantly cytoplasmic localization has also been reported for Pex5p in oleate-grown S. cerevisiae cells (Elgersma et al., 1996a). In this study we reinvestigated the Pex5p localization in S. cerevisiae under different growth conditions and applied GFP-tagging of Pex5p to identify candidate proteins that play a crucial role in targeting or tethering Pex5p at the peroxisomal membrane. We show that in glucose-grown wild-type cells, Pex5p could specifically be recovered from the 25,000 x g organellar pellet fraction, whereas only a minor portion of Pex5p was detectable in the 25,000 x g supernatant fraction (Figure 1A). In glucose-grown pex3Δ cells, which are characterized by the absence of peroxisomal membrane remnants (Baerends et al., 1996; Hetteima et al., 2000; Wiemer et al., 1996), Pex5p was mainly detected in the supernatant fraction, implying that the subcellular distribution of Pex5p in glucose-grown wild-type cells is related to the presence of peroxisomes. The relatively low amount of Pex5p in the 25,000 x g supernatant fraction of differentially centrifugated glucose-grown wild-type cells was not be caused by instability of cytosolic Pex5p, since the abundance of Pex5 protein in glucose-grown wild-type and pex3Δ cells was comparable (data not shown). Furthermore, overexpression of Pex5p does not change the distribution of the protein (Figure 3), indicating that protein abundance is not an important determinant in the subcellular distribution of Pex5p.

By changing growth conditions from glucose to oleate, Pex5p gradually appeared in the supernatant fraction, and 8 hours after the shift the protein showed a distribution comparable to that of cells grown overnight on oleate (Figure 1B). The shift from glucose to oleate growth conditions results in massive transcriptional upregulation of genes involved in fatty acid metabolism (Kal et al., 1999). By contrast, transcription of most genes encoding proteins involved in the biogenesis of the peroxisome (peroxins) remains unchanged. These differences in transcriptional regulation result in a different ratio of peroxins over peroxisomal matrix proteins in the two growth conditions: in glucose-grown cells there are relatively more peroxins than peroxisomal matrix proteins, while in oleate-grown cells matrix proteins are far more abundant than peroxins. It was therefore tempting to speculate that the cytoplasmic localization of Pex5p in oleate-grown cells would correlate with the increased number of newly synthesized PTS1 proteins. To test this hypothesis we made use of the S. cerevisiae pip2Δ strain, which is unable to induce expression of
most peroxisomal proteins in oleate-grown cells (Karpichev and Small, 1998; Rottensteiner et al., 1996). Surprisingly, in oleate-grown pip2Δ cells Pex5p was primarily localized in the 25,000 x g supernatant fraction, a distribution that is comparable to that of Pex5p in oleate-grown wild-type cells (Figure 2B). Moreover, overexpression of a PTS1 protein (Mdh3p) in cells grown on glucose, a condition with low levels of endogenous PTS1 proteins, also did not change the distribution of Pex5p (Figure 2A). Together, these data indicate that distribution of Pex5p is not dependent on the presence of PTS1 protein cargo in the cell. However, the distribution of Pex5p must be tightly regulated, since overexpression or underexpression does not change the proportion of Pex5p in the 25,000 x g pellet and supernatant fractions, whereas absolute amounts of Pex5p in the pellet and supernatant fractions can vary enormously (Figure 3) without seriously affecting function (i.e. ability to complement oleate growth of pex5Δ cells). How the distribution of Pex5p between peroxisome and cytoplasm is regulated remains to be analyzed. It is tempting to speculate that posttranslational modification of the receptor and/or its binding partners at the peroxisomal membrane could be involved in this regulation.

Genes involved in peroxisomal location of Pex5p

To identify proteins involved in Pex5p localization we screened the known pex deletion strains of S. cerevisiae with the reporter GFP-Pex5p. This screen identified two proteins, Pex14p and Pex17p that are essential for targeting of Pex5p to or tethering of Pex5p at the peroxisomal membrane. Flotation gradient analysis of glucose-grown pex14Δ cells confirmed that both the GFP-Pex5p and the endogenous Pex5p were unable to associate to membranous structures (Figure 5). Since all PEX deletion strains containing peroxisomal membrane remnants still displayed GFP-Pex5p association with peroxisomal ghosts (Table 1), there is no apparent correlation between Pex5p binding to peroxisomes and a functional matrix protein import system. Similar observations have been reported for human Peroxisome Biogenesis Disorder (PBD) cell lines with mutations in the RING finger-containing proteins Pex2p and Pex10p. In those mutant cells Pex5p still targeted to peroxisomal ghosts (Chang et al., 1999; Dodt et al., 1995) and mutations in the third RING finger-containing protein Pex12p, and also in Pex13p of CHO cells, even resulted in accumulation of Pex5p at peroxisomal ghosts (Dodt and Gould, 1996; Otera et al., 2000). Such an accumulation was not observed in yeast pex12Δ and pex13Δ cells, since the cytoplasmic pool of GFP-Pex5p was always clearly detectable in these cells.

A direct correlation between Pex14p expression and Pex5p localization at the peroxisomal membrane was obtained by expressing a galactose-inducible form of Pex14p in pex14Δ cells. Fluorescence and biochemical analysis showed that at early time points after induction of Pex14p expression (2 hours) GFP-Pex5p appeared at the peroxisome. This redistribution of GFP-Pex5p coincided with the import of the PTS1 reporter protein GFP-PTS1 (Figure 6B). However, the GFP-PTS1 import appeared to be very inefficient since a large fraction of the cells showed a diffuse
cytosolic labeling even after eight hours of Pex14p induction (Figure 6B). The inefficient PTS1 protein import in these cells is most likely caused by the dramatic overexpression of Pex14p. Overexpression of Pex14p has previously been reported to inhibit peroxisomal protein import both in the yeasts *S. cerevisiae* (Bottger et al., 2000) and *H. polymorpha* (Komori et al., 1997), and in CHO cells (Otera et al., 2000). It is remarkable that despite the considerable overexpression of Pex14p in our galactose-inducible system, Pex5p still exhibited a predominantly cytoplasmic localization (data not shown). Increasing the absolute amount of Pex14p therefore appears not to affect the distribution of Pex5p between peroxisome and cytoplasm. Similar results have been obtained in oleate-grown *S. cerevisiae* cells overexpressing Pex14p and/or Pex13p (A. Stein and G. Bottger, unpublished observations). Apparently Pex14p provides a potential binding site for Pex5p, but the amount of Pex5p at the peroxisomal membrane is also determined by other factors.

**Involvement of Pex14p and Pex17p in Pex5p localization**

Our data suggest that Pex14p and Pex17p are strictly required for peroxisomal localization of Pex5p, and that Pex5p distribution does not depend on a functional PTS1 protein import system. Although Pex14p has previously been identified as part of the PTS receptor docking complex (Albertini et al., 1997; Fransen et al., 1998; Girzalsky et al., 1999; Otera et al., 2000; Shimizu et al., 1999; Subramani et al., 2000; Urquhart et al., 2000), our data now indicate that Pex14p is the primary binding factor for Pex5p at the peroxisomal membrane. Interestingly, Pex17p is the second component required for peroxisomal targeting of Pex5p. *Sc PEX17* was isolated in a genetic screen for oleate non utilizers and was shown to be required for the import of both PTS1 and PTS2 proteins (Albertini et al., 1997). Pex14p and Pex17p interact with each other and are both peripheral membrane proteins facing the cytoplasmic side of the peroxisomal membrane (Erdmann et al., 1997; Huhse et al., 1998). Two-hybrid experiments in a *pex14Δ* strain revealed that Pex5p and Pex17p only display a positive two-hybrid interaction in the presence of Pex14p, indicating that the three proteins can form a complex in which Pex14p bridges the interaction between Pex5p and Pex17p (Huhse et al., 1998). In the absence of Pex17p, Pex14p is still associated to the peroxisomal membrane (Girzalsky et al., 1999). Cytosolic localization of Pex5p in *pex17Δ* cells is therefore not caused by loss of Pex14p at the peroxisomal membrane. The requirement of Pex17p in peroxisomal association of Pex5p in the absence of a direct interaction between these proteins, suggests that Pex17p and Pex14p must associate to establish stable contact between Pex5p and Pex14p *in vivo*. In addition to Pex14p, two other proteins, Pex8p (Rehling et al., 2000) and Pex13p (Barnett et al., 2000; Bottger et al., 2000; Urquhart et al., 2000) have been shown to directly interact with Pex5p. The interaction between Pex13p and Pex5p is mediated by the SH3 domain of Pex13p, which is exposed to the cytosolic face of the peroxisomal membrane. Based on these observations Pex13p has been implicated as a component of the PTS1 receptor docking complex. Our observation that GFP-Pex5p still associates with peroxisomal membranes of *pex13Δ* and *pex8Δ* cells
suggests that the interactions of Pex5p with the SH3 domain of Pex13p and with Pex8p play a role in a post-docking event. In support of this we have previously reported that disruption of the interaction between Pex5p and Pex13-SH3 did not affect the peroxisomal location of Pex5p, but did affect protein import into the peroxisome (Bottger et al., 2000). In addition, disruption of the interaction between Pex13-SH3 and Pex14p severely disturbs peroxisomal matrix protein import, but has no effect on peroxisomal localization of Pex5p (Bottger et al., 2000) and our unpublished observations). The Pex13p-Pex14p complex at the peroxisomal membrane may therefore not function in the primary docking of the PTS1 receptor, but may play a role downstream of receptor docking. In support of this view, Urquhart et al. (2000) showed in vitro that Pichia pastoris Pex14p preferentially binds a PTS1-protein loaded Pex5p, whereas Pex13-SH3 prefers to bind an unloaded receptor, suggesting that Pex5p loses its PTS1 protein cargo between its interaction with Pex14p and Pex13p.

Together, these results suggest that the interaction of Pex5p with the Pex14p-Pex17p complex is either the first contact of Pex5p with the peroxisome, or is the rate-limiting step in PTS1 protein delivery to the translocation machinery and is likely to occur prior to complex formation of Pex14p and Pex13p. The sequential binding of Pex5p to the different Pex proteins at the peroxisomal membrane may induce conformational changes in the receptor itself or in the interacting proteins, thereby regulating the matrix protein import process. However, an additional level of regulation must exist since protein-protein interaction alone does not explain the subcellular localization of Pex5p under different growth conditions. Since environmental changes can trigger signal transduction pathways, the distribution of Pex5p between peroxisomes and the cytoplasm may be regulated via post-translational modifications of the receptor itself, or one of its interacting partners. In the yeast H. polymorpha phosphorylation of Pex14p has been reported (Komori et al., 1999). It remains to be determined whether Pex5p itself and/or other peroxins undergo post-translational modifications in order to fine-tune the regulation of the import of proteins into peroxisomes.

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ABBREVIATIONS
GFP: green fluorescent protein; SH3: Src homology 3; PTS: peroxisomal targeting signal; CHO: Chinese hamster ovary; PGK: Phosphoglycerate kinase; PMP: peroxisomal membrane protein; NH: haemeagglutinin
REFERENCES:


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