Redox regulated cargo binding and release by the peroxisomal targeting signal receptor, Pex5*

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Background: Pex5 transports PTS1 proteins to peroxisomes, releases them there and returns to the cytosol.

Results: Several steps of the import cycle are controlled by redox-sensitive oligomeric states of Pex5.

Conclusion: Cargo release from Pex5 is achieved by a redox-regulated oligomer to dimer transition of Pex5 and aided by Pex8.

Significance: This redox-regulation of Pex5 function provides the first mechanistic view of cargo release.

Abstract

In its role as a mobile receptor for peroxisomal matrix cargo containing a peroxisomal targeting signal called PTS1, the protein, Pex5, shuttles between the cytosol and the peroxisome lumen. Pex5 binds PTS1 proteins in the cytosol via its C-terminal tetratricopeptide domains and delivers them to the peroxisome lumen, where the receptor/cargo complex dissociates. The cargo-free receptor is exported to the cytosol for another round of import. How cargo release and receptor recycling are regulated is poorly understood. We found that Pex5 functions as a dimer/oligomer and that its protein interactions with itself (homo-oligomeric) and with Pex8 (hetero-oligomeric) control the binding and release of cargo proteins. These interactions are controlled by a redox-sensitive amino acid, cysteine 10 of Pex5, which is essential for the formation of disulfide bond-linked Pex5 forms, for high-affinity cargo binding and for receptor recycling. Disulfide bond-linked Pex5 showed the highest affinity for PTS1 cargo. Upon reduction of the disulfide bond by dithiothreitol, Pex5 transitioned to a non-covalent dimer, concomitant with the partial release of PTS1 cargo. Additionally, dissipation of the redox balance between the cytosol and the peroxisome lumen caused an import defect. A hetero-oligomeric interaction between the N-terminal domain (aa 1-110) of Pex5 and a conserved motif at the C-terminus of Pex8 further facilitates cargo release, but only under reducing conditions. This interaction is also important for the release of PTS1 cargo. Additionally, we suggest a redox-regulated model for Pex5 function during the peroxisomal matrix protein import cycle.

INTRODUCTION
Peroxisomes are organelles dedicated to lipid metabolism and scavenging of reactive oxygen species. The role of these intriguing organelles has recently been extended to aging, as well as to antiviral defense by activation of the rapid interferon-independent signaling pathway and/or stimulation of invariant natural killer T cells (1-4).

From yeast to plants and mammals, peroxisomes contain from several dozen to several hundred luminal proteins (5). Most peroxisomal matrix proteins contain a conserved C-terminal PTS1 (peroxisomal targeting signal 1), which is comprised of a tripeptide, SKL, or its variants. The PTS1 receptor, Pex5, captures PTS1 proteins in the cytoplasm by a direct interaction between the C-terminal tetratricopeptide repeat (TPR) domains of Pex5 and the PTS1 motif of the cargo (6). The N-terminal disordered region (aa 1-276) of Pex5 is essential for interaction with other peroxins, such as Pex14 and Pex13, but is not required for its binding to canonical PTS1 proteins (7). In the absence of cargo, the TPR domains of Pex5 organize into an open, snail-like conformation and form a tunnel-shaped binding pocket. Upon binding of the PTS1 motif to the tunnel, the TPR domains transition to a closed, circular conformation (6). However, there are conflicting reports regarding the oligomeric state of Pex5 and the functions assigned to these states (8-11). Specifically, the mechanism and nature of the oligomeric species of Pex5 to which PTS1 proteins are bound are unclear.

The PTS1 receptor/cargo complex translocates across the peroxisomal membrane through an unusual protein channel, which opens up to 9 nm in diameter, and is composed of at least two proteins, Pex14 and the PTS1 receptor itself (12,13). After unloading the peroxisomal cargo in the organelle lumen, Pex5 is exported by an unknown mechanism to the cytosolic side of the peroxisomal membrane, where it is mono-ubiquitinated on a conserved cysteine residue (Cys10 in *Pichia pastoris* Pex5) near the N-terminus (14,15). The mono-ubiquitinated receptor is released to the cytosol by the action of AAA ATPases, Pex1 and Pex6, for another round import (16). When the receptor-recycling pathway is blocked, Pex5 is poly-ubiquitinated at one (K22 in *P. pastoris* Pex5) or two conserved N-terminal lysine residues and degraded by the proteasome (17). How the receptor recycling and degradation pathways are regulated is not known.

Unlike the ER, chloroplast and mitochondria, whose matrix protein import receptors are integral membrane proteins, the peroxisomal receptors, Pex5 and the PTS2 receptor, Pex7, shuttle between the cytosol and the peroxisomal lumen (18,19). Therefore, the peroxisomal receptors face a challenging problem because they must ensure high affinity binding to the PTS1 proteins in the cytosol, but must not rebind them after their release in the peroxisomal lumen. How cargo is released is poorly understood. Pex8 from lower eukaryotic cells and Pex14 from higher eukaryotic cells have been suggested to be involved in the release of PTS1 proteins from Pex5, respectively (8,20). However, the underlying molecular mechanism is not evident.

The redox state of the peroxisome lumen is more reducing than in the cytosol (21). Consistent with this observation, we show that the oligomeric states of Pex5 and their differential ability to bind cargo are under redox regulation. We identified Cys10 in *P. pastoris* Pex5 as a redox-sensitive amino acid, which regulates the oligomeric state of Pex5, cargo binding, cargo release, and receptor recycling. We found that a disulfide bond-linked Pex5 (homo-oligomeric state) demonstrates the highest affinity for PTS1 cargo. Upon reduction of the disulfide bond *in vitro* by dithiothreitol (DTT), thereby mimicking the intraperoxisomal reducing environment, Pex5 transitions to a non-covalent dimer (homo-dimer) concomitantly with the partial release of PTS1 cargo. Dissipation of the redox balance between the cytosol and the peroxisome lumen with...
H$_2$O$_2$ causes a cargo import defect. The cargo release process is facilitated and driven to completion by the interaction between Pex5 and Pex8 (hetero-oligomeric state). We have identified the domains of Pex5 and Pex8 involved in this interaction and we suggest a mechanistic model for Pex5 function during the import cycle for PTS1-containing proteins into the peroxisome matrix.

**EXPERIMENTAL PROCEDURES**

**Yeast strains, plasmids, and culture conditions**

The *P. pastoris* strains used are listed in Table 1. Growth media components were as follows: rich medium YPD, 1 % yeast extract, 2 % peptone, 2 % glucose; synthetic medium YNM, 0.67 % yeast nitrogen base, 0.1 % yeast extract, 0.5 % (vol/vol) methanol; mineral oleate medium YNO, 0.67 % yeast nitrogen base, 0.1 % yeast extract, 0.2 % (vol/vol) oleate and 0.02 % (vol/vol) Tween-40. Yeast cells were grown at 30 °C in YPD for 6-7 h, washed with distilled H$_2$O, and shifted either to synthetic methanol medium (YNM), or mineral oleate medium (YNO) for biochemical experiments or fluorescence microscopy.

**Plasmid construction**

The *PpPEX5* promoter was amplified and cloned into the pIB1 vector at SmaI-PstI sites, creating pCM256. The PpPEX5 ORF was amplified and transferred to pMY69 (lab stock) at ClaI-AflII sites to generate PpPex5 with a C-terminal HA tag. The Pex5-HA fragment was amplified and cloned downstream of the PpPEX5 promoter in pCM256 at PstI-HindIII sites, creating pCM259. Based on pCM259, the corresponding cysteine mutants (C10S, C338S, C444S, C338S/C444S, and N460K) and the truncated fragment (amino acids 1-110) were made by site-directed mutagenesis based on pCM258 and used for protein purification.

The *PpPEX8* promoter and ORF were amplified and cloned into the pIB1 vector between the KpnI-BamHI sites, creating pSGP01. Based on pSGP01, the corresponding mutants, Pex8A36, Pex8ΔAKL, Pex8ΔWWYΔAKL, were generated by site-directed mutagenesis.

GFP with a N-terminal FLAG tag and a C-terminal PTS1 (SKL) was cloned into the pETDuet-1 vector (Novagen) at NcoI-NotI Sites, creating FLAG-GFP-SKL for co-purification with His-Pex5 or the pACYCDuet-1 vector at Ascl-NotI, creating His-FLAG-GFP-SKL for estimation the size of GFP-SKL in the absence of receptor (used only in Fig. 4C).

**Subcellular fractionation and protease protection**

Subcellular fractionation from oleate-induced yeast cells and the protease protection assay were performed as described previously (12).

**Fluorescence microscopy**

Cells were grown in YPD medium and switched to YNM during exponential phase growth. Images were captured on a Zeiss Axioskop fluorescence microscope (AxioSkop 2 Plus, motorized) coupled to a cooled CCD monochrome camera (AxioCam MRM, Zeiss) and analyzed using AxioVision 4 software.
Protein purification

The P. pastoris Pex5 and its cysteine mutants were expressed in E. coli. Cultures were grown in LB medium to mid-log phase and induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and incubated for 3 h at 30 °C. Cells were collected by centrifugation and resuspended in lysis buffer containing 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF), complete™ protease inhibitor (Roche Applied Science) and 1 mg/ml lysozyme. Cells were broken by sonication, followed by centrifugation to remove cell debris and other insoluble material. Supernatants were loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) column (HisTrap FF column, GE Healthcare, USA) and the proteins were purified using an AKTA purifier (GE Healthcare, USA). To estimate the size of the recombinant His-Pex5 and His-Pex5 C10S, gel filtration analyses were performed on a gel filtration column (Superdex 200 10/300, GE Healthcare, USA) equilibrated with buffer containing 50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8.0. Fractions (250 µl) were collected and analyzed by SDS-PAGE, followed by Western blotting.

P. pastoris Pex8 with a C-terminal HA tag or Pex8Δ36 was amplified and transferred to pETM11 vector (a gift from Matthias Willmanns, EMBL, Germany), which contains a cleavable tobacco etch virus (TEV) protease site after the His-tag. The recombinant His-Pex8-HA and His-Pex8Δ36 were purified as described for His-Pex5 and their N-terminal His-tag was cleaved by incubation with His-TEV protease overnight at 4 °C. The samples were incubated with Ni-NTA beads and the supernatant were collected after centrifugation.

To generate His-Pex5/FLAG-GFP-SKL, proteins were co-expressed in E. coli. The complexes were first affinity purified by HisTrap FF column, followed by gel filtration analysis using a gel filtration column (HiLoad Superdex 200 16/60) in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl in the absence or presence of 10 mM DTT. Fractions (1 ml) were collected and analyzed by SDS-PAGE, followed by Protoblue staining or Western blotting.

GST-Pull down assay

For assaying protein-protein interaction, around 1 µg of GST-SKL was immobilized on 20 µl packed glutathione Sepharose 4B slurry (GE Healthcare, USA), incubated with whole cell lysates expressing equal amount of His-Pex5 or its relevant mutants in the binding buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 1 mM PMSF, complete™ protease inhibitor, Roche Applied Science) for 2 h at 4 °C. The beads were washed five times with binding buffer. Bound proteins were analyzed by SDS-PAGE, followed by Western blotting with anti-His antibody.

Cargo release experiments

2 µg of His-Pex5/GFP-SKL complex was immobilized on 80 µl Ni-NTA beads incubated overnight at 4 °C in binding buffer in the absence of DTT (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, 0.1% Triton-X-100 and protease inhibitors). The beads were washed three times with binding buffer. The beads were aliquoted into four reactions and treated with or without 2 mM DTT or 4 µg each of Pex8-HA or Pex8Δ36 for 2 h at RT. Subsequently, the supernatants were aliquoted into fresh tubes and heated in sample buffer to 100 °C for 5 min. The beads were washed three times (50 mM NaH$_2$PO$_4$, 150 mM NaCl, 20 mM imidazole, 0.01% Triton-X-100 and protease inhibitors) and eluted in sample buffer. Equal volumes of the supernatant and bead fractions were analyzed by SDS-PAGE, followed by Western blotting.

RESULTS

P. pastoris Pex5 is linked by disulfide bonds
In order to investigate whether Pex5 undergoes an oxidative modification, PpPex5 was TCA precipitated from cells grown in methanol medium and analyzed using reducing and non-reducing SDS-PAGE. The anti-Pex5 antibody is specific since, under reducing conditions (+DTT), only a single band around 72 kDa was detected in wild-type *P. pastoris*, but not in the Δpex5 strain (Fig. 1A, left panel). Under non-reducing SDS-PAGE (-DTT), in addition to the 72 kDa band, a band around 140 kDa (corresponding to a Pex5 homodimer, see below) and two bands larger than 170 kDa (corresponding to Pex5 oligomers, see below) were observed (Fig. 1A, right panel). A band around 80 kDa found upon SDS-PAGE in non-reducing conditions is likely the mono-ubiquitinated Pex5 (22). The high molecular weight (HMW) Pex5 species visible by non-reducing SDS-PAGE were sensitive to 2 mM DTT and 2 mM glutathione (GSH), indicating they are linked through disulfide bonds (Fig. 1B).

The HMW species of PpPex5 found in *P. pastoris* could either be a homo- or a hetero-oligomeric complex with other peroxins or unknown proteins. Therefore, we expressed PpPex5 in *E. coli* to determine whether Pex5 can form a disulfide bond-linked dimer or oligomer. PpPex5 with an N-terminal His-tag was first affinity-purified and then loaded onto a gel filtration column. During gel filtration chromatography, His-tagged PpPex5 appeared in two peaks as detected by anti-His antibody, corresponding to molecular masses around 140 kDa and 280 kDa, respectively (Fig. 1C). In non-reducing gels, Pex5 in the 140 kDa peak ran as a single band (72 kDa), indicating it is a non-covalent dimer. In contrast, Pex5 in the 280 kDa peak ran as two bands (140 kDa and 72 kDa) of similar intensity, indicating that Pex5 in the 280 kDa peak is likely to be a tetramer composed of two homo-dimers linked by intermolecular disulfide bonds. We refer to the two forms of PpPex5 as disulfide bond-linked Pex5 (dslPex5) and non-covalent Pex5 (ncPex5), noting that different oligomeric states of Pex5 (monomer, dimer or tetramer) have been reported based on the use of different organisms, buffers and methods (9,11,23).

When ncPex5 was concentrated in non-degassed buffer, around half of this species was converted to dslPex5 (Fig. 1D), corresponding to a similar band seen in the extracts of methanol-grown yeast cells (Fig. 1A, right panel).

The redox states between the cytosol and the intraperoxisomal matrix change when cells are grown in different media (21). To investigate whether the dslPex5 correlates with the redox conditions under peroxisome proliferative conditions, Pex5 expressed from a constitutive glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter was generated. The dslPex5 was barely discernible in rich glucose medium (Fig. 1E, 0 h), but appeared when cells were grown in methanol medium (Fig. 1E, 6 h). Therefore, the formation of dslPex5 was enhanced in peroxisome proliferation conditions.

**PpPex5 C10 is essential for the formation of disulfide bond-linked Pex5**

PpPex5 contains three cysteines, all of which are conserved among different Pex5 homologues. PpPex5 Cys10 (C10) is located in the N-terminal disordered region, while Cys338 and Cys444 reside in the second and fifth TPR motif, respectively (Fig. 1F).

To investigate which cysteine/s is/are required for the formation of dslPex5, the three cysteines were mutated to serine. The Pex5 cysteine mutants with a C-terminal HA tag, each expressed from their own promoters, were transformed independently into the Δpex5 strain. In contrast with wild-type PpPex5, the PpPex5 C10S mutant completely lost the ability to generate dslPex5 when induced in methanol medium overnight (Fig. 1G, left panel). However, when compared to wild-type Pex5, the expression level of PpPex5 C10S was low, but even in long exposures we still did not see any dslPex5 (Fig. 1G, right panel). PpPex5 C338S and PpPex5 C444S did not
show significant loss of dslPex5 (Fig. 1G, left panel). However, when both C338 and C444 were replaced by serine, the Pex5 bands above 170 kDa decreased significantly, suggesting these two cysteines have a redundant role in the formation of intermolecular disulfide bond-linked Pex5 oligomers. Consistent with the in vivo data, in vitro purified PpPex5 C10S completely lost the disulfide bond-linked forms as well, and instead showed only the ncPex5 (Fig. 1H). When compared to wild-type Pex5, PpPex5 C10S may have a different conformation that is more resistant to the attack of proteases, since the affinity-purified Pex5 contained several degradation products that were almost non-existent in the affinity-purified Pex5 C10S (Fig. 1I).

PpPex5 C10S has a defect in importing peroxisomal PTS1 cargo

We reasoned that the three cysteines and the dslPex5 might be important for fulfilling the function of Pex5 as a PTS1 receptor. As shown by the growth curve in methanol medium, PpPex5 C10S did not complement Δpex5 cells (Fig. 2A). Although PpPex5 C338S and PpPex5 C444S eventually grew as well as the wild-type cells, both grew more slowly. Moreover, the PpPex5 C338S/C444S double mutant showed a more severe growth defect than the respective single mutants.

The inability of these Pex5 cysteine mutants to complement Δpex5 cells under peroxisome proliferation conditions indicates they may have a defect in importing PTS1 proteins into peroxisomes. Using fluorescence microscopy, we found that in Δpex5 cells complemented with wild-type Pex5 expressed from its own promoter, the peroxisomal PTS1 cargo, BFP-SKL, was targeted to punctate structures co-localizing with Pex3p-mRFP-labeled peroxisome clusters (Fig. 2B). However, in Δpex5 cells transformed with PpPex5 C10S, BFP-SKL was completely cytosolic. The targeting of BFP-SKL to peroxisomes was normal in Δpex5 cells transformed with either PpPex5 C338S or PpPex5 C444S. However, similar to the situation seen with PpPex5 C10S cells, BFP-SKL was predominantly cytosolic in the Pex5 C338S/C444S double mutant. These results indicate that the three cysteine residues of PpPex5 are important for its function as a PTS1 receptor and that C338 and C444 may perform redundant functions.

Disulfide bond-free PpPex5 C10S enters peroxisomes and is degraded via the RADAR pathway

To check whether the PpPex5 C10S mutant enters and exits peroxisomes, we performed differential centrifugation assays. As shown in Fig. 2C, in Δpex5 cells complemented with wild-type Pex5, the receptor was primarily in the cytosol fraction, while only a small amount was associated with the organelle fractions. Pex17, a peroxisomal membrane control, was associated with the organelle fractions, as expected. However, PpPex5 C10S was distributed more in the pellet than in the supernatant fraction, suggesting that it probably cannot be efficiently recycled. Moreover, consistent with the growth curve and fluorescence microscopy data, the peroxisomal import of catalase, a PTS1 cargo, was impaired in Δpex5 cells transformed with PpPex5 C10S.

To distinguish whether PpPex5 C10S entered the peroxisome lumen, we performed protease protection experiments (Fig. 2D). Similar to what was observed in the protease protection assay from the Δpex5 cells complemented with wild-type Pex5, PpPex5 C10S was resistant to protease treatment in the absence of detergent and was degraded only after addition of Triton X-100. In contrast, Pex17 was susceptible to proteases even without detergent. However, thiolase, serving as a peroxisomal matrix protein control and targeted to the matrix in a Pex5-independent manner, was resistant to protease treatment in the absence of Triton.

The instability of PpPex5 C10S relative to wild-type Pex5 (Fig. 2E) suggests it may be degraded by entering the RADAR (Receptor
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Accumulation and Degradation in the Absence of Recycling) pathway, because it cannot be mono-ubiquitinated on C10 and recycled (22). If this hypothesis is true, mutating lysine 22, an amino acid predicted to be important for poly-ubiquitination on Pex5, should rescue the instability of PpPex5 C10S. Indeed, as shown in Fig. 2E, PpPex5 C10S/K22R was stabilized relative to PpPex5 C10S. However, stabilized Pex5 C10S/K22R could not rescue the import defects of Pex5 due to its inability to be recycled or degraded (Fig. 2B). Therefore, PpPex5 C10S can enter the peroxisome lumen and be transferred to the peroxisome membrane where it is degraded by the RADAR pathway.

**Pex5 cysteine mutants have lower binding affinity for PTS1 cargo**

In contrast to PpPex5 C10S, PpPex20 C8S, which similarly blocks mono-ubiquitination and recycling of Pex20 showed only a mild growth defect, a result in line with the hypothesis that a deficiency in receptor recycling alone would result in a milder peroxisomal import defect, as long as the RADAR pathway is able to clear the receptor from the peroxisome membrane (24). The dysfunction of Pex5 C10S in peroxisome biogenesis could not be solely attributed to its instability. When Pex5 C10S was expressed from the GAPDH promoter in methanol medium for 3 h, it had a similar expression level as WT Pex5 (Fig. 3A). However, BFP-SKL, expressed from the alcohol oxidase promoter, could only be imported efficiently in cells harboring a WT Pex5, but not Pex5 C10S (Fig. 3B). The severe PTS1 import defect observed in the PpPex5 C10S mutant suggests that PpPex5 Cys10 may be involved in other steps in the peroxisomal matrix protein import cycle.

As a test of this hypothesis, the binding abilities of cargo to PpPex5 and PpPex5 C10S were tested by a GST-pull down assay. GST-SKL, a known peroxisomal matrix cargo, was bound to glutathione beads. Then, cell lysates containing equal amounts of Pex5, or its cysteine mutants, were mixed with the beads to test their binding ability to the peroxisomal cargo in the absence of DTT. A Pex5 N460K mutant, which has been shown from different Pex5 homologues to be essential for cargo binding (25), was used as a negative control. As shown in Fig. 3C, PpPex5 C10S (right panel), similar to PpPex5 N460K (left panel), showed very low binding affinity to GST-SKL when compared to the wild-type Pex5. The other Pex5 cysteine mutants had mildly reduced cargo-binding affinities relative to wild-type Pex5.

**PTS1 cargo/receptor complex dissociates upon DTT treatment**

To see whether reducing conditions play a role in cargo release, we first co-purified the His-Pex5/GFP-SKL complex under non-reducing conditions by affinity chromatography. Then we ran two separate gel filtrations, with and without 10 mM DTT. In the absence of DTT, GFP-SKL was mainly associated with dslPex5 in the first peak (Fig. 4A, fractions 23-33 and Fig. 4D). A small portion of the GFP-SKL was associated with the second peak (Fig. 4A, fractions 36-40), which has a mixture of mostly non-covalent and some covalent dimers (Fig. 4D). However, the purified receptor/cargo complex was dissociated partially under reducing conditions (Fig. 4B). The dslPex5 was reduced to ncPex5 concomitant with the appearance of free GFP-SKL. Under reducing conditions, less than 50% of GFP-SKL was still associated with ncPex5. GFP-SKL itself is a monomer (Fig. 4C) and no intermolecular bond was found between Pex5 and GFP-SKL, as judged by the absence of this hetero-oligomeric species (Fig. 4E).

To see the kinetics of cargo release, the His-Pex5/GFP-SKL complex was first captured on Ni-NTA beads in the absence of reducing agents and then treated with or without 1 mM DTT for up to 90 min. In the presence of DTT, around five-fold more cargo was released compared to treatment without
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DTT (Fig. 4F), indicating the receptor/cargo complex dissociates under reducing conditions.

Dissipation of the redox balance between the cytosol and the peroxisome lumen causes an import defect

In glucose medium, the peroxisomal import machinery of wild-type cells was not sufficient to transport all GFP-SKL into peroxisomes when it was overexpressed from the GAPDH promoter. However, all the GFP-SKL was transported from the cytosol to peroxisomes after the cells were transferred from glucose medium to methanol medium for 3 h since there was no remaining cytosolic staining (Figure 5A, top panel, compare 1 h and 3 h). In contrast, a significant amount of GFP-SKL remained in the cytosol when cells were exposed to 100 μM H$_2$O$_2$ for 3 h, indicating that maintaining a reducing peroxisomal lumen environment is important for cargo import (Fig. 5A, lower panel). The addition of 100 μM H$_2$O$_2$ caused a growth defect in methanol medium, but not in glucose or lactate medium, an indicator of functional mitochondria (data not shown). Moreover, the exposure of cells to 100 μM H$_2$O$_2$ did not significantly affect the expression of peroxins that are directly involved in peroxisomal matrix protein import (Fig. 5B). Surprisingly, we found less dslPex5 in cells treated with 100 μM H$_2$O$_2$ (Fig. 5C). This is likely caused by the activation of the RADAR pathway because, in the presence of 100 μM H$_2$O$_2$, the use of Pex5 K22R rescued the level of dslPex5 by blocking the RADAR pathway. These data are consistent with the hypothesis that oxidative stress impairs the recycling of Pex5.

Pex8 facilitates cargo release

Next, we investigated whether PpPex8, an intraperoxisomal peroxin only found in yeast, has a role in mediating cargo release. PpPex8 contains a PTS1, but is fully functional in the absence of its PTS1 (26). Therefore, if Pex8 has any role in cargo release, it is unlikely to be executed by direct displacement of PTS1 cargo by binding of the PTS1 on Pex8 to the TPR domains of Pex5.

Zhang et al. (2006) showed that Pex8 interacted, independently of its PTS1, with the N-terminal disordered region of Pex5 (26). We further localized the binding region of Pex8 on Pex5 to the N-terminal 110 amino acids by using co-purification experiments (Fig. 6A).

A pex8 mutant, per3-1, described earlier appeared to efficiently import thiolase, a PTS2 protein, but was specifically impaired in the targeting of PTS1 proteins (21,27), whereas in Δpex8 cells, both the PTS1 and PTS2 pathways were completely blocked. By sequencing the Pex8 locus of per3-1, we found that it lacks the last 36 amino acids of Pex8. A multisequence alignment indicates the existence of a short conserved motif (WWY) within the last 36 amino acids of Pex8 (Fig. 6B). When Pex8Δ36 was expressed from its own promoter in Δpex8 cells, the import of thiolase, but not catalase, was restored, confirming the importance of the C-terminal of Pex8 in the targeting of PTS1 proteins (Fig. 6C). Moreover, similar to Pex8Δ36, we found that Pex8ΔWWYΔAKL could selectively restore the PTS2, but not the PTS1, import pathway (Fig 6C). Expression of neither Pex8Δ36 nor Pex8ΔWWYΔAKL could complement the growth defects of Δpex8 cells in methanol medium (Fig. 6D).

We investigated whether Pex8 could cause cargo release by addition of an excess of recombinant Pex8 to the His-Pex5/FLAG-GFP-SKL complex, which was pre-bound to Ni-NTA beads in the absence of DTT. If Pex8 caused cargo release, one would expect GFP-SKL to be displaced into the supernatant. GFP-SKL was not released into the supernatant by dissociation from Pex5 when incubated with Pex8 under non-reducing conditions (Fig. 6E, compare GFP-SKL in Sup, lanes 1 and 3 and bar graph). Thus, Pex8 on its own does not displace
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PTS1 proteins from the Pex5-cargo complex. However, more cargo was released upon addition of DTT and Pex8, but not Pex8∆36, in comparison to the addition of DTT alone (Fig. 6C, compare GFP-SKL on beads and in Sup, lanes 2, 4 and 6, and bar graph). Note that this difference in the behaviour of Pex8 versus Pex8∆36 is attributable to the ability of Pex5 in the beads fraction to bind Pex8, but not Pex8∆36 (Fig. 6E, compare lanes 3, 4 versus 5, 6). Therefore, while Pex8 does not directly cause PTS1 protein release from Pex5, it facilitates cargo release by interacting with the N-terminal domain of Pex5. In contrast, because Pex8∆36 cannot bind Pex5, it fails to promote cargo release beyond the level caused by DTT alone (Fig. 6E, bar graph, last column).

DISCUSSION

In this manuscript we show that the formation and reduction of disulfide bonds via Cys10 regulate the oligomeric state of Pex5, which allows PTS1 protein binding to, and release from, Pex5. Homo-oligomeric Pex5 linked by intermolecular disulfide bonds binds GFP-PTS1 cargo with the highest affinity and can be purified as a complex. Upon addition of DTT to reduce the disulfide bonds, thereby mimicking the reducing environment of the peroxisome matrix (21), Pex5 transitions to a non-covalent homo-dimeric form with a much lower affinity for PTS1 cargo, thereby resulting in partial cargo release. The subsequent hetero-oligomeric interaction between the N-terminus of Pex5 with Pex8 shifts the equilibrium towards complete cargo release within the peroxisome. These studies provide insights into the molecular mechanism of cargo binding by Pex5 in the cytosol, shuttling to the peroxisome matrix where disulfide bond reduction and cargo release occur, and freeing up Cys10, which is mono-ubiquitinated by Pex4, thereby allowing receptor export from the peroxisome membrane to the cytosol.

We show that dsLPex5 accumulates under peroxisome proliferation conditions and can be reduced by 2 mM glutathione (GSH). GSH is present in the peroxisomes of methylotrophic yeasts and is necessary for the metabolism of methanol (28) and known to be one of the important components involved in the plant peroxisomal ascorbate-glutathione cycle for scavenging reactive oxygen species (29). It is quite likely that GSH is also required to reduce the dsLPex5 in the peroxisome lumen to facilitate cargo release and to make Cys10 available for mono-ubiquitination. Failure to reduce the dsLPex5 in the peroxisome lumen, or further oxidizing Cys10 under oxidative stress conditions, may trigger an intracellular signal for poly-ubiquitination of Pex5, activating a quality-control cascade whereby non-functional Pex5 is degraded through the RADAR pathway. This may be the true physiological reason for the existence and evolutionary conservation of the RADAR pathway, which is generally non-essential for peroxisome biogenesis, as long as receptor recycling is efficient. Consistent with this hypothesis, in the absence of Hansenula polymorpha PMP20, concomitant with the increase of oxidative stresses, the steady state level of HpPex5 was reduced when induced in methanol medium (30).

The C-terminal TPR domains of Pex5 are directly involved in cargo binding, while the N-terminal domain of Pex5 (NTD) contains motifs that interact with Pex14, Pex13 and Pex8. The NTD of Pex5 may also be involved directly or indirectly in modulating cargo binding. The role of the NTD in cargo binding could be explained by a direct interaction between the NTD and the TPR domains as suggested in mammalian Pex5: a competition between the PTS1 cargo and the NTD for binding to the TPR domain might result in the displacement of the PTS1 from the TPR domains (23). On the other hand, the reduction of the disulfide bond-linked NTD of Pex5 and the interaction between the NTD and other peroxins like Pex14 (20) and Pex8 (see below) may induce a conformational change in the C-terminal region of Pex5 corresponding to the TPR
domains, which probably decreases the affinity to cargo and ultimately causes cargo release (6). We favor the second hypothesis since we did not see a significant interaction between the PpPex5 NTD and the TPR domains under reducing conditions (data not shown). The formation and disruption of the intermolecular disulfide bonds via Cys10 may initiate an interdomain communication within Pex5 that regulates the affinity of cargo binding to, or release from, the TPR domains. Indeed, we found that the PpPex5 C10S mutant, which showed decreased binding capacity to cargo, may have a different conformation compared to PpPex5 since it is more resistant to proteases. It remains to be seen whether Pex14 interacts with the NTD of Pex5 in a manner that prevents disulfide bond formation, and in doing so, perhaps facilitates cargo release.

It has been suggested that Pex8 is involved in PTS1 protein release, but the molecular mechanism underlying this process is not known (8). Cargo release is not achieved by direct competition between Pex8 and PTS1 proteins in binding to the TPR domains of Pex5 because Pex8 can cause the PTS1 peptide to dissociate from Pex5 independent of its PTS1 (8). We found Pex8 binds to the N-terminus of Pex5, which may induce a conformational change of the TPR domains and decrease their binding affinity to cargo. Furthermore, a conserved motif (WWY) at the C-terminus of Pex8 was found to be essential for the targeting of PTS1 proteins. The binding of Pex8 to Pex5 in the peroxisome lumen makes the cargo release reaction irreversible and may further facilitate the subsequent receptor recycling process by maintaining the Cys10 in its reduced state.

The intraperoxisomal redox state has been shown to be more reducing than that in the cytosol (21). Maintaining a reduced environment in the peroxisome lumen is crucial for Pex11-mediated peroxisome proliferation and the activity of peroxisomal 3-ketoacyl-CoA thiolase, an enzyme involved in fatty acid beta-oxidation (31-33). Deletion of S. cerevisiae glutathione peroxidase 1, an antioxidant using thioredoxin or glutathione for its reducing power, impaired peroxisome biogenesis (34). Knockout of PMP20, a gene encoding a peroxiredoxin in H. polymorpha, impaired the targeting of peroxisomal matrix proteins, apparent consequences resulting from the reduced levels of Pex5 and phosphorylated Pex14 and damaged peroxisomal membranes. Legakis et al. (2002) showed that peroxisomal PTS1 protein import is compromised in aging fibroblast cells, which produce an increased amount of reactive oxygen species (35). Furthermore, they showed using an in vitro import assay that exposing early passage cells to 125 μM H₂O₂ significantly reduced the import of PTS1 protein. Although knockout of genes encoding either glutathione peroxidase or PMP20 in P. pastoris showed neither a significant decline in PTS1-import (data not shown) nor reduced levels of Pex5, we found that exposing P. pastoris cells to 100 μM H₂O₂ caused a clear delay in the import of PTS1 proteins. Our studies indicate that intraperoxisomal reducing conditions are important for peroxisomal cargo import, e.g. intraperoxisomal cargo release depends on reduced Pex5, a conformation that has a reduced binding affinity for cargo and a greater competence for undergoing monoubiquitination, fulfilling a necessary step for receptor recycling. Furthermore, the formation of dslPex5 through Cys10 in the cytosol prevents irreversible oxidation, which reduces the binding ability of Cys10 in the cytosol. Pex5 subsequently activates the RADAR pathway.

How PTS2 cargo is released in peroxisome is still elusive. However, when a conserved cysteine (Cys204) of Pex7 was mutated to serine or modified by NEM, the binding ability of Pex7 to the PTS2 cargo was significantly decreased (36). Whether Cys204 is involved in PTS2 cargo releasing from Pex7 needs further investigation.
REFERENCES


Redox regulated cargo binding and release


Redox regulated cargo binding and release


ACKNOWLEDGEMENTS

We thank the members of the Subramani lab for helpful discussion.

FOOTNOTE

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2. The abbreviations used are: dslPex5, disulfide bond-linked Pex5; DTT, dithiothreitol; GSH, glutathione; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ncPex5, non-covalent Pex5; NTD, N-terminal domain; PTS, peroxisomal targeting signal; RADAR, Receptor Accumulation and Degradation in the Absence of Recycling; TPR, tetratricopeptide.

Figure legends

Fig. 1. Intermolecular disulfide bond-linked PpPex5 was found in *P. pastoris* and in *E. coli*. A. Cell lysates from wild-type (PPY12) and ΔPex5 cells, grown overnight in methanol medium, were TCA precipitated and separated by reducing (+DTT) or non-reducing (-DTT) SDS-PAGE, followed by immunoblotting with anti-Pex5 and anti-F1β (the loading control). B. Cell lysates of wild-type cells from A were treated with reducing agents - 2 mM DTT, 2 mM GSH, or 5 mM GSH for 1 h at RT and analyzed by non-reducing SDS-PAGE, followed by immunoblotting with anti-Pex5 and anti-F1β. C. The affinity-purified recombinant His-Pex5 from *E. coli* was analyzed by gel filtration. Fractions collected were analyzed by non-reducing SDS-PAGE, followed by immunoblotting with anti-His antibodies. D. The non-covalent dimer of His-Pex5 purified from *E. coli* is oxidized to a covalent dimer. After gel filtration, the non-covalent dimer of Pex5 was concentrated and exchanged to a non-degassed buffer. The concentrated proteins treated without (lane 1) or with (lane 2) 2 mM DTT were analyzed by non-reducing SDS-PAGE, followed by immunoblotting with anti-His antibodies. The doublet of bands around 43 kDa (lane 2) was either due to degradation of full-length Pex5 or its truncated translation products. E. Disulfide bond-linked PpPex5 was observed in *P. pastoris* under peroxisome proliferation conditions. F. PpPex5 contains three cysteines, Cys 10 located in the N-terminal domain, and Cys 338 and Cys 444
located in the TPR domains. G. PpPex5 and its cysteine mutants were expressed from their own promoters in Δpex5 cells. Cell lysates of methanol grown cells were TCA precipitated for non-reducing SDS-PAGE, followed by immunoblotting with anti-Pex5 and anti-F1β (left panel). The film was overexposed to show the absence of disulfide bond-linked Pex5 in Δpex5 cells transformed with PpPex5 C10S (right panel). H. The affinity-purified recombinant His-Pex5 C10S from E. coli was analyzed by gel filtration. Fractions collected were analyzed by non-reducing SDS-PAGE and immunoblotted with anti-His antibodies. I. Wild-type Pex5 is more susceptible to proteolysis than is Pex5 C10S during expression and purification from E. coli cells.

Fig. 2. The PpPex5 cysteine mutants have peroxisomal matrix protein import defects. A. Growth curve of cells in methanol medium. The Δpex5 cells harboring an overexpressed peroxisomal marker, BFP-SKL, were transformed with wild-type Pex5 or the relevant cysteine mutants expressed from the endogenous Pex5 promoter. The GS115 (his4) parent cells transformed with the pIB1 empty vector were used as a wild-type control. B. Fluorescence microscopy analysis of Δpex5 cells expressing wild-type Pex5 or the relevant cysteine mutants from their endogenous promoter. Pex3p-mRFP and BFP-SKL are peroxisomal membrane and matrix markers, respectively. DIC (differential interference contrast). Scale bar, 2 µm. C. Differential centrifugation analysis. Equal proportions of 200,000g supernatant and pellet fractions from Δpex5 cells expressing wild-type Pex5 or Pex5 C10S were separated by SDS-PAGE and immunoblotted with the indicated antibodies. D. Protease protection assay of a P200 pellet fraction isolated from Δpex5 cells expressing wild-type Pex5 or PpPex5 C10S. E. Cell lysates from Δpex5 cells expressing wild-type PpPex5, PpPex5 C10S, and PpPex5 C10S/K22R were TCA precipitated and separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Fig. 3. PpPex5 C10S has a lower cargo binding affinity compared to wild-type Pex5. A. The expression levels of Pex5 and Pex5C10S expressed from the GAPDH promoter in methanol medium were analyzed by Western blots. B. Fluorescence microscopy analysis of Δpex5 cells co-expressing wild-type Pex5 or Pex5 C10S with BFP-SKL from the AOX promoter in methanol medium for 3 h. Scale bar, 2 µm. C. Immobilized protein GST-SKL was incubated with E. coli cell lysates expressing PpPex5, C10S, C338S, C444S, C338/444S or N460K mutants of PpPex5. After washing, bound proteins were eluted and analyzed by SDS-PAGE and Western blotted using anti-His antibodies. The GST-SKL was detected by Ponceau S staining.

Fig. 4. Cargo release upon DTT treatment. A. His-Pex5/GFP-SKL was co-expressed in E. coli and affinity-purified through a HisTrap FF column and then split into two aliquots: one was loaded onto a gel filtration column in buffer without DTT. B. The second aliquot was treated with 10 mM DTT for 1 h at room temperature and loaded onto a gel filtration column in buffer containing 10 mM DTT. The fractions were collected, separated by SDS-PAGE and immunoblotted with the indicated antibodies. C. Affinity-purified GFP-SKL was analyzed by gel filtration in the absence of DTT. The fractions were separated by SDS-PAGE and the membrane was stained with Ponceau S. D. The affinity-purified His-Pex5/GFP-SKL complex was analyzed by gel filtration and samples were collected for non-reducing SDS PAGE (the same samples were used for reducing SDS PAGE in A) and stained by Proteoblue (A) or immunoblotted with anti-GFP (E). The immunoblot was overexposed intentionally to confirm that there is no intermolecular disulfide linkage between Pex5 and GFP-SKL. F. His-Pex5/GFP-SKL complex was rebound to the Ni-NTA beads and incubated in the absence or presence of 1 mM DTT. At each time point, the beads were spun down and the supernatant was collected and separated by SDS-PAGE to check the release of GFP-SKL from His-Pex5 by Western blot.

Fig. 5. Peroxisomal protein import was inhibited upon hydrogen peroxide treatment. A. Fluorescence microscopy analysis of methanol-grown wild type cells expressing GFP-SKL from the GAPDH promoter. Cells were treated with H2O2 upon shift to the methanol medium. Scale
bar, 2 µm. Arrows showed the clustered peroxisomes. B and C. Immunoblotting of cell lysates from methanol-grown cells used in A with the indicated antibodies. Cells were treated with 0, 50, 100 µM H$_2$O$_2$. C. Non-reducing SDS-PAGE followed by Western blot to analyze the expression level of dslPex5.

Fig. 6. Pex8 binds the NTD of Pex5 and facilitates the release of cargo. A. Co-purification of GST-Pex8 with His-Pex5 (1-110) via HisTrap FF column. GST-Pex8, but not the truncated products of GST-Pex8, is specifically co-purified with His-Pex5 (1-110) B. Multisequence alignment of the C-termini of Pex8 from *C. dubliniensis* (Cd), *L. elongisporus* (Le), *P. angusta* (Pa), *P. guilliermondii* (Pg), *P. pastoris* (Pp), *P. stipitis* (Ps), *S. cerevisiae* (Sc), *Y. lipolytica* (Yl). C. Differential centrifugation fractions from oleate-grown ∆pex8 cells expressing Pex8, Pex8∆36, or Pex8∆WWY∆AKL were immunoblotted with the indicated antibodies. D. Growth curve of WT, ∆pex8, and ∆pex8 cells co-expressing Pex8 or the relevant Pex8 mutants in methanol medium. E. Immobilized protein complex His-Pex5/GFP-SKL was incubated with Pex8 or Pex8∆36 in the absence (-) or presence (+) of DTT. The beads were pelleted after 2 h and the amounts of His-Pex5, GFP-SKL, and Pex8-HA in the supernatant and pellet were assessed (left panel). Bar graph on the right represents a quantification of the fraction of GFP-SKL released into the supernatant. Error bar represents the standard deviation of three independent experiments. * indicates a p value of <0.05 for the samples being compared.
### Table 1. P. pastoris strains used in this study

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Figure 3

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B

BFP-SKL  DIC

Δpex5 +P GAP-Pex5-HA
Δpex5 +P GAP-Pex5 C10S
-Δpex5 +P GAP-Pex5 C10S
-Δpex5 +P GAP-Pex5 C10S

C

Input  Pull-down  Input  Pull-down

Pex5  N460K  Pex5  N460K

Pex5 C10S  C338S  C444S
Pex5 C338S  C444S

α-His  GST-SKL
Figure 4

A
His-Pex5/GFP-SKL, 0 mM DTT

B
His-Pex5/GFP-SKL, 10 mM DTT

C
GFP-SKL, 0 mM DTT

D
dslPex5—
ncPex5—

E

F
-DTT +DTT

α-His

α-GFP
Figure 5

A

GFP-SKL

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Downloaded from http://www.jbc.org by guest on October 30, 2017
Figure 6

A

Input Fractions of affinity purification

His-Pex5 (1-110)

GST-Pex8

Truncated products of GST-Pex8

B

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D

\[
\text{O.D.}_{600}
\]

Time (h)

\[\text{WT} \quad \text{ΔPex8} \quad \text{Pex8ΔAKL} \quad \text{Pex8Δ36} \quad \text{Pex8ΔWWY ΔAKL}\]

E

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\* \*
Redox regulated cargo binding and release by the peroxisomal targeting signal receptor, Pex5
Changle Ma, Danielle Hagstrom, Soumi Guha Polley and Suresh Subramani

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